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Electron Spin Resonance Spin Trapping for Analysis of Lipid Oxidation in Oils: Inhibiting Effect of the Spin Trap α-Phenyl-*N*-*tert*-butylnitrone on Lipid Oxidation

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The electron spin resonance (ESR) spin trapping technique was investigated as an analytical approach to follow lipid oxidation of rapeseed oil, sunflower oil, and fish oil during storage at 40 °C. Unlike previous investigations, α-phenyl-*N*-*tert*-butylnitrone (PBN), used as spin trap, was added to the fresh oils and formation of radicals was monitored during storage. Results were compared with the development in peroxide value (PV) and the thiobarbituric acid index (TBA). Increasing radical development was detected during the initial stages of oxidation, during which no significant changes in PV and TBA were observed. Evidence of spin adduct depletion was found during prolonged storage, suggesting that although spin trapping of radicals may be used to follow early events in lipid oxidation, it is not a suitable parameter for long periods of time. Addition of the spin trap after sequential samplings is recommended for getting an insight of oxidative changes during storage. Further, the influence of the spin trap (PBN) on lipid oxidation was studied in detail by application of PV and TBA and by following the depletion of naturally occurring tocopherol. PBN was found to possess a profound inhibiting effect on lipid oxidation. Such an effect was found to be dependent on the nature of the oil, and it was observed that the lower the oxidative stability, the larger the effect of PBN on lipid oxidation. This effect was interpreted in terms of the capability of PBN to react with peroxyl radicals, which in turn depends on the initial tocopherol content of the oils.

KEYWORDS: ESR; spin trapping; lipid oxidation; oils; PBN

INTRODUCTION

Lipid oxidation is a major cause of deterioration of the lipids of foods during processing and storage, affecting quality and determining in many cases the shelf life of the food. Autoxidation, the reaction of oxygen with unsaturated lipids, is generally accepted to proceed through a radical chain mechanism in which three steps are differentiated as initiation, propagation, and termination. Analytical indices such as peroxide value, anisidine value, and the TBA index, among others, which account for relatively stable compounds formed in the propagation and termination steps, are commonly applied to evaluate oxidative changes in food lipids.

Detection of transient radicals by electron spin resonance (ESR) spectroscopy has been recently applied as an indication of early events of lipid oxidation in different foodstuffs (*1*−*15*). The steady-state concentrations of lipid-derived radicals during autoxidation fall below the ESR detection limit, and techniques such as spin trapping are required for their detection. The ESR spin trapping technique is based on the reaction of radicals with diamagnetic compounds (spin traps) added to the system to form more stable radicals (spin adducts), which accumulate at detectable concentrations ($>10^{-7}-10^{-6}$ M). Detection of these new radical species allows the indirect detection of radicals involved in lipid oxidation.

Application of ESR spin trapping has provided qualitative results that correlated with sensory analysis for mayonnaise enriched with fish oil (*4*). In meats, results obtained by ESR spin trapping were found to be in agreement with TBARS determination (*1*, *8*), oxygen consumption measurements (*12*), and peroxide value (*14*). ESR spin trapping has also been applied as a rapid test to determine oxidative stability of food lipids under mildly accelerated conditions. Oxidative stability was defined as the resistance to formation of radical species as detected by spin trapping. Radical development exhibited a very short induction period in which radicals were formed very slowly before a sudden linear increase was observed. In storage experiments of rapeseed oil at 60 °C and mayonnaise at 50 °C for 4 weeks, oxidative stability of rapeseed oil and the lipid fraction of mayonnaise was determined after different samplings. As expected, the induction period decreased and the amount of radicals at fixed time increased with storage time (*6*). In a recent report, oxidative stability of different vegetable oils, including rapeseed oil, sunflower oil and different mixtures of both, was

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approached by ESR spin trapping at 60 °C, and results were compared with those obtained at 100 °C by the Rancimat test and by another accelerated method based upon differential scanning calorimetry (DSC). While results obtained by the ESR method are indicative of the onset of primary oxidation, results obtained by the Rancimat and DSC methods account for the onset of advanced oxidation. Although different aspects of the oxidative process are assessed and different oxidation conditions were applied, the results obtained by the ESR method showed satisfactory linear correlations with those provided by the Rancimat test and also with those by DSC (*15*).

In the present study, the ESR spin trapping technique was investigated as an analytical approach to follow lipid oxidation in oils during storage at 40 °C. Unlike previous investigations, α-phenyl-*N*-*tert*-butylnitrone (PBN), used as spin trap, was added to fresh oils and radical formation was monitored during storage. Preliminary investigations had shown detectable amounts of radicals in oils containing the spin trap PBN after exposure to oxidation conditions for long periods of time. If detection of radicals provided objective information about oxidative changes during storage of oils, application of ESR would be of great utility to evaluate in situ lipid oxidation in other more complex food systems in which the spin trap would be incorporated during their elaboration. Thus, extraction of the lipid phase to be evaluated in a solid food would be not necessary since ESR spectroscopy can be applied directly on nontransparent systems. Detailed investigation was accordingly conducted in order to examine PBN spin trapping as a suitable method to follow lipid oxidation of oils. Results were compared with those obtained by application of peroxide value and the TBA index, both commonly applied as standard analytical determinations. Quantitative analysis of radicals was approached by using external calibration with a stable radical dissolved in a saturated oil nonsusceptible to oxidation. Furthermore, the influence of the spin trap (PBN) on lipid oxidation was studied in detail by following the peroxide value, the TBA index, and the depletion of naturally occurring tocopherol.

MATERIALS AND METHODS

Chemicals. α-Phenyl-*N*-*tert*-butylnitrone (PBN) (purity ≥97%) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (purity approximately 98%) were purchased from Fluka Chemie GmbH (Buchs, Germany) and Sigma−Aldrich Chemie GmbH (Steinheim, Germany), respectively, and used as received. Saturated medium-chain triacylglycerol (MCT) oil was provided by Brøste A/S (Lyngby, Denmark). All other chemicals and reagents were of analytical grade and used without further purification.

