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ORIGINAL PAPER

Oxidative Stability of Lipids by Means of EPR Spectroscopy and Chemiluminescence

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Abstract The aim of this study was to investigate the oxidation of selected vegetable oils (CLSO, CCSO, CBO) at accelerated oxidation rates. Several seldom used analytical methods were applied including electron paramagnetic resonance (EPR), spin trapping with α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), Fe^{2+}-induced chemiluminescence, Rancimat tests, and the determination of conjugated diens at $\lambda = 233$ nm. The antioxidative properties of POBN and DMPO were also investigated. The time required for each method was determined. EPR spectrometry of trapped radicals generated during oxidation turned out to be the fastest method to determine oxidative stability. Chemiluminometric determination of oxidation kinetics showed that POBN has a very strong anti-oxidative potential: it significantly (by 160–277%) lengthened the time to the chemiluminescence peak, as well as the induction time in the Rancimat test (by 110–140%). Photo-oxidation studies showed that superoxide anion radicals are the main factor responsible for the oxidation of lipids in the investigated oils.

Keywords EPR · Chemiluminescence · Antioxidants · Vegetable oils

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Abbreviations

CBO	Crude borage oil
CCSO	Crude *Camelina sativa* oil
CLSO	Crude linseed oils
EPR	Electron paramagnetic resonance
DMSO	Dimethyl sulfoxide
POBN	α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone
DMPO	5,5-Dimethyl-1-pyrroline-*N*-oxide

Introduction

The oxidative stability parameter allows the assessment of the nutritional value of oils/fats and their usefulness for consumption. Several traditional methods commonly used to determine that parameter generally employ oxidation of lipids at accelerated rates. Specific products of auto-oxidation and the rates of their formation have also been investigated [1]. However, techniques that would allow the unambiguous determination of oxidative stability of plant/animal-origin lipids within a short analysis time are still being sought for [1–4].

Improvement of lipid oxidative stability is another noteworthy aspect. The stability may be increased by anti-oxidative additives; there are several classes of such chemical compounds. Some of them intercept oxidation factors (like free radicals) and protect double bonds of fatty acids linked in triacylglycerides [5]. Ideal anti-oxidants should have appropriate anti-oxidative activity, do not exhibit any reactivity in the oxidized form, and not harm humans, animals or the environment. Selection of a proper anti-oxidant requires knowledge of the lipid auto-oxidation process, particularly of its initial stage when the first radicals form. Therefore, that stage is very important from the

point of view of lipid oxidative stability. However, in spite of numerous studies devoted to that stage no theory of the process has been formulated.

Oxygen is fundamentally important in auto-oxidation of lipids. Low-reactivity triplet oxygen (ground state of the $^3O_2$ oxygen molecule) is not likely to initiate the oxidation process. Superoxide anion radicals (which may originate from single-electron reduction of oxygen catalyzed with ramped temperature or electromagnetic radiation) are much more reactive. They may be formed in reactions between triplet oxygen and metals present in vegetable oils, pigments (considered as sensibilizers of the oxidation process), phenolic compounds etc. Singlet oxygen form ($^1O_2$) formed in reactions of lipid oxidation (especially in the presence of UV radiation) shows significantly higher reactivity. Formation of $^1O_2$ requires excitation of $^3O_2$ with energy necessary to move electron from an anti-bonding molecular orbital π^* to the second one and reverse its spin, or to reverse spin of an electron on the same molecular orbital. There are two singlet oxygen $^1O_2$ forms, S_1 and S_2, of various lifetimes ($\tau_{1/2}$) and excitation energies. $^1O_2$ molecule in the S_2 state has a very short lifetime and does not play any significant role in lipid oxidation processes. $^1O_2$ molecule in the S_1 state lifetime is significantly longer [6–8]. Triplet oxygen does not react directly with triacylglycerides due to the high activation energy of the reaction, 146–273 kJ/mol [5]. Transformation of triplet oxygen into the singlet form and the reaction with triacylglycerides in this latter form is a much more energetically preferred process, requiring only about 92 kJ/mol of energy (delivered by UV radiation or thermally). Light and, to some extent, also elevated temperatures catalyze the oxidation of lipids by singlet oxygen, especially in the presence of chlorophyll, carotenoid pigments, or polyaromatic hydrocarbons. Such sensibilizers are present in both refined and cold-pressed oils [9]. A sensibilizer is a compound which in its ground singlet state may absorb light or thermal energy to form an excited singlet form. Sensibilizers de-excite by means of light photon emission (fluorescence), an internal radiation-less decay, or through an inter-system crossing. Such crossing may result in the formation of an excited triplet form capable of generating a singlet form of oxygen and of lipid hydroperoxides [5]. Generation of singlet oxygen molecules is a multi-stage process. The $O_2^{\cdot-}$ radicals formed in the first stage decay through electron reduction (e.g. in a reaction with triacylglyceride) to $^1O_2$ (S_1) [10]: $O_2^{\cdot-} \rightarrow {}^1O_2 + e^-$.

