Electron Paramagnetic Resonance spin trapping of sunflower and olive oils subjected to thermal treatment: optimization of experimental and fitting parameters.

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Abstract

Sunflower oil (SO) and extra virgin olive oil (EVOO) were heated at 90 °C in the presence of PBN. The radical species formed during thermal treatment, trapped by PBN, were revealed by Electron Paramagnetic Resonance (EPR) spectroscopy, a widely used method to study oils' oxidation.

The effect of the experimental parameters on the intensity of PBN adduct was analyzed with the aim to standardize the spin trapping protocol for oils. A modification of the Boltzmann sigmoidal equation was proposed to fit the experimental points representing the changes of the EPR intensity of the PBN adduct vs. time. The fitting parameters allowed for distinguishing between SO and EVOO and to obtain more reliable Induction Period (IP) values. The fitting parameters and the shape of the curve depend on the diameter of the sample holder. The IP and the time at which maximum intensity is reached, $t(I_{max})$, in thin capillary tubes (IP 35.92 min, $t(I_{max})$ 186 min) were shifted at longer times in comparison with flat cell (IP 69.54 min, $t(I_{max})$ 106 min). The peroxide values (PV) were measured in SO and EVOO samples, with and without PBN, at specific points of the curve and related to the intensity of the EPR signals. PBN inhibits the propagation of the chain reaction and the extent of inhibition is lower in EVOO than in SO maybe due to the effect of phenolic compounds that in SO are lacking. The phenolic compounds are also the responsible for the lower PBN adduct intensities observed in EVOO than in SO. This study further highlights the power of EPR spectroscopy in the evaluation of oil oxidation and provides a guide for EPR experimental and fitting parameters for oils.

Keywords:-Electron Paramagnetic Resonance (EPR), *N-tert*-butyl-α-phenylnitrone (PBN), 5,5-dymethyl-1-pyrroline N-oxide (DMPO), spin trapping, Extra Virgin Olive Oil, sunflower oil.

INTRODUCTION

Oil oxidation, triggered by oxygen, light, and metals, is an important challenge for food industries. Byproducts produced during oil oxidation can reduce the nutritional quality of food and may be harmful to human health. In oils, lipid oxidation is a chain reaction that involves three steps: initiation, propagation, and termination.[1-3] Hydroperoxides are the primary oxidation products. At room temperature, they are relatively stable; however, at high temperatures they rapidly decompose to alkoxyl radicals, which, due to the homolytic β-scission of the carbon-carbon bond, form alkyl radicals.[2] The extent of oil oxidation depends on oil fatty acid composition, the presence of antioxidants, and external factors like temperature and oxygen.[2]

The methods used to study the oil oxidation processes and to assess the oxidation status of oils are based on the analysis of primary or secondary oxidation products. Peroxide value (PV), P-anisidine value (AV), the ThioBarbituric Acid Reactive Substances (TBARS), conjugated dienes, and trienes are the most frequently used. Together with these methods, other methodologies have been developed to analyze, on different oils, the oxidation process. The EPR spectroscopy coupled with the spin trapping method has been employed to study the thermo-induced oxidation processes of plant extracts, alcoholic beverages, food lipids, bulk oils, and oil emulsions.[4-7] Peroxyl and alkoxyl radicals formed during oil oxidation are trapped by *N*-*tert*-butyl-α-phenylnitrone (PBN), the most widely used open-chain nitrone spin trap, forming a PBN radical adduct, that being relatively stable is easily detected by EPR. The EPR spin trapping technique has been applied to olive, peanut, rapeseed, soybean, sunflower, and fish oils to study oils' oxidative stability and correlate it with oils' shelf life.[4, 8-12] In these papers the oxidative stability experiments were carried out by subjecting oils to mild thermal treatments, with temperatures not higher than 70 °C, heating the oils outside the EPR cavity, then measuring the adduct intensity at room temperature.[4, 8-12] The oxidative stability is measured as induction period (IP), defined as the time at which the radical adducts increase suddenly after a slow increase of their concentration. The IP is calculated by a bilinear regression that involves the points with a low PBN adduct intensity and those with a sharp increase of the adduct signal. The drawback is that this calculation is rather subjective and may provide different results.

Other authors investigated, by EPR spectroscopy, the evolution of lipid oxidation products of bulk oils and fatty acids methyl esters during controlled thermal treatments at different temperatures.[13-15] In a kinetic study of the oxidation products of peanut oil treated at 180 °C, Silvagni *et al*.[13] followed the formation of PBN adducts for 40 minutes and determined the contribution of the identified radical species for each point of the kinetic curve. On grape seed oil and on fatty acids methyl esters, some authors described, with the EPR spin trapping technique, the formation and the decomposition of the radical adducts produced during oils thermal treatment at 105 °C.[14, 15] More recently on peanut oil Jiang *et al.*,[11] followed by EPR the formation of PBN radical adducts at different temperatures (60, 80, 90 °C) with the aim to identify the IP value with the Boltzmann sigmoidal equation according to Barr *et al*.[16] In these experiments the changes of

intensity of the PBN-adduct over the time were generally studied for short periods. Even when the formation and decomposition of the radical adducts were studied for a longer period of time, no fitting of the experimental points was provided.

The role of oxygen in oils' oxidation has been widely studied;[3] at temperatures lower than 60 °C the amount of oxygen dissolved depends on oil's composition and on the oxygen partial pressure above oil surface. By contrast at temperature higher than 60 °C the amount of oxygen dissolved decreases with increasing temperatures. In this context the headspace oxygen becomes the supply for reaction with lipids.[3] In EPR experiments, aimed at determining oils shelf life or monitoring the amount of radicals in oils subjected to thermal treatment, the effects of the exposed surface of oil in the sample holder on the changes of intensity of PBN-adduct have never been taken into consideration. As a matter of fact, in papers dealing with the effects of thermal treatments on oil oxidation, authors generically indicated that experiments were performed in EPR tubes, which usually have an internal diameter of 3 or 4 mm[11, 17] or in capillary tubes[5].

In this paper, we followed by EPR spin trapping technique the evolution of PBN adducts over time in SO and EVOO subjected to thermal treatments at 90 °C. The aim is to describe the evolution pattern of the PBN adduct and to fit it with mathematical functions characterized by parameters that distinguish between SO and EVOO and to easily identify the IP value. For this purpose, a modification of the Boltzmann sigmoidal equation is proposed to improve the fitting of the curve and to give more reliable IP values.

Moreover, with the aim to standardize the spin trapping protocol for oils, the effect on the kinetic curve of the shape of the sample holder, where the experiments were performed, has been investigated.

Finally, the peroxide values have been measured in thermally treated samples with and without PBN, and these values have been related to the PBN signal intensity.

MATERIALS AND METHODS

Materials

Hydroxytyrosol (≥98%) and tyrosol (≥98%), oleuropein (≥98%), vanillin (≥99%), vanillic acid (≥97%), p-coumaric acid (≥98%), pinoresinol (≥95%), luteolin (≥98%), apigenin (≥95%) methanol for HPLC (≥99.9%), ethanol, PBN (*N-tert*-butyl-α-phenylnitrone), isooctane and ammonium thiocyanate were purchased from Sigma-Aldrich (Milan, Italy), and acetonitrile by ChemLab (Zedelgen, Belgium). DMPO (5,5-dymethyl-1-pyrroline N-oxide) was purchased from Enzo Life and used without further purification. Ultrapure water was prepared using a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

SO was purchased from the local market, while EVOO was obtained from a local producer. Upon arrival at the laboratory, oils were stored at – 20 °C until analysis.