Samples. Rapeseed oil (RO), conventional sunflower oil (SO), and crude fish oil (FO) were used in this study. RO and SO were acquired from a local supermarket, while FO was kindly supplied by TripleNine Fish Protein amba. (Esbjerg, Denmark). The three oils were used as received and characterized by chemical analysis as described below. PBN was dissolved in the oils at concentration of 1 mg/g of oil (5.64 mmol/kg of oil) by stirring at room temperature. Samples without PBN were used as control samples.

Oxidation Conditions. Samples were oxidized in air at 40 °C in the dark under 0% relative humidity by using P_2O_5. The oils were divided into independent aliquots of 1.25 mL placed in Eppendorf tubes, which were left open. The tubes were placed into a desiccator containing P_2O_5, and the desiccator was placed in an oven. The air in the headspace of the desiccator was replaced during each sampling. Two tubes of each sample were taken periodically and the contents were mixed. In selected samplings, duplicates of samples were considered by taking four tubes and mixing their contents two by two. Replicates of samples were taken into account only in ESR analyses.

ESR Spectroscopic Analyses. Samples were placed into ESR tubes with 0.4 mm i.d. (700-PQ-7, heavy wall; Wilmad Glass, Buena, NJ) and analyzed by ESR immediately after sampling. The amount of oil in the tubes was that allowing the sample cavity of the spectrometer to be completely full with the sample. Thus, analyses were performed at constant volume of sample, determined by the height of the cavity (max. sample access 11.5 mm, according to technical specifications), by taking amounts reaching around 9 cm high in the tubes. An ECS 106 ESR spectrometer (Bruker, Rheinstetten, Germany) was used with an X-band resonator (ER 4103TM). Analyses were performed at room temperature, and special care was taken to avoid the presence of light. The parameters used in all ESR measurements were kept constant in each determination, and only the receiver gain was adjusted and measurements accordingly corrected. The parameters applied were as follows: center field, 3475.5 G; sweep width, 100 G; resolution, 1024 points; microwave power, 20 mW; modulation amplitude, 1.1 G; modulation frequency, 100 kHz; conversion time, 41 ms; time constant, 328 ms; and accumulated scans, 2. The area under the curve of the absorption spectra and the peak-to-peak amplitude were determined by the Winepr software program (Bruker, Rheinstetten, Germany). Determination of the area was approached by double integration of the spectra over the entire scan sweep (100 G). Analyses were performed in duplicate.

Simulation of ESR Spectra. Computer simulation of experimental ESR spectra was used for the calculation of hyperfine coupling constants. Simulation was performed by the Public EPR Software Tools (PEST) from the National Institute of Environmental Health Sciences, available on the Internet (http://epr.niehs.nih.gov/). Optimization parameters were obtained when correlation exhibited regression coefficients above 0.996. Hyperfine coupling constants were obtained as average values of ESR parameters from computer simulations of 24 experimental spectra, which provided standard deviation below 0.03 G.

Calibration Curve of TEMPO. TEMPO dissolved in MCT oil by stirring at room temperature was used as external standard. Solutions of TEMPO with concentrations ranging from 0.34 to 6.40 mmol/kg of oil were prepared in duplicate by dilution of a stock solution containing TEMPO at 1 mg/g of oil. One determination of each duplicate was performed. ESR analyses were carried out by applying the same procedure and parameters described above.

Chemical Analyses. *Fatty Acid Composition.* Fatty acid composition was analyzed by gas−liquid chromatography after derivatization to fatty acid methyl esters with 2 M KOH in methanol at room temperature according to the IUPAC standard method (*16*). A 5890 A-II chromatograph (Hewlett-Packard Co., San Fernando, CA) with a split injector operating with a 10:1 split ratio at 250 °C, a HP-FFAP capillary column, 25 m × 0.20 mm i.d. and film thickness 0.33 μm (Hewlett-Packard Co., San Fernando, CA), and a flame ionization detector at 300 °C were used. Analyses were performed with helium (0.95 mL/min) as carrier gas and applying the following temperature program: 50 °C held for 1 min, 15 °C/min to 180 °C, 5 °C/min to 220 °C held for 10 min. Analyses were performed in duplicate on duplicate samples.

Analysis of Tocopherols. Quantification of α- and γ-tocopherol was carried out by reverse-phase liquid chromatography (HPLC) with fluorescence detection by using as external standard solutions of known concentrations of α- and γ-tocopherol. Oil samples (25 mg) were dissolved in 3 mL of ethanol/1-butanol (75:25 v/v) and directly analyzed. A HPLC analytical column (125 mm × 4.0 mm) (Agilent Technologies, Karlsruhe, Germany) packed with a C18 phase and containing a mean particle size of 5 μm was used. Methanol/water (94:6 v/v) was used as mobile phase with a flow of 1 mL/min. The fluorescence detector operated with the excitation wavelength set at 292 nm and the emission wavelength at 330 nm. Analyses were performed in duplicate.

Peroxide Value. Peroxide value (PV) determination was carried out by means of the iodometric assay according to the AOCS (*17*) with minor modifications. Samples of 200 mg of oil and a 1.25×10^{-3} N $Na_2S_2O_3$ solution were used. Determinations were performed in duplicate.

Thiobarbituric Acid Index. Thiobarbituric acid index (TBA) was determined by the direct method of AOCS (*18*). Determinations were performed in duplicate.

Oxidative Stability Index. The oxidative stability index (OSI) was determined on a Rancimat instrument, model 679, from Metrohm

Table 1. Fatty Acid Composition, α- and γ-Tocopherol Content, Peroxide Value (PV), and Oxidative Stability Index (OSI) of the Oil Samples

	rapeseed oil	sunflower oil	fish oil
fatty acid[a] (%)			
C14:0	0.05 ± 0.00	0.05 ± 0.00	5.23 ± 0.06
C16:0	5.15 ± 0.10	6.07 ± 0.26	15.14 ± 0.12
C16:1	0.19 ± 0.00	0.08 ± 0.01	5.87 ± 0.12
C17:0	ND	ND	1.13 ± 0.02
C17:1	ND	ND	0.98 ± 0.07
C18:0	1.67 ± 0.00	3.52 ± 0.16	2.08 ± 0.01
C18:1	59.58 ± 0.13	27.29 ± 1.20	15.82 ± 0.27
C18:2	20.85 ± 0.09	61.61 ± 1.71	2.24 ± 0.04
C18:3	9.43 ± 0.02	0.12 ± 0.00	2.03 ± 0.04
C20:0	0.57 ± 0.00	0.25 ± 0.01	ND
C20:1	1.40 ± 0.02	0.30 ± 0.01	12.55 ± 1.18
C20:5	ND	ND	6.71 ± 0.09
C22:0	0.34 ± 0.01	0.71 ± 0.04	ND
C22:1	0.48 ± 0.01	0.00 ± 0.00	8.06 ± 0.17
C22:6	ND	ND	9.00 ± 0.14
others	0.29 ± 0.00	0.00 ± 0.00	13.16 ± 0.18
α-Toc[a] (mg/kg of oil)	232 ± 1	489 ± 2	83 ± 1
γ-Toc[a] (mg/kg of oil)	316 ± 3	26 ± 2	ND
PV[a] (mequiv of O_2/kg of oil)	2.51 ± 0.34	3.55 ± 0.53	1.53 ± 0.11
OSI[a] (h)	19.05 ± 0.35	11.95 ± 0.21	1.45 ± 0.04

[a] Results are expressed as mean ± standard deviation ($n = 2$). ND, not detected.

Nordic ApS (Glostrup, Denmark). Samples of 2.00 ± 0.01 g of oil were analyzed at 100 °C by applying an air flow of 20 L/h.

Statistical Analysis. Statistical analysis was applied by using SPSS 11.8 software (SPSS Inc., Republic of Ireland). One-way ANOVA, using the general linear model procedure, and Tukey's test at the 5% level were applied to analyze the influence of oxidation time and the kind of oil on the coupling constants, the influence of the concentration of TEMPO on the line width, and the effect of the addition of PBN on PV, TBA, and tocopherol content. Pearson correlation analysis was applied at the 1% level to study correlations of PBN adduct concentration with PV and TBA.

RESULTS

Characterization of Oils. Table 1 lists fatty acid composition, tocopherol content, peroxide value (PV), and oxidative stability index (OSI) of the oils assayed in the present study. RO and SO showed the typical fatty acid profile of low-erucic rapeseed oils and high-linoleic sunflower oils, respectively. Fatty acid composition of FO indicated the presence of a mixture of different fish species. According to the provider, this mixture was mainly composed of sand eel oil. Tocopherol content was within normal range. PV was low and typical of fresh oils. The Rancimat test indicated that the three oils presented very different oxidative stabilities.

ESR Analyses. ESR signals were detected only in samples containing PBN. All spectra recorded consisted of three broad lines, although very slight shoulders were also observed in FO samples (**Figure 1**). Computer simulation of the ESR spectra revealed a hyperfine splitting made up of a triplet of doublets, whose splitting constants were determined to be 14.88 and 1.81 G, respectively. No significant differences ($p > 0.05$) were found in the coupling constants of spectra obtained from different oils, as well as over oxidation time. These coupling constants are characteristic of nitroxyl radicals and account for the coupling of the unpaired electron with the nitrogen nucleus (^{14}N, $I = 1$) and the hydrogen (^{1}H, $I = {}^{1}/_{2}$) on the carbon adjacent (**Figure 2**), respectively. Nevertheless, the hydrogen splitting was not observed in this study due to line broadening effects. Such effects may originate from restrictions in rotational mobility

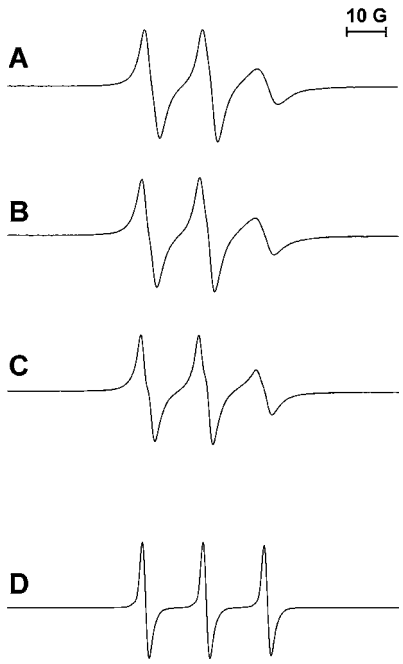

Figure 1. ESR spectra obtained in (**A**) rapeseed oil, (**B**) sunflower oil, and (**C**) fish oil containing PBN, and (**D**) ESR spectrum of TEMPO dissolved in MCT oil.

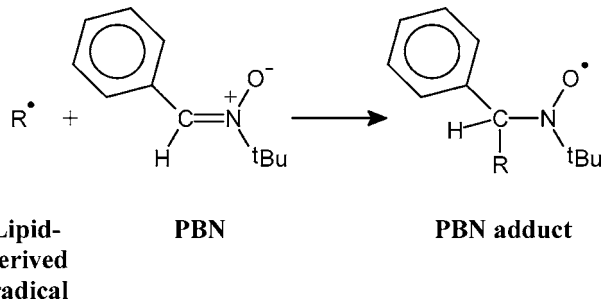

Figure 2. Representative scheme of spin trapping with PBN.

leading to partial averaging of the ESR spectra from different orientations of the radicals with respect to the magnetic field. As a result, the spectrum observed is that formed by the superposition of the spectra of all individual orientations (*19*). In particular, the high-field line ($M_I = -1$) was mainly affected by such restriction, and loss in intensity due to remarkable line broadening was observed (**Figure 1A−C**). In this respect, molecular tumbling rates depend on the shape and size of molecules, the solvent viscosity, and temperature (*19*). To examine the viscosity of the oils as the main cause of broadening, a nitroxyl radical with free molecular mobility, TEMPO, was dissolved in the fresh oils and its spectra were recorded. Three narrow lines of similar intensities were detected in the three oils under study (results not shown), as well as in MCT oil (**Figure 1D**). Thus the completely averaged spectra showed by TEMPO suggest that the line broadening was due to restricted mobility caused by the high molecular volume of the radical added to PBN, that is, lipid-derived radicals (**Figure 2**).