Identification and quantification of phenolic compounds

The profile and the concentration of phenolic compounds were analyzed in SO and EVOO. Phenolic compounds were extracted according to Deiana *et al*.[18] mixing 4 g of oil sample with 5 ml of methanol/water (8:2, v/v). The mixture was shaken (30 min) and centrifuged at 5000 rpm (15 min), then the polar supernatant was separated and analyzed to determine the concentration of phenols. The extraction process was performed twice and the extracted polar fraction filtered through 0.45 µm PVDV filters. The separation and quantification of phenolic compounds were performed by HPLC using an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311A), a degasser, column thermostat, an auto-sampler (G1313A), and a diode array detector (G1315 B, DAD). A C-18 Luna column (250 x 4.6 mm, 5 µm; Phenomenex, Torrance, CA, USA) with a security guard cartridge (4 × 2 mm) was used for chromatographic separation. The flow rate was set at 1 mL/min, column temperature 30 °C, injection volume was 20 µL. Phenolic compounds were quantified based on their respective standard (mg L^{-1}), whereas secoiridoids and 1-acetoxypinoresinol were quantified using oleuropein and pinoresinol as standards. Results were expressed as mg of phenolic compounds kg^{-1} of oil.

Thermal treatment and EPR spin trapping analysis of SO and EVOO

EPR measurements were carried out with a Bruker EMX spectrometer operating at the X-band (9.4 GHz) equipped with an HP 53150A frequency counter and a variable temperature unit ER 4111 VT. Spectra were acquired with Bruker WinEPR Acquisition Version 4.33 and simulated with Bruker WINEPR SimFonia Version 1.26.

SO and EVOO containing the spin trap PBN were heated at 90 °C inside the EPR cavity.

Five µL of a 2.5 M PBN solution in absolute ethanol were dried under a nitrogen flow to avoid any interference of ethanol during spin trapping experiment. Hundred µL of oil were mixed with the solid PBN. To evaluate the effect of the shape of the sample holder on the kinetic of the PBN adduct formation, a flat cell and two capillary tubes differing for the diameter of the capillary were tested. The flat cell was a cylindrical quartz tube with an inner diameter of 2.5 mm (exposed oil surface 5 mm2) with a 25 mm terminal flat part at the bottom. The large capillary tubes had a diameter of 1.6 mm and an exposed oil surface of 2 mm2, whereas the thin capillary tubes had a diameter of 1.1 mm and an oil exposed surface of 1 mm2. In each sample holder, 100 µL of oil with PBN were transferred.

EPR spectra were acquired every 5 minutes for 5 hours. The EPR instrument was set under the following conditions: modulation frequency 100 kHz, modulation amplitude 0.106 mT, receiver gain 5 x 104, microwave power 20 mW (which is with the ER 4119HS cavity, below the saturation limit), resolution 1024 points, sweep time 167.772 s, time constant and conversion time 163.84 msec. The selected values of time constant, sweep time, resolution, and sweep width allow to resolve the narrowest line corresponding to 0.049 mT. These parameters were optimized with preliminary analysis on different oils.

The intensity of the PBN-adduct was estimated from the double integration of the spectra and was plotted against time.

The experimental points followed a double sigmoidal growth and decrease pattern. The first part of the curve, from the beginning of the experiment to the achievement of the maximum intensity, was fitted with a modified Boltzmann sigmoid equation. The modified equation, proposed here for the first time, is the following:

$$Y = Bottom + (Top - Bottom)/(1 + \exp(V_{50} - x)/slope) + rise*x$$

The second part of the curve that includes the points from the maximum of intensity to the end of the experiment was fitted with a reverse Boltzmann modified sigmoid equation:

$$Y = (((Top - Final)/(1 + \exp(x - Mid_2)/slope)) + Final) - decline*x$$

The experimental points were fitted with GraphPad Prism8 for Windows software (GraphPad Software Inc. La Jolla, CA92037, USA).

DMPO spin trapping experiments

100 µL of a DMPO solution (0.125 mM) in absolute ethanol were dried under a nitrogen flow. One hundred µL of SO were mixed with the spin trap, placed in a flat cell, and inserted into the EPR cavity heated at 90 °C. EPR spectra were acquired every 5 minutes for 4 hours.

Effect of PBN and oxygen availability on lipid oxidation

To draw a more complete picture of the reactions taking place during oil's thermal treatment and to evaluate the effect of PBN on lipid oxidation, SO and EVOO were heated in a thermostatic bath set at 90 °C. One hundred and fifty µL of oil with and without PBN were placed in 1.5 ml safe-lock tubes. For samples with PBN, aliquots of 7.5 µL of a 2.5 M PBN solution in absolute ethanol were dried under a nitrogen flow and mixed with the oil. At fixed time intervals, oil samples were analyzed for the concentration of peroxides, spectrophotometric constants, and PBN adduct concentration. SO and EVOO were heated for 30 h. For sunflower oil, samples were taken at 0.5, 1, 2, 3, 4, 6, 12, 15, 20 and 30 hours, whereas for olive oil they were withdrawn at 6, 12, 15, 20, and 30 hours.

To evaluate the effects of oxygen availability and oil surface exposed to air (S) on peroxide values and on PBN adduct intensity, SO was placed in 1.5 mL or 0.5 mL sample tubes, with or without PBN (final concentration 125 mM). Tubes of 1.5 mL capacity and an air-exposed surface of 33.18 mm^2 were filled with 200 µL of oil, whereas 0.5 mL tubes with an air-exposed surface of 25.50 mm^2 were filled with 150 µL of oil.

In half of the sample tubes (both 1.5 and 0.5 mL), the headspace volume has been reduced by inserting a small metal cylinder to limit oxygen availability. All tubes were heated at 90 °C for 15 hours.

Spectrophotometric detection of peroxide value and conjugated dienes

Peroxides were determined according to the International Dairy Federation method described by Shantha *et al*.[19] The method is based on the oxidation of Fe(II) to Fe(III) by hydro-peroxides and on the formation of a red Fe(III)-thiocyanate complex. Briefly, about 10 mg of EVOO or SO were mixed with 9.8 mL of chloroform-methanol 7:3 (v/v) and vortexed. Fifty µL of ammonium thiocyanate solution (394 mM, 30 g in 100 ml of H_2O) were added to the mixture, vortexed, then 50 µL of a $FeSO_4 \cdot 7H_2O$ (18 mM, 30 g in 100 ml of H_2O containing 2 ml of HCl 10 M) were mixed. After 15 minutes, the absorbance was measured at 507 nm with a Perkin-Elmer Lambda 35 spectrophotometer. Peroxide value was expressed as micro-equivalents of peroxides$\cdot g^{-1}$ of oil, based on a calibration curve built using $FeCl_3 \cdot 6H_2O$ as Fe(III) source (Fe(III): 6.1×10^{-5} – 4.6×10^{-4} M; $R^2 = 0.99$).