Determination of radical concentration was approached by external calibration, with a stable radical as standard, TEMPO, dissolved in a saturated oil nonsusceptible to oxidation (MCT oil). As a first attempt, TEMPO was tested in the fresh oils; however, significant losses were promptly detected over time. In contrast, TEMPO dissolved in MCT oil showed high stability. The range of concentrations of TEMPO was selected according

to the initial concentration of PBN in the oils (5.64 mmol/kg of oil). An excellent linear correlation ($r = 0.9999$, $p < 0.0001$) was found between the area under the curve of the absorption spectrum, that is, the double integration of the ESR spectrum, and the concentration of TEMPO (**Figure 3B**). For illustrative purposes, the absorption spectra obtained are given in **Figure 3A**. On the other hand, when the peak-to-peak amplitude of the low-field line ($M_I = +1$) of the ESR spectrum was taken instead, significant deviation from linearity was found at high concentrations of TEMPO (**Figure 3C**). In this regard, analyses of the spectra indicated that the line width was significantly enhanced as the concentration of TEMPO was increased from 1.30 mmol/kg of oil. Such concentration-dependent broadening can be ascribed to electron-spin exchange phenomena consisting of bimolecular interactions in which the unpaired electrons of two radicals exchange their spin orientations (*19*). Since the peak-to-peak amplitude is inversely proportional to the square of the line width, a slight broadening causes a notable decrease in the height of the line (*19*). As can be observed in **Figure 3C**, the values obtained when an increase in the line width took place were lower than those expected in the absence of line broadening. These results suggest that the peak-to-peak amplitude is directly proportional to the radical concentration only in the absence of intermolecular spin exchange, that is, at low radical concentrations.

All spectra obtained in the oils containing PBN were free of spin-exchange broadening, as no change in the line width was observed with the increase of the ESR signal. As can be observed in **Figure 4A**, the peak-to-peak amplitude was directly proportional to the area under the curve of the absorption spectrum in the three oils. Even though a satisfactory linear correlation common for the three oils was obtained, the line width was found to be oil-dependent, probably because of differences in radical mobility. In the case of MCT oil containing TEMPO, deviation from linearity was observed as the spin-exchange broadening took place (**Figure 4B**).

For comparative purposes, concentrations of PBN adducts were determined by taking both the area under the curve of the absorption spectra and the peak-to-peak amplitude in the absence of spin-exchange broadening. Results obtained from the peak-to-peak amplitude were around 4 times lower than those obtained from the area. When the peak-to-peak amplitude is considered, it is supposed that both the standard and the species to be quantified possess the same peak-to-peak height for the same value of the area. In this respect, it is important to emphasize that the different hyperfine splitting of the PBN adducts with respect to that of TEMPO was not taken into account. The hyperfine splitting of the former corresponds to a triplet of nonresolved doublets against the triplet shown by the standard. Therefore, for the same value of the area, the peak-to-peak amplitude is expected to be 2 times larger in the case of TEMPO. Further, as mentioned above, the line broadening caused by restricted radical mobility in the oil samples leads to a decrease in the peak-to-peak amplitude. As a result, for the same value of the area, the peak-to-peak amplitude was lower in the oil samples than in MCT oil containing TEMPO (**Figure 4C**). Therefore, the peak-to-peak amplitude was discarded and the area under the curve of the absorption spectrum was used for the calculation of the absolute concentrations.

No ESR signal was detected in the oils prior to storage. However, the double integration of the spectra revealed a very slight signal that was proven to come from the sample cavity, probably due to the presence of paramagnetic impurities. Therefore, the area under the curve of all absorption spectra

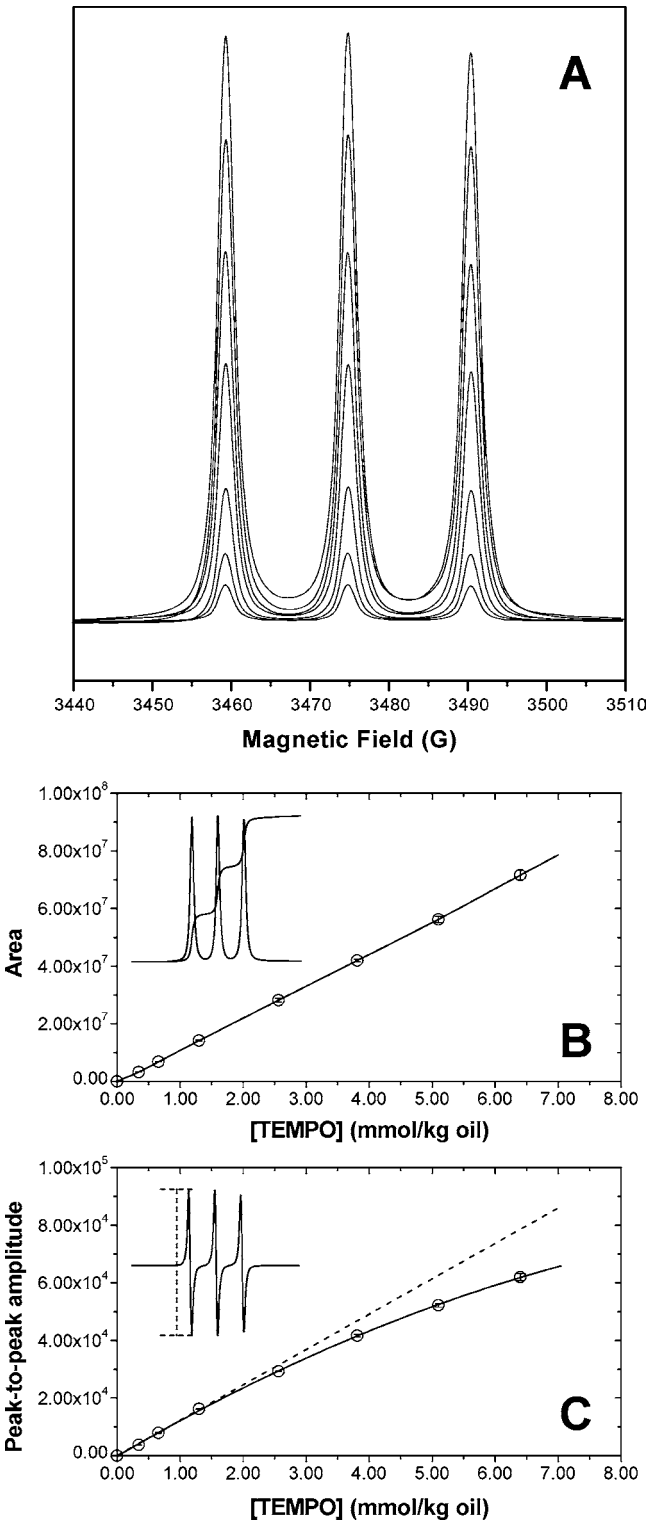

Figure 3. (**A**) ESR absorption spectra of TEMPO in MCT oil at concentrations of 0.34, 0.66, 1.30, 2.56, 3.81, 5.10 and 6.40 mmol/kg of oil. (**B**) Calibration curve of TEMPO (area $= 1.11 \times 10^7$ ($\pm 0.04 \times 10^7$) [TEMPO] (mmol/kg of oil), $r = 0.9999$) by use of the area under the curve of the ESR absorption spectrum. An example of the integration has been included as an inserted graph. (**C**) Calibration curve of TEMPO (amplitude $= 62 \times 10^3$ ($\pm 1 \times 10^3$) [TEMPO] (mmol/kg oil), $r = 0.9993$) taking the peak-to-peak amplitude of the low-field line ($M_I = +1$) of the ESR spectrum. An example of this parameter has been included as an inserted graph.

was accordingly corrected. Even so, the FO sample showed an additional initial value that was taken into account because it

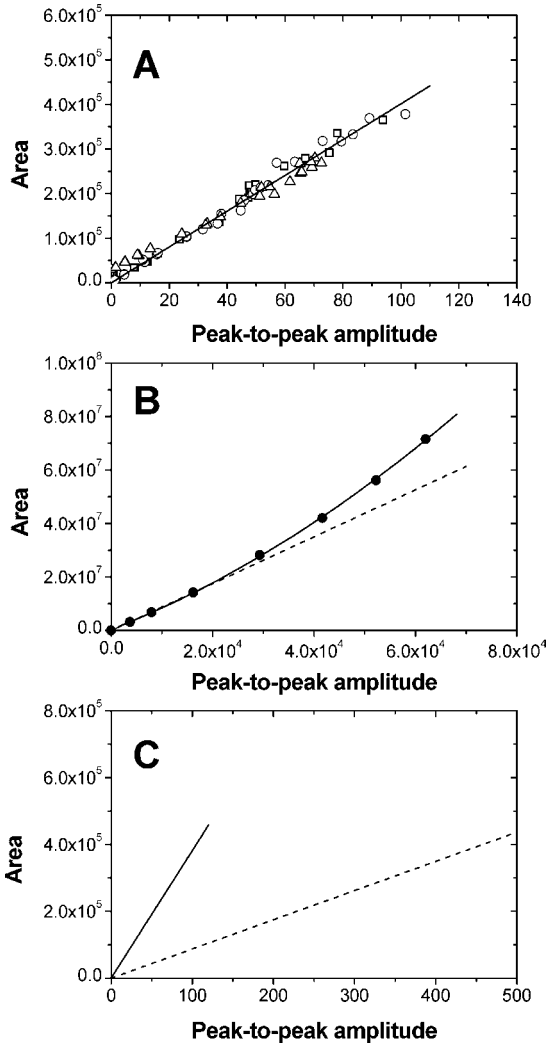

Figure 4. (**A**) Linear correlation between the area under the curve of the ESR absorption spectra and the peak-to-peak amplitude of the low-field line ($M_I = +1$) of the ESR spectra of rapeseed oil (□), sunflower oil (○), and fish oil (△) containing PBN (area = 4014 (±35) amplitude, $r = 0.9925$). (**B**) Linear correlation between the area under the curve of the ESR absorption spectra and the peak-to-peak amplitude of the low-field line of the ESR spectra of TEMPO in MCT oil in the absence of electron spin exchange (area = 873 (±5) amplitude, $r = 0.9999$). (**C**) Comparison between the linear correlations obtained in the oils containing PBN (—) and in MCT oil containing TEMPO (---).

showed high reproducibility. The origin of this detectable value in the area of the absorption spectrum of the initial FO sample was not further investigated.

Figure 5 illustrates concentrations of PBN adducts over oxidation time. In comparison with the initial concentration of PBN (5.64 mmol/kg of oil), the concentration of the radicals detected was found to be very low, within a range of 35 μmol/kg of oil. On the other hand, results showed high reproducibility. In terms of relative standard deviation, reproducibility of the ESR determination was found to be below 5%, while replication of samples showed values below 6%.

Adducts of PBN were detected at day 1 in FO and SO, whereas RO exhibited no ESR signal until day 2. The three samples showed a first period of fast formation of PBN adducts followed by a period in which the formation was slowed, as in RO and SO, or leveled off, as in FO. Furthermore, a decrease in the concentration of radicals was found in FO over oxidation time. Even though concentrations of radicals were significantly

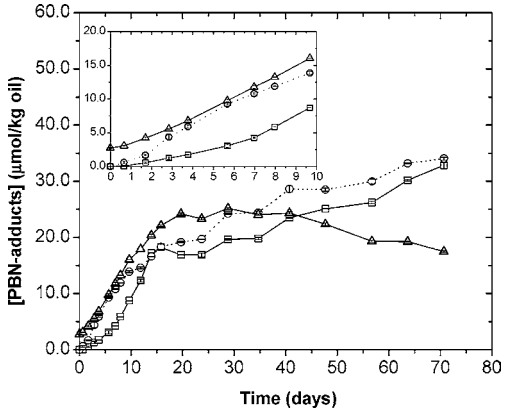

Figure 5. PBN adduct concentrations obtained in rapeseed oil (□), sunflower oil (○), and fish oil (△) during oxidation at 40 °C in the dark. Inserted graph, magnification during the first 10 days of oxidation. Error bars express standard deviation ($n = 2$).

lower in RO during the first period, the profiles of the curves were very similar within this period (**Figure 5**).