Peroxide values were also determined in SO containing PBN at the final concentrations of 62.5, 125, and 250 mM. Sample tubes (1.5 mL capacity) containing 100 µL of SO and PBN were heated at 90 °C for 5 hours (300 min), then the peroxide value was determined

Conjugated dienes and trienes absorb in the ultraviolet region of the spectrum and are frequently used to determine the oils spectroscopic index (K232 and K268). The spectroscopic index was assessed according to the method proposed by the International Olive Oil Council.[20] Samples were prepared using 1% w/v solutions of the oils in isooctane. The specific absorbance values at 232 and 268 nm were recorded with an Agilent spectrophotometer (8453 UV-Visible Spectrophotometer, Agilent Technologies, Palo Alto, CA, USA) against a blank of pure isooctane in 1 cm optical path-length UV-cuvettes. Since PBN absorbs in the ultraviolet region of the spectrum, the absorbance of samples containing PBN was corrected for the absorbance of an equimolar PBN solution in isooctane.

Statistical analysis

Statistical analysis was performed with GraphPad Prism8 for Windows software (GraphPad Software Inc. La Jolla. CA92037, USA). A one-way ANOVA was used to compare the results of peroxides, conjugated dienes, and kinetic parameters calculated from sunflower and olive oils kinetic curves. Three replications were performed for each analysis. In addition, means separation was calculated by Tukey's test or t-test at $P \leq 0.05$.

RESULTS

Oil phenolic composition

EVOO is rich in phenolic compounds (Table S1), as expected, in the SO no phenolic compounds were detected. Oleocanthal (p-HPEA-EDA), oleacin (3,4-DHPEA-EDA), and the aglyconic derivatives of oleuropein (3,4-DHPEA-EA) and ligstroside (p-HPEA-EA) represent over 90% of the total phenols detected in olive oils. These compounds own antioxidant properties and are responsible for EVOO health properties.[21] Other phenolic compounds such as hydroxytyrosol (3,4-DHPEA), tyrosol (p-HPEA), 1-acetoxypinoresinol, vanillin, phenolic acids (*o*- and *p*-coumaric and vanillic acid), and flavonoids (luteolin and apigenin) are also present.

Thermal treatment of sunflower oil at 90 °C with PBN and DMPO

PBN (*t*-butyl-α-phenyl nitrone) is by far the most frequently employed open-chain nitrone spin trap. It is used to trap 1-hydroxyethyl radicals in wines, beer, or ethanolic plant extracts and lipid-derived radicals in oils.[7, 22-24] Figure 1A shows the EPR spectra of PBN adduct generated during the thermal treatment of sunflower oil at 90 °C. When different radical species are contemporaneously present and trapped by PBN, their identification is not easy because the variation of the spectroscopic parameters of the spin adducts is little.

The only observable coupling constant, besides that of the nitrogen itself, is that with the β hydrogen. These two coupling constants vary in a small range a_N 1.415 – 1.496 mT and a_H 0.210 – 0.375 mT for alkyl/alkoxyl radical adducts. Moreover, there is no ~~any~~ evident trend in relation to the trapped radical.[25]

Yamada *et al*.[26] reported the a_N and a_H values of 13.44 and 1.63 G for the adducts of peroxyl radical of methyl linoleate; for the corresponding alkoxyl radical, the values were 14.22 and 2.10 G, and finally, for the carbon-centered radical, the values were 15.03 and 2.83 G.

Figure 1

During the thermal treatment of sunflower oil at 90 °C in the flat cell, three different spectra are distinguishable, although their hyperfine constants vary very little (Figure 1A). The first spectrum (black line in Figure 1) obtained after 36 min has been satisfactorily simulated with a_N 1.488 mT, a_H 0.205 mT, and *g* 2.00610; the second one (in red line Figure 1A), obtained after 106 min, has a_N 1.494 mT, a_H 0.210 mT and *g* 2.00614; finally, the species present after 146 min (in blue in Figure 1A) is characterized by a_N 1.490 mT, a_H 0.215 mT and *g* 2.00617. The selected time intervals correspond approximately to the lag time (36 min), to the time at which the maximum EPR intensity was reached (106 min), and to the final time (146 min), see Figure 2 and Table 2. Thus, the parameters of these three species are very similar, but, as we previously mentioned, it very hard to distinguish between the various types of adducts based on these variations.

245 At the beginning of the experiments performed with PBN, a species with a_N 1.4950 mT, a_H 0.4900 mT, and g
246 2.00555 has been identified (see Figure S2); this species could be an adduct of MNP (2-methyl-2-
247 nitrosopropane) derived from the decomposition of the PBN-OOR adduct, as reported in the literature.[5, 15]
248 Vicente *et al*.,[15] in the thermal treatment at 105 °C of fatty acids methyl esters, detected three adducts:
249 peroxyl and alkyl adducts of PBN and one of MNP, a degradation product of PBN. The importance of
250 peroxyl adduct was more significant than the other adducts at lower temperatures, while the alkyl adduct
251 increased its concentration when oxygen was lacking.
252 To identify the radical species produced, the sunflower oil was heated at 90 °C in the presence of DMPO
253 instead of PBN. With the cyclic spin trap DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), the variation of the
254 splitting due to the β hydrogens is much larger and depends on the nature of the trapped radical being ≥ 20
255 G for the alkyl radicals and between 0.6 and 0.8 mT for the alkoxyl radicals.
256 Unlike the adducts with the PBN, those with the DMPO give more information on the nature of the trapped
257 radical. However, there are two problems connected with the use of this spin trap: i) the first is that PBN has more
258 frequently been used in the literature studies of oils; ii) the second is that the adducts formed with DMPO are less
259 stable in comparison with those of PBN and in fact, the intensity of the EPR signals in the first case is much lower. In
260 agreement with our results, Xie *et al*.[17] showed that it is more easy with DMPO, in comparison with PBN, to distinguish
261 between different types of adduct formed by peroxyl, alkoxyl and alkyl radicals formed during the thermal treatment
262 at 90 °C of oleic and linoleic acids. Two main adducts can be identified (see Figure 1B).
263 These DMPO-adducts were simulated with the following parameters: a_N 1.4876 mT, a_H 0.2081 mT and a_N
264 1.425 mT, a_H 2.010 mT while g 2.0078 for both (see Figure 1Bb).
265 The DMPO-adduct with a_H 2.01 mT can be identified as an alkyl adduct, probably formed after the
266 displacement of the allylic hydrogen on the carbon adjacent to one (or two) double bond(s) of oleic (or
267 linoleic) acid(s). Alberti and Macciantelli[25] identified a species with an a_H value in the range 6-8 G assigned
268 to the trapping of an alkoxyl radical. In our experiment, this species was not detected, but we identified a
269 species with a_H 0.2081 mT. Similarly, other authors[17, 27] observed, during the thermal oxidation of oleic acid,
270 a DMPO adduct having a_H 0.212 mT and 0.19921 mT, respectively, and interpreted this splitting as due to
271 the γ hydrogen of an oxidized DMPO adduct where the β hydrogen was lost. Besides the species with a_H
272 0.2081 mT, we observed another species with only three lines. This species, more likely an oxidized DMPO
273 adduct where the β hydrogen has been lost, increases its intensity up to 11 min, then decreases without
274 disappearing and can be distinguished again at the end of the experiment (a_N 1.400 mT, g 2.00553, see
275 Figure 1Ba).
276 Other minor adducts are present at the beginning of the experiment, but these quickly disappear and are
277 difficult to be identified (see peaks indicated by * in Figure 1Ba), similarly as observed with PBN.
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Kinetic analysis of PBN adduct