Peroxide Value. No linear correlation ($p < 0.01$) was found between the concentration of radicals and PV. Pearson correlation coefficients were determined to be 0.740, 0.887, and 0.816 in RO, SO, and FO, respectively. As can be observed in **Figure 6**, remarkable concentrations of radicals were obtained before detection of significant increases in PV. In comparison with the control samples, the presence of PBN led to a marked decrease of PV over oxidation time. One-way ANOVA revealed a significant interaction between the addition of PBN and the kind of oil. The influence of PBN on the decrease of PV appeared therefore to be oil-dependent. Post hoc analysis based upon Tukey's test was applied to the differences in PV between the oils and the corresponding controls. Results revealed that the lesser the oil stability (RO > SO > FO), the larger the effect of PBN on the decrease of PV (RO < SO < FO). In particular, FO showing the largest effect of PBN exhibited PV ranging between 1.5 and 15.4 mequiv/kg of oil, whereas the control reached PV as high as 167 mequiv/kg of oil.

Thiobarbituric Acid Index. RO and FO showed a slight development in TBA over oxidation time, whereas no significant detection of TBA was found in SO, neither in the sample containing PBN nor in the control (**Figure 7**). No linear correlation ($p < 0.01$) was found between the concentration of radicals and TBA. The addition of PBN had a significant effect on the TBA values. Larger TBA values were found in the control samples than in the samples containing PBN. Tukey's test was applied on the differences between the values obtained in the oils containing PBN and their corresponding controls. Results showed that the effect of PBN on TBA was higher in FO than in RO.

Tocopherol Content. A significant effect of PBN was also found on the depletion rate of naturally occurring tocopherol. Samples containing PBN exhibited larger tocopherol concentrations over oxidation time than the corresponding controls (**Figure 8**). When tocopherol content was completely depleted in the control samples, the oils containing PBN showed remaining amounts as high as 57%, 72%, and 90% in RO, SO, and FO, respectively. On the loss of tocopherol, one-way ANOVA indicated a significant interaction between the presence of PBN and the type of oil. Within the time range in which the total depletion of the antioxidant took place in the control samples, results showed that the lesser the oil stability (RO > SO > FO), the larger the effect of PBN (RO < SO < FO) on the tocopherol content.

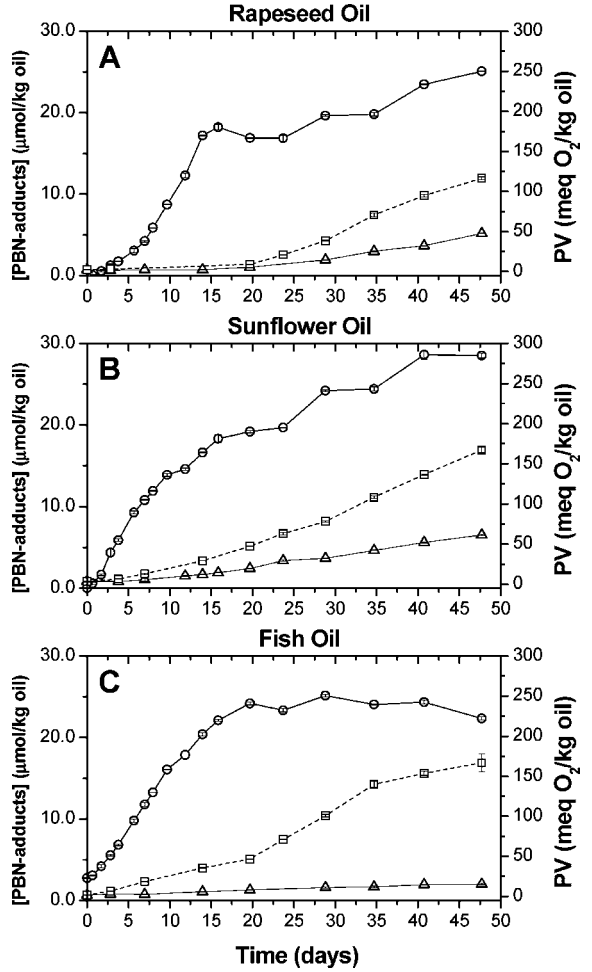

Figure 6. Peroxide value of samples containing PBN at 5.64 mmol/kg of oil (△) and samples without PBN (control samples) (□) in (**A**) rapeseed oil, (**B**) sunflower oil, and (**C**) fish oil during oxidation at 40 °C in the dark. For comparative purposes, the concentration of the detected PBN–adducts (○) has been included. Error bars express standard deviation ($n = 2$).

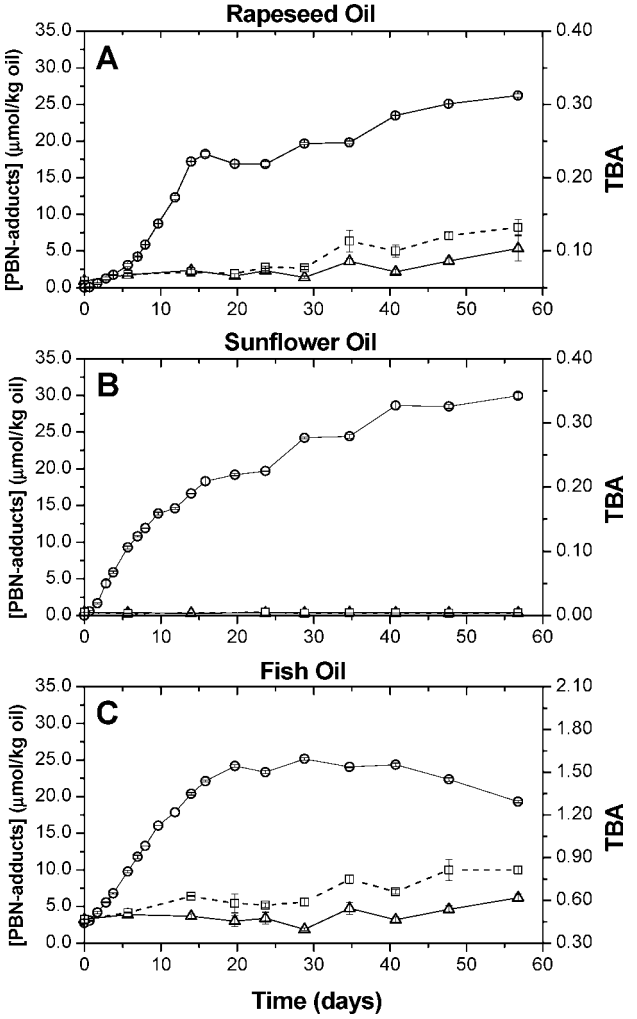

Figure 7. TBA development of samples containing PBN at 5.64 mmol/kg oil (△) and samples without PBN (control samples) (□) in (**A**) rapeseed oil, (**B**) sunflower oil, and (**C**) fish oil during oxidation at 40 °C in the dark. For comparative purposes, the concentration of the detected PBN adducts (○) has been included. Error bars express standard deviation ($n = 2$).

DISCUSSION

The concentration of a radical species is known to be proportional to the area under the curve of the ESR absorption spectrum, which is obtained from double integration of the first-derivative spectrum. Extreme care must be taken, however, in evaluating the area as serious errors may be introduced, such as those resulting from failure to include measurements sufficiently far from the center of the line, especially in spectra with a Lorentzian shape because of their extensive wings (*19*). As a result, this parameter is not usually considered and the peak-to-peak amplitude of the ESR line, better known as the ESR signal intensity, is taken instead and considered to be proportional to the radical concentration. Our present results have shown that while at low concentrations the ESR signal intensity is in fact directly proportional to the radical concentration, at concentrations high enough for spin−spin interactions, linearity is lost.

Quantification of radicals by ESR can be approached by external calibration with stable radicals. The chemical nature of the standard has no effect on the calibration provided that the area under the curve of the absorption spectra is used rather than the peak-to-peak amplitude. Results have shown that differences in hyperfine splitting, which might be not resolved, and radical mobility between the standard and the species to be quantified can introduce significant errors if the peak-to-peak height is used for the calculation of absolute concentrations. These results emphasize the importance of using the double integrals of the ESR spectra for quantitative purposes.

Although very low concentrations of radicals were found in the oil samples, increasing concentrations were observed during the first stages of oxidation, suggesting that the spin adducts formed had high stability. Barclay and Vinqvist (*20*) reported that a significant loss of PBN as detected by UV absorption was not observed until tocopherol was completely exhausted during oxidation of methyl linoleate. In the present study, a low but significant development of radicals was observed when tocopherol was still present at high concentrations. Despite the low concentration of radicals trapped by PBN, the presence of PBN had a profound inhibiting effect on the course of lipid oxidation.

The profiles of the curves in **Figure 5** appear to be useful only during the initial stages of radical formation. In agreement with other studies (*6*, *15*), detection of a significant ESR signal or a sudden increase of radical formation indicated a different oxidative stability of the three oils, which was in accordance with the results obtained by the Rancimat test (**Table 1**). Nevertheless, subsequent decay of spin adducts seems to become

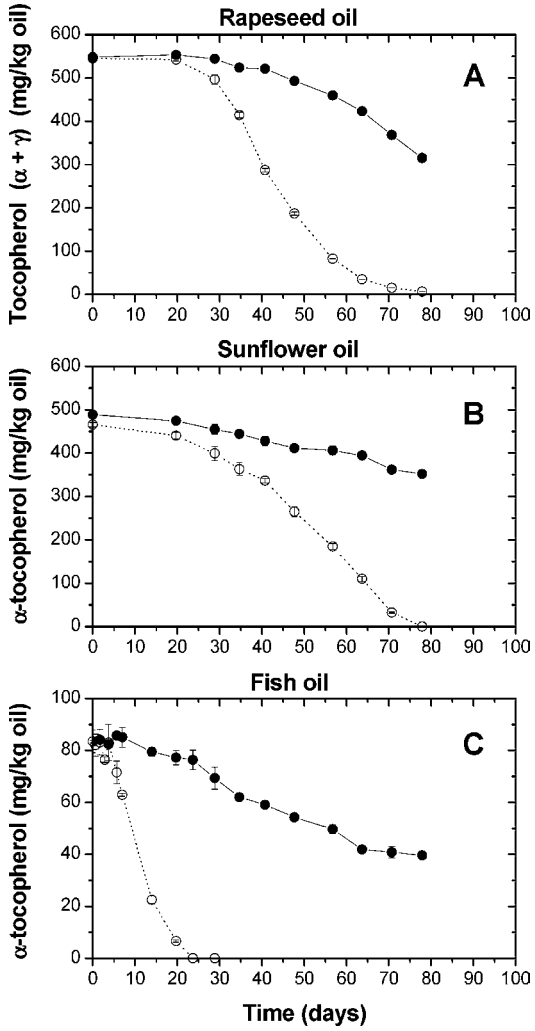

Figure 8. Tocopherol depletion in (**A**) rapeseed oil, (**B**) sunflower oil, and (**C**) fish oil samples containing PBN at 5.64 mmol/kg of oil (●) and samples without PBN (control samples) (○) during oxidation at 40 °C in the dark. Error bars express standard deviation ($n = 2$).

significant with oxidation time, leading to different behavior of the three oils depending on the relative rates of formation and decay reactions. In this respect, Qian et al. (*21*) pointed out that stability of spin adducts depends on the subsequent radical formation as they react with new radicals to form diamagnetic species. These authors found that the removal of the spin adducts from the reaction system by suitable extraction enhanced their stability considerably. In addition, in previous studies concerning application of ESR spin trapping to monitor oxidation of cream cheese during storage, it was recommended that experiments should be limited to few days in order to avoid the influence of subsequent reactions involved in losses of spin adducts (*3*).