Sunflower oil thermal treatment: fitting of the kinetic curve

SO was used as a model to study the kinetic of PBN-adduct of oils subjected to thermal treatment at 90 °C. Figure S3 reports the EPR intensity of the PBN-adduct as a function of time. The resulting pattern can be associated with a double sigmoidal curve. This kinetic pattern described by Caglar *et al*.[28] is quite common in biological systems and can be considered as the sum of two phases: an exponential increase followed by a decay. In the oxidation process of sunflower oil, at t_0 the beginning of the experiment, the intensity of the adduct is rather low and increases continuously until the intensity of the signal rises sharply. After the lag time, the intensity of the PBN-adduct increases exponentially until the achievement of a maximum beyond which a decay of the signal intensity is observed. On oil, the changes of PBN-adduct intensity over time were only partially studied without analyzing in detail the shape of the resulting curve [13]. On wine, the changes of the 1-hydroxyethyl radical (HER) –POBN adduct over time were followed from the beginning of the experiment to achieving the maximum adduct intensity. Wines were classified according to the parameters extracted from the experimental curve: I_{max} (the maximum intensity of the adduct) and $r_{POBN\text{-}HER}$ (the rate of increase of the adduct).[24] Similarly, on soybean oil, the PBN-adduct signal growth was suggested as a tool to measure oil rancidity.[16]

The kinetic pattern of the PBN spin trap adduct described in this paper for SO is similar to that described by Samouilov *et al*.[29] for DEPMPO-OOH adduct (5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide superoxide adduct). According to these authors, the kinetic pattern of the radical adduct over time is the result of two different and independent processes: the formation and the decomposition of the adduct. In the formation process, the accumulation of the adduct over time depends on the number of radicals produced in the system and trapped by the spin trap if no other reactions quench the radical. In complex systems like oils, beer, wine, or plant extracts, it is likely that the endogenous antioxidants quench the radicals thus competing with the spin trap and slowing down the rate of adduct formation. The adducts have limited time stability, lower than the experiment duration, so once formed, the adducts decay relatively rapidly in no EPR detectable products.[30] For this reason, the concentration of the adduct measured in the kinetic curve is the result of a continuous equilibrium between formation and decay. On vegetable oils, the heat-induced radical formation is generally characterized by an initial slow adduct increase phase, whose length depends on oils, temperature, and the presence of antioxidants, followed by a sharp increase of the adduct signal.[11, 16, 31] The occurrence of the two phases is essential to calculate the IP (induction period), a parameter correlated to oils oxidative stability.[4, 11, 32]

Recently Jiang *et al*.[11] calculated IP fitting the experimental points with the Boltzmann function according to Barr *et al*.[16] Due to its shape, the Boltzmann sigmoidal equation fits the part of the kinetic that describes the formation of the adduct, but it is unable to describe the last part of the kinetic, which concerns its decomposition. Moreover, even in the first part, the Boltzmann sigmoid equation could not match the

experimental points since the intensities of the adducts constantly increase with time, while the asymptote of the Boltzmann sigmoid equation is parallel to the x axis. To overcome this problem, we propose a modification of the Boltzmann sigmoid equation. The modified Boltzmann sigmoid equation is described in the materials and methods section. The new parameter introduced in the equation is the rise, which considers the slope of the experimental points of the first part of the curve (Figure S4). The rise is not directly involved in the calculation of the IP but its introduction in the curve equation determines a modification of the parameters used to calculate it. As can be observed in Figure S4 and in Table 1, the better fitting of the experimental points corresponding to the slow adduct increase phase provided by the new equation resulted in significantly higher IP values. The parameters used for the fitting are reported in Table S2.

In previous papers dealing with the determination of the oxidative stability of oils, the kinetic of the PBN adduct was followed for a short period of time without reaching the point of maximum intensity [11]. In the present paper, for the first time, the kinetic was followed beyond the point of maximum intensity until the achievement of equilibrium. Similarly to DEPMPO-OOH adduct kinetic described by Roubaud *et al*.[30] and Samouilov *et al*.,[29] the last part of the kinetic describes the decomposition of the adduct. According to these authors, the decay of the spin adduct does not follow any particular decay law but depends on the experimental conditions of the reaction. In this paper, the differences observed between EVOO and SO might be due to the different oil compositions rather than to the experimental conditions since the same conditions were applied to both oils. The experimental points of the decomposition part of the kinetic were fitted with a reverse Boltzmann sigmoid equation, which is reported in the materials and methods section. Even in this case, a correction for the slope of the last points has been applied to the equation. These parameters were used to calculate the width of the kinetic curve and the final time that is analogous to the IP.

Sunflower and olive oil kinetic parameters

Table 1 reports the IP values calculated with the Boltzmann and the modified Boltzmann equations and the curve width and final time calculated from the fitting of the Boltzmann reverse equation for EVOO and SO. Both in EVOO and SO, the Boltzmann equation significantly underestimates the IP. As already highlighted, the introduction of the new parameter in the Boltzmann equation is not involved in calculating the IP, but it slightly modifies the parameters used to calculate it. The IP and the other parameters extracted from the PBN-adduct intensity curve vs. time were significantly different between SO and EVOO. As expected, the IP of EVOO is significantly higher than that of SO. Papadimitriou *et al*.[32] calculated the induction period (lag time) of some Greek olive oils and observed a strong correlation between the oxidative stability and the concentration of polyphenols, whereas no correlation was found with tocopherols. Our results agree with

the hypothesis of polyphenols' involvement in enhancing oils' oxidative stability since no phenolic compounds were detected in SO. At the same time, the different lag time of EVOO and SO may be the result of a different oxygen diffusivity into the oil since, as it will be discussed later, the induction period (i.e., lag time) was demonstrated to be indirectly proportional to the oxygen concentration in the oil.[33] Along with the lag time, even the curve width and the final time, calculated from the part of the kinetic related to the decomposition of the adduct, can discriminate between SO and EVOO.

In the SO, the chain reactions occurring during lipid oxidation run out faster than in the EVOO, as demonstrated by the greater slope of the curve and the narrower curve width (Figure S5).

A comparison of the slope of the two curves shows that with olive oil, the increase of the adduct signal intensity is slower than with sunflower oil, probably because polyphenolic compounds compete with PBN for the quenching/trapping of radical species. The lower maximum intensity value observed for olive oil could be explained in the same way. Therefore, the amount of antioxidants in oils affects the slope of the kinetic curve and the value of its intensity maximum. In sunflower oil, no phenolic compounds were detected, whereas olive oil was rich in secoiridoids and derivatives that are responsible for its high antioxidant properties.