It is very interesting to note that a significant development of radicals was detected before a significant increase in PV. This fact may be attributed to the high sensitivity provided by ESR spectroscopy, as radicals formed react very quickly and do not accumulate prior to formation of hydroperoxides. In this respect, the lack of correlation between the detection of radicals and PV during the initial stages of oxidation would be justified.

The application of the TBA determination is limited to oils with high degree of unsaturation, and only oxidation compounds arising from fatty acids with three or more double bounds are detectable (*22*). For the SO sample studied, the most unsaturated fatty acid is linolenic acid (C18:3), whose concentration as low as 0.1% (**Table 1**) could explain the absence of TBA development in SO.

Even though low concentrations of radicals were detected, the presence of PBN led to a remarkable inhibiting effect on lipid oxidation as assessed by PV, TBA, and tocopherol depletion. These results are in agreement with the numerous reports attributing to the spin trap PBN an important role in protecting biological systems from lipid peroxidation (*23*−*31*).

Barclay and Vinqvist (*20*) reported, however, that PBN does not really act as a chain-breaking antioxidant, but it acts as a weak retarder of lipid oxidation. In experiments conducted at 760 Torr of oxygen, different concentrations of PBN did not show any induction period as detected by oxygen consumption. Under the same conditions, chain-breaking antioxidants are known to inhibit oxygen uptake by the lipid substrate for a certain period of time, known as the induction period, until they are completely depleted. Then the induction period is followed by a return to the uninhibited rate of oxygen consumption. In comparison with effective amounts of antioxidants, larger amounts of PBN were necessary to observe only a retardation effect consisting of a decrease in the rate of oxygen uptake. In combination with antioxidants, PBN exhibited only a slight cooperative effect. It was proposed that the effect of PBN on the rate of oxidation results from trapping carbon-centered radicals, as the reaction between PBN and peroxyl radicals was suggested to be ineffective. Under reduced oxygen partial pressure, larger effects of PBN on the rate of oxidation were accordingly observed. Further, experiments combining PBN and antioxidants of the chromanol class showed a definite cooperative effect that was not observed at high oxygen partial pressure. These authors concluded that the effect of PBN consists of trapping initial carbon-centered radicals, especially at low oxygen conditions, and that this spin trap should be considered as a retarder or preventative antioxidant rather than a chain-breaking antioxidant.

In contrast, the results obtained in the present study indicate that, under conditions of high oxygen availability, PBN caused a profound effect on the rate of lipid oxidation. Under such conditions, it is expected that PBN reacts with peroxyl radicals to a greater extent than with alkyl radicals, as the reaction between the latter with oxygen to form peroxyl radicals is known to be very fast ($k = 10^9$ M^{-1} s^{-1}) and controlled by diffusion (*32*). Maeda and Ingold (*33*) reported that the rate constant for the reaction of PBN with secondary alkyl radicals in organic solvent was on the order of 10^5 M^{-1} s^{-1}, being therefore around 4 orders of magnitude lower than that between oxygen and alkyl radicals.

The reaction between PBN and peroxyl radicals has been reported to give unstable spin adducts that rapidly decompose at temperatures above 230 K (*34*, *35*). Pfab (*34*) suggested that peroxyl spin adducts of PBN decompose, generating alkoxyl radicals that in turn are trapped by new molecules of PBN. Dikalov and Mason (*36*) supported this very early hypothesis expressed by Pfab (*34*) and suggested that this is a general phenomenon for nitrone spin traps that does not depend on either the spin trap or peroxyl radical structure. Later, Dikalov and Mason (*37*) concluded that only alkoxyl spin adducts are detected in polyunsaturated lipids at temperatures higher than 250 K.

The effect of PBN on lipid oxidation has been found to depend on the type of oil, being related to the oxidative stability. Thus, it was found that the lower the oil stability as detected by the Rancimat test, the larger the effect of PBN on the rate

of oxidation. This effect may be interpreted in terms of the different capability of PBN to react with peroxyl radicals in the three oils, which is affected by the concentration of tocopherol. Therefore, the different tocopherol content occurring naturally in the oils may result in different effectiveness of PBN to react with peroxyl radicals. Barclay and Vinqvist (*20*) found that, in the presence of tocopherol, amounts of PBN much larger than that of tocopherol were required to observe an effect on the rate of oxygen uptake during oxidation of methyl linoleate, indicating that peroxyl radicals are mainly scavenged by tocopherol. In addition, these results are supported by the fact that significant losses of PBN were not detected until the complete exhaustion of tocopherol (*20*). In the present study, PBN was added to the three oils at the same concentration, and the results obtained could be explained in terms of the PBN-to-tocopherol concentration ratio. Thus, on a molar basis, relative concentrations of PBN were 4.3, 4.7, and 29.3 for RO, SO, and FO, respectively. As a result, the effect of PBN increased with the PBN-to-tocopherol ratio, that is, as the competition of PBN for peroxyl radicals became more significant in the presence of lower amounts of tocopherol. Although this fact may be attributed to differences in reaction rates between PBN and tocopherol, that is, to the faster scavenging of peroxyl radicals by tocopherol, the different scavenging mechanism of both molecules must be also taken into account. In this respect, it is known that tocopherol is able to scavenge two radicals per molecule, while PBN has been suggested to react with peroxyl radicals to form spin adducts that decompose, generating secondary radicals (*36*).

Two major conclusions can be inferred from the results obtained in the present study. First, application of ESR spin trapping to follow oxidative changes during storage of oils containing the spin trap is not suitable for long periods of reaction time. Addition of the spin trap after successive samplings is recommended instead for getting an insight of oxidative changes during storage (*6*). Second, PBN interferes with the oxidation chain, modifying both the pathway and rate of lipid oxidation. The extent to which PBN is able to inhibit lipid oxidation depends on the content of naturally occurring antioxidants. These results should be taken into account even in the application of ESR spin trapping to determine oxidative stability of oils (*15*).

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