Effect of the sample holder on the kinetic of PBN adduct in SO

The presence of an IP and the shape of the kinetic curve depend on the conditions applied during the experiment. Temperature is one of the main experimental factors affecting it. On grape seed oil, Vicente *et al*.,[14] studying the effect of temperature on the formation and decomposition of PBN adduct, observed that the adduct half-life significantly decreased with increasing temperature, and at temperatures higher than 180 °C, the stability of the adduct was too low to allow any time-course experiment. The same authors, plotting against time the EPR spectral intensities of oils heated at temperatures ranging from 105 to 180 °C, observed increasing rates of adduct formation with rising temperatures, suggesting the involvement of different oil-derived radicals in the reaction.[14] Similarly, Jiang *et al*.[11] studied the effect of temperature on PBN-adduct intensity during EPR experiments demonstrating that the higher the temperature, the greater the intensity of the adduct. In this paper, the choice of the temperature of 90 °C was based on preliminary results on oils and on other plant extracts;[7] agreeing with Jiang *et al*.,[11] who identified 90 °C as the best choice for IP determination in EPR experiments following the evolution over the time of the PBN adduct intensity. It is important to notice that the experiments cited above were performed heating the oil or the extracts in situ in the spectrometer cavity, while other experiments on olive or soybean oils were performed heating the oils outside the cavity, then measuring the adduct intensity at room temperature.[4, 9, 34]

Even if the temperature can increase lipid oxidation rates, alone is not sufficient to affect oils' oxidative stability. According to several authors, oxygen is the main responsible for the oxidation process of edible oils;[3, 33] in particular the amount of dissolved oxygen that can react with lipids and the oxygen reacting at oil surface are the main responsible for lipid oxidation.

Figure 2 shows the kinetic pattern of SO analyzed in three different sample holders and heated at 90 °C inside the EPR cavity. The IP is not easily identifiable in the flat cell as the adduct increases rapidly from the beginning of the experiment. By contrast, the adduct grows more slowly in capillary tubes: the narrower the capillary, the more slowly the adduct grows. The kinetic curves in Fig. 2 also differ for the maximum intensity of the adduct (I_{max}). However, the Imax values obtained with the three sample holders cannot be directly compared since the volume of oil contained inside the EPR cavity decreases in the order: flat cell > large capillary tube > thin capillary tube, because of the different diameter of these sample holders (see Experimental).

The SO contained in flat cell had a significantly shorter IP than the same oil contained in capillary tubes (Table 2). As can be noticed in Table 2, the IP increased in the order: flat cell < large capillary tube < thin capillary tube. Besides the IP, the sample holder affected the time at which I_{max} is reached: oils inside the flat cell reached it earlier than those in capillary tubes. At the same time, the shape of the sample tube affects the parameters that describe the decomposition of the adduct in the kinetic curve. The width of the curve of oil in thin capillary tubes was significantly larger than that in the other two sample tubes. Similarly, the "final time", which can be considered the opposite of the IP, was significantly higher in thin capillary tubes than in flat cells or large capillary tubes.

As explained above, oxygen has a critical role in free radical generation in heated lipids.[15] In degassed samples of linolenate, linoleate, and oleate heated at 105 °C, the shape of the spectral intensities over time were similar to those of aerated samples but with lower rates of generation and with lower spectral intensities.[15] Šimon *et al*.[33] observed that the length of the induction period was inversely proportional to the concentration of oxygen in the oil. According to these authors, oils' oxygen concentration depends on the equilibrium between oxygen amount that reacts with lipids and the one present in the headspace. At equilibrium, the oxygen concentration in the oil is determined by the diffusion coefficient, an oil characteristic, and the oils' surface (S) exposed to air. In the absence of mechanical agitation, diffusion is the only way for oxygen to be dissolved in oils. However, at temperatures higher than 60 °C, the oil oxygen concentration decreases as a consequence of the enhanced oxidation rates and the low oxygen solubility. Our experiments were carried out all at the same temperature (90 °C) and with the same oil volume (100 µL), but the oil's surface exposed to oxygen was different among sample tubes. In the flat cell, oil had an exposed surface of 5 mm^2, while in large and thin capillary tubes, it had a surface exposed to air of 1 and 2 mm^2; thus, the oil to air interface seems the key point for the different curve shapes observed for the

heated oils. The greater the contact surface with the air, the greater the amount of oxygen reacting on the oil surface and the amount of adduct formed.

Figure 2

Table 2

Effect of oxygen availability on the formation of PBN adduct in SO

To evaluate the effect of oxygen availability and oil surface exposed to air on peroxide value and on PBN adduct intensity, sunflower oil was heated at 90 °C for 15 h in 1.5 and 0.5 mL volume safe-lock sample tubes. In half of the sample tubes, the headspace volume was reduced to limit oxygen availability. In a recent paper, Velasco *et al*.[12] reported that low oxygen diffusion from the headspace of the EPR tube and the low surface-to-volume ratio contributed to limit oxidation. However, the temperature has a great effect on lipid oxidation rates and on oxygen solubility in bulk oils. For this reason, the results of this study cannot be directly compared with ours because they were obtained at 40 °C.

In SO heated at 90 °C with PBN, the headspace volume reduction had no effect either on peroxide values or on PBN adduct intensities measured by EPR. By contrast, in samples without PBN, SO in full volume tubes had significantly higher peroxide values than oil in reduced volume tubes (Figure 3A). Similar results were achieved when SO was heated with or without PBN in 0.5 mL safe-lock tubes (Figure 3C, 3D). In this case, however, in SO with PBN, some differences, albeit not significant, can be observed between full and reduced volume. The effect of oxygen availability is more clear observing the values of the adduct intensity: when the availability of oxygen is higher, the intensity of the adduct is significantly higher.

When PBN is present in the reaction mixture, it hinders the radicals' chain reaction by trapping lipid radicals and likely reduces the effect of oxygen on the oxidation process. There is competition between oxygen and PBN for the reaction with free radicals, which affects the extent of oxidation.

The reaction between radicals and PBN is favored over the reaction with oxygen because at the temperature of 90 °C oxygen solubility is rather low. This scenario changes when PBN is not present, so the reaction is controlled by oxygen availability only. As highlighted by Crapiste *et al*.[35] if oxygen supply is unlimited, oxidation rates depend on oil surface exposed to air and sample volume. In that paper, oil was heated at 47 °C, so the oil's surface exposed to air determined the amount of oxygen which dissolved in the oil volume. At higher temperatures (90 °C), as in our experiment, the oxygen solubility in oil is much lower, and the oxidation processes take place at the air/oil interface.

To evaluate the effect of the exposed surface on peroxide amount and on PBN adduct intensity, we paired off data of 1.5 mL sample tubes with those of 0.5 mL in both full volume and reduced volume. Under full oxygen availability conditions (full volume), no differences in peroxide value and EPR intensity were

observed between oils with a different exposed surface, neither with PBN nor without (supplementary materials Figure S6A, S6B). Conversely, Crapiste *et al*.[35] observed, in SO stored at 47 °C for 60 days, an enhancement of peroxide values with increasing S/V ratios.

In our experiments, when oxygen availability was reduced (reduced volume), the oils with a higher exposed surface (1.5 mL sample tubes) had significantly higher PBN adduct signal intensity. When there are no restrictions on oxygen availability, the concentration of dissolved oxygen in bulk oil is lower than the amount available in the headspace because, at 90 °C, its solubility is low.

On the contrary, when oxygen availability is limited, oxygen solubility in oil is likely higher than its availability in the headspace, so the exposed surface regulates oxygen uptake: the lower the exposed surface, the lower is the amount of oxygen reacting with lipids. Thus the PBN adduct signal intensity decreases.

Figure 3

Peroxides, conjugated dienes and PBN-adduct intensity of SO and EVOO treated at 90 °C

To understand what was happening during the thermal treatment at 90 °C, we simultaneously measured the peroxide value, the adduct's EPR intensity, and the conjugated dienes and trienes of SO and EVOO at different time intervals during a time-course experiment (Figure 4). The EPR spectra were obtained collecting 100 μL of oil containing PBN, kept at 90 °C in 1.5 mL Eppendorf safe-lock tubes for the desired time interval; on the same samples, PV and conjugated dienes and trienes were also measured (Figure 4). Peroxide values, dienes, and trienes were also measured in samples subjected to the same thermal treatment but without the addiction of PBN. While designing the experiment, the choice of the time intervals for the analysis was based on the kinetic curve of the PBN adduct measured in the flat cell heated at 90 °C into the EPR cavity. In particular, the points corresponding approximatively to the IP, the V_{50}, the I_{max}, and the point of equilibrium in the final part of the kinetic curve have been chosen. However, as shown in Figure 4, the reaction time in 1.5 mL safe-lock tubes is much longer than that measured in the flat cell or in the capillary tubes. For this reason, we decided to continue with the thermal treatment beyond 5 hours, the time at which the kinetic EPR experiment was ended. Results show that even after 1800 min (30 hours), the maximum of the EPR intensity was not reached, while in the flat cell, the maximum was at *ca*. 106 min. This further confirms the importance of the sample holder and the oil exposed surface to air during the oil thermal treatment.

As pointed out by some authors,[4, 34] the use of spin trapping agents like PBN during oil accelerated ageing interferes with the free radical mechanism of oxidation since, once trapped by PBN, the free radical species cannot propagate the chain mechanism inhibiting the oxidation process.

Figure 4

This is clear comparing the peroxide values measured in sunflower and olive oils with and without PBN. The presence of PBN significantly decreased the peroxide values from the beginning of the experiment, but its effect grew over time. The inhibiting effect of PBN was observed in SO, rapeseed, and fish oils heated at 40 °C by Velasco *et al*.[34] According to these authors, the extent of PBN inhibition was dependent on the type of oil. In particular, they observed that the effect of PBN on lipid oxidation was related to oils' oxidative stability: the lower the oxidative stability, the larger the effect of PBN on oxidation rate. Even at 90 °C, PBN shows an inhibiting effect. Moreover, our results on EVOO confirm the differences among oils. Up to 6 hours of heating treatment at 90 °C, in EVOO, no differences could be detected between samples with and without PBN. By contrast, in SO PBN inhibited lipid oxidation from the beginning of the experiment.

Velasco *et al*.[34] ascribed the extent of the PBN inhibiting effect to the presence of tocopherols, showing that the PBN inhibiting effect of lipid oxidation was higher in oils with low amounts of tocopherols in comparison with the added PBN. In the literature is reported that the amount of tocopherols in SO is nearly four-fold higher than in olive oil.[36] For this reason, we should expect a higher PBN's inhibiting effect in EVOO. It is evident from Figure 4A and 4C that the PBN inhibiting effect is higher in SO because in this oil is larger the difference in PV values in samples with and without PBN, while the EPR intensity in SO is higher than in EVOO (the higher the PBN inhibiting effect, the higher is the intensity of the EPR signals of the PBN adducts). Tocopherols alone do not explain the inhibiting effects of PBN observed on SO and EVOO. Therefore, other polyphenolic compounds, of which EVOO is rich (see Table S1), compete with PBN and decrease its inhibiting effect of lipid oxidation.

To understand whether the PBN concentration might increase the PBN inhibiting effect of lipid oxidation, peroxide values were measured in sunflower oil containing PBN at different concentrations and heated at 90 °C for 5 hours (Figure S7). The results indicate that PBN concentration had no effect on peroxide value, at least in the range of concentrations (62.5 – 250 mM) examined here.

The evolution of the conjugated dienes confirmed the peroxide values obtained for sunflower and olive oils (figure 4B and 4D). Conjugated trienes were not affected by either thermal treatment or PBN (data not shown). The values of conjugated dienes are an index of secondary products of lipid oxidation. Their levels are associated with the oxidative stability of the oils: high levels of dienes reflect low oxidative stability, as confirmed in this work, in which EVOO had the lowest values. In the present study, conjugated dienes of SO and EVOO increased during the thermal treatment. The presence of PBN diminished the values of conjugated dienes with a different extent of depletion between oils.

In this paper, for the first time, an analysis of the kinetic curve of the PBN adduct was performed. When sunflower and virgin olive oil are heated at 90 °C in the presence of PBN, the generated radical species could react with the antioxidants or be trapped by spin trapping molecules (PBN or DMPO) originating relatively stable radical species, which can be easily detected by EPR. The kinetic curves representing the EPR intensity of the PBN adducts vs. time can be divided in two parts considering the formation of the adduct and its decomposition. Both adduct formation and decomposition were fitted with two modified Boltzmann sigmoidal equations.

The new equation, proposed here for the first time, allows obtaining a more realistic value of the lag time (or final time) because the asymptotes of the Boltzmann sigmoidal equation are not perfectly horizontal, but slightly "rise". The lag time, the curve width and the final time, calculated from the fitting parameters allow for distinguishing SO from EVOO.

The experimental conditions affecting the kinetic analysis of the PBN adduct were also studied. In this paper it was demonstrated that the same oil heated in different sample holders produce kinetic curves of different shape, thus affecting the parameters proposed to discriminate oils. This aspect should be carefully taken into account when comparing results from different laboratories. In experiments performed at 90 °C, where the oxygen solubility is low, a key factor which influences the experimental results is the oil surface exposed to air. In these conditions the reactions with oxygen take place at the oil/air interface and not in the bulk oil where the oxygen solubility is limited. The results obtained in this work allow for reaching a better knowledge of the factors which influence thermal treatment coupled with spin trapping experiments. The proposed Boltzmann modified sigmoidal equation can be applied to other fields, like the study of lag time of beers, exploring the whole kinetic curve and not only the first part, which allows for determining the lag time value.

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Figure captions

Figure 1. A: EPR spectra measured at a) 36 min; b) 106 min and c) 146 min during the thermal treatment of sunflower oil at 90 °C in the presence of PBN in the flat cell; B: Experimental and simulated EPR spectra of sunflower oil containing DMPO obtained during thermal treatment at 90 °C. a) experimental (full line) and simulated (dotted line) spectrum at 11 min; b) experimental (full line) and simulated spectra (dotted lines) at 81 min. Asterisks indicate minor adducts present at the beginning of the experiment.

Figure 2. Kinetic curves of sunflower oil heated with PBN (125 mM final concentration) at 90 °C and placed in a flat cell (•), large capillary tubes (○) and thin capillary tubes (▲).

Figure 3. Peroxide values and PBN adduct intensity of sunflower oil heated for 15 h at 90 °C in safe-lock sample tubes of 1.5 (A and B) and 0.5 mL (C and D). White bars indicate tubes where internal volume has been reduced, while black bars indicate tubes without any volume reduction. Bars with different letters are statistically different according to Tukey's test ($P \leq 0.05$).

Figure 4. Evolution of peroxide value (A and C, fuchsia and black lines), EPR intensity (PBN adduct signal intensity (AU)) (A and C, purple line) and conjugated dienes (B and D) during the time course of thermal treatment of sunflower oil (A and B) and Olive oil (C and D) at 363 K in 1.5 mL safe-lock sample tubes. In all graphs fuchsia lines indicate oil samples without PBN whereas black lines indicate oil samples with the addition of PBN.

Table 1. Kinetic parameters calculated from the kinetic curves of sunflower and olive oil heated with PBN (125 mM final concentration) at 90 °C in flat cell.

	Kinetic parameters											
	Adduct formation								Adduct decomposition			
	IP				Slope				Curve width		Final time	
Oils	Boltzmann		Boltzmann modified		Boltzmann		Boltzmann modified					
Olive	47.71 ± 4.7	bA	60.36 ± 5.2	aA	37.74 ± 6.1	aA	32.10 ± 5.1	aA	101.63 ± 3.8	A	228.6 ± 13.3	A
Sunflower	22.63 ± 3.5	bB	35.92 ± 2.7	aB	26.43 ± 2.6	aB	18.68 ± 2.9	bB	72.64 ± 4.5	B	144.00 ± 0.1	B

Data are presented as mean ± SD. Capital letters relate to differences between oils while lower case letters relate to differences between lag times calculated with Boltzmann and modified Boltzmann equations within the same oil. Differences were calculated according to Student's *t*-test ($P \leq 0.05$).

1 Table 2. Parameters extracted from the kinetic curves of sunflower oil heated at 90 °C and
2 placed in different sample tubes.

Sample tubes	**IP (min)**	**curve width (Mid_2 - V_{50})**	**Final time (min)**
Flat cell	35.92 ± 2.7 **c**	72.64 ± 4.5 **b**	144.00 ± 0.1 **b**
Large capillary tubes	47.08 ± 5.2 **b**	78.18 ± 2.4 **ab**	181.77 ± 16.2 **b**
Thin capillary tubes	69.54 ± 0.3 **a**	89.10 ± 0.1 **a**	231.08 ± 1.7 **a**

3 Each value is the mean of three replicates ± standard deviation. Means with different letters within the same column are statistically different by
4 Tukey's test (P≤ 0.05).

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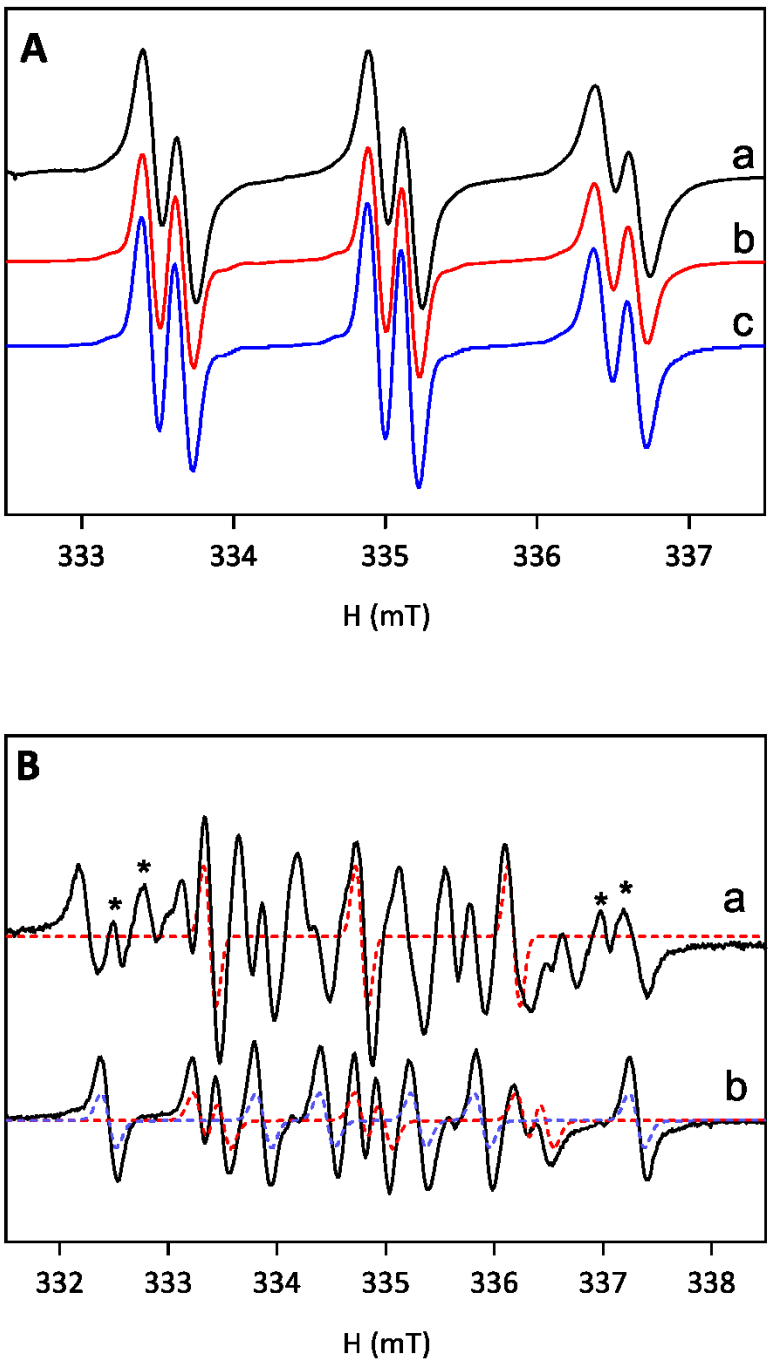

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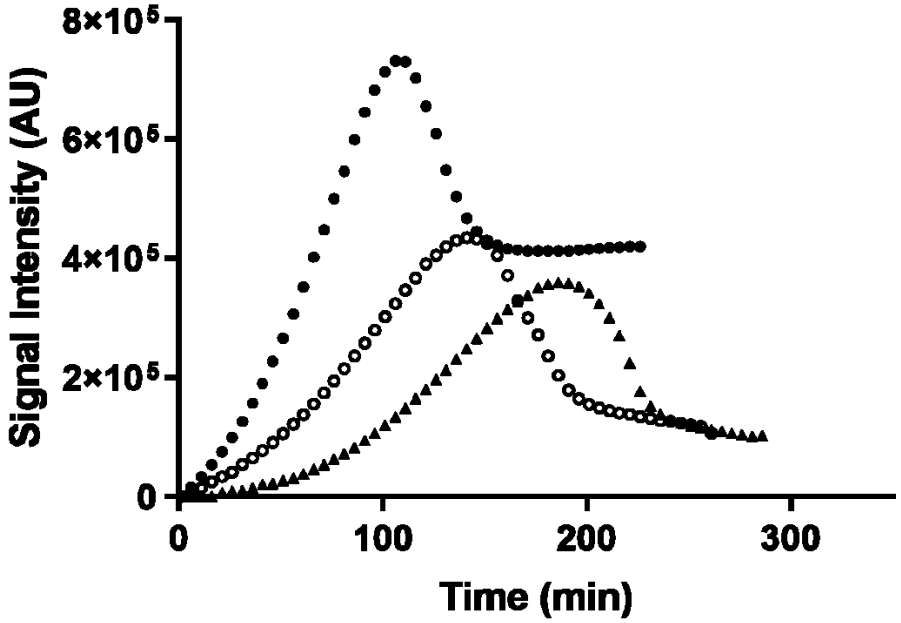

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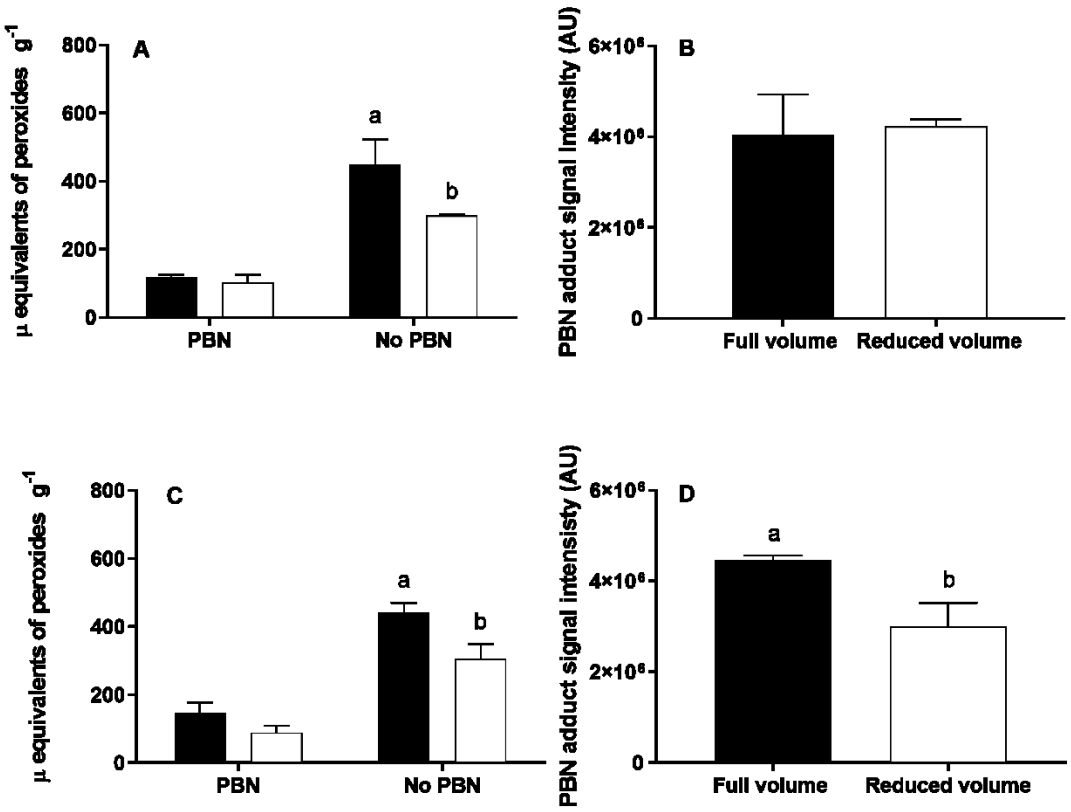

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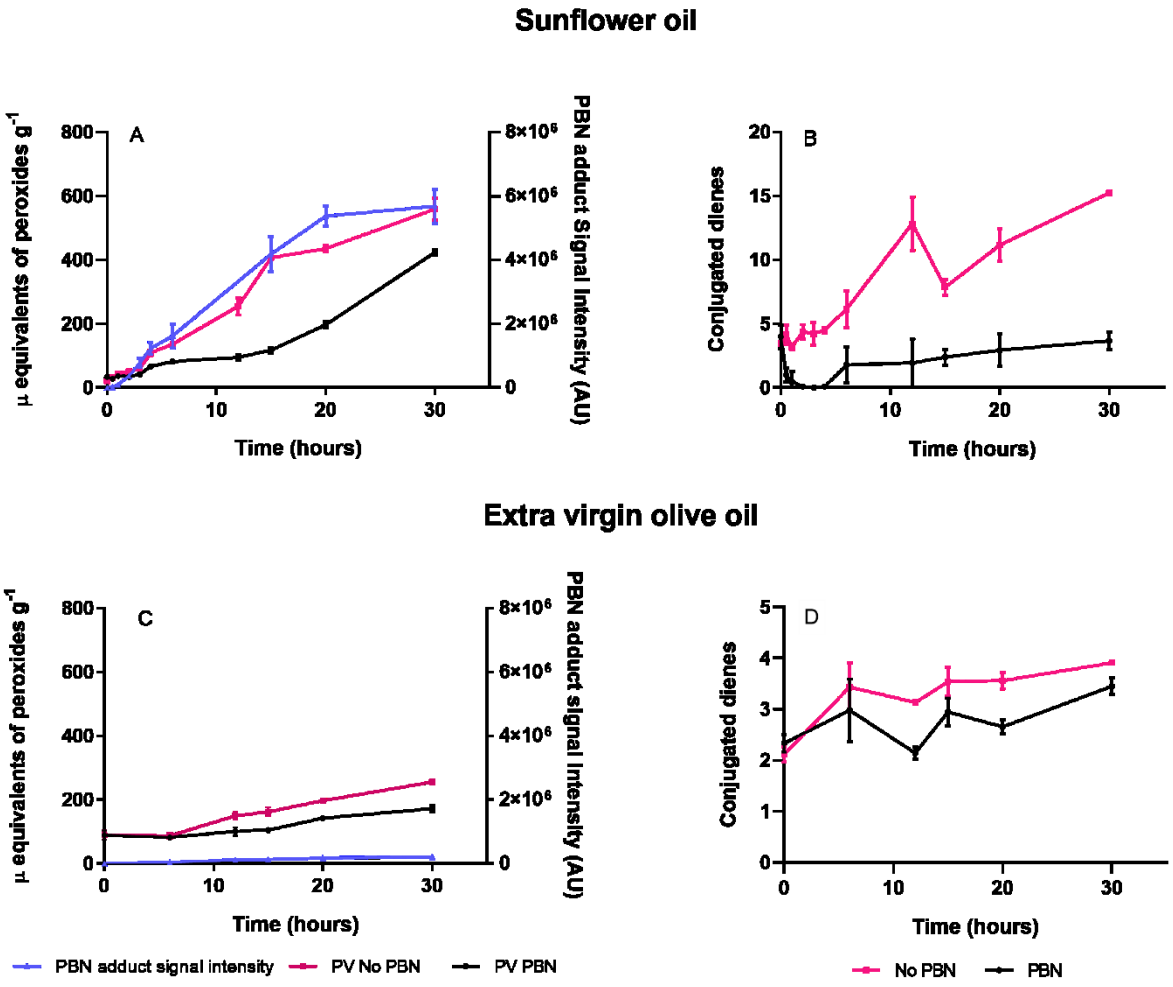

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