Lipid oxidation is a multi-stage process including a radical generation stage, a radical reaction stage (propagation phase), and a final stage, in which some non-reacting oxidation products are formed. Most researchers are of the opinion that generation of radicals, radical reactions and formation of final oxidation products phenomena occur at every stage of the process. However, it is suspected that one of these phenomena dominates at any particular stage of the process. Undesirable changes in lipid characteristics are related mainly to the initial stage of their oxidation process [5].

Spin traps may be used to pick up short-living radicals formed during various biological/chemical processes, including the process of lipid oxidation [11, 12]. They enable identification of the radicals and determination of their concentration. In principle, the traps are not radical-specific, nevertheless particular traps are considered more or less useful for trapping particular radicals. POBN [α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone] is often used to capture the superoxide anion radicals $O_2^{\cdot-}$, hydroxyl radicals OH· and carbon-based radicals (i.e. lipid radicals) [13–16], whereas DMPO [5,5-dimethyl-1-pyrroline-N-oxide] is useful for trapping such radicals as OH·, CH_3·, and $O_2^{\cdot-}$. The latter trap is much less radical-specific and significantly less stable after trapping a radical [13–21]. The POBN trap is not as commonly used as DMPO. It is in fact a modified version of the commonly used PBN trap (α-phenyl-*tert*-butylnitrone) with improved stability and radical trapping efficiency [14].

The aim of this study was to investigate the oxidative stability of selected plant oils (CLSO, CCSO, CBO). Several analytical methods were applied including EPR spectroscopy, POBN/DMPO spin traps, Fe^{2+}-induced chemiluminescence, Rancimat tests, and the determination of conjugated dienes. We also investigated the influence of atmospheric oxygen on the initial stage of the lipid oxidation process, as well as inhibition effects exhibited by several anti-oxidant additives.

Methods and Apparatus

Chemicals

Commercially available samples of crude oils were supplied by Szarłat s.c. (Warsaw, Poland). POBN and DMPO spin trapping agents were purchased from Sigma–Aldrich Chemicals (Bellefonte, PA, USA). DMPO was stored under freezing conditions under an argon atmosphere, while DPPH was distilled before use. HPLC-grade $FeCl_2$, $CuSO_4$, DMSO, dichloromethane, and hexane chemicals were purchased from POCH (Gliwice, Poland).

EPR Measurements of Radicals ($O_2^{\cdot-}$, OH·, CH_3·)

A 2.5-g sample of homogenized oil was placed in a 15-mL quartz glass test tube and spiked with 100 μL of POBN or

DMPO solution (100 and 250 mg in 10 mL of dichloromethane, respectively). The samples were placed under a 50 W quartz (UV) lamp and irradiated for 20 s. Samples for analyses were collected after 0/10/15 and 20 s of irradiation. Irradiated samples were transferred into a flat EPR cell. For the EPR measurements, the standard X-band (~9 GHz) spectrometer, produced in Wroclaw, with digital registration of the spectra was used. The temperature measurements were done using the digital temperature control system (BRUKER ER 4131VT), which allows a temperature range from 100 to 500 K. Spectra were recorded at 4 min intervals using an Aspect 2000 computer system connected to the spectrometer. The *g*-values were determined with an uncertainty of ±0.0001 using the manufacturer's internal reference marker containing solid DPPH.

Hydroxyl radical generation was initiated by addition of 100 μL $CuSO_4$ solution (10 mM) to a mixture of 100 μL H_2O_2 (5 mM in 0.1 M phosphate buffer pH 7.4) and 100 μL DMPO (100 mM in 0.1 M phosphate buffer pH 7.4) [22, 23]. All $CuSO_4$ solutions were prepared immediately prior to use.

Superoxide anions were generated immediately before EPR measurements by mixing 50 μL DMSO with 50 μL POBN solution in DMSO (180 mM), then 50 μL of KOH solution (25 mM) and 50 μL hydrogen peroxide (25 mM) were added. Formation of short-living radicals, especially the superoxide anion, hydroxy radical, and methylene ones ($O_2^{\cdot-}$, OH·, CH_3·) was confirmed [18]. All analyses were done in triplicate.

Rancimat Test

Conductometric analysis of the total volatile compounds formed during lipid hydroperoxide degradation was carried out using the Methrom (Herisau Switzerland) Rancimat device [24, 25]. Briefly, a 2.5-g sample of the oil with/without 100 μL of the POBN/DMPO additive (100/250 mg in 10 mL of dichloromethane, respectively) was placed in a reaction cell and oxidized in a hot air stream (flow rate 10 L h^{-1}, temperature 90 °C). All analyses were carried out in triplicate.

Chemiluminescence

Chemiluminescence at 90 °C was measured using a chemiluminometer built by the first author [4]. Test tubes with oil samples containing Fe^{2+} ions (20 ± 0.5 mg of $FeCl_2$ added to a 50-ml glass tube) were put to the chemiluminometer cell. A 2.5-g sample of the oil at room temperature with/without the POBN/DMPO additive (the same solutions as those used in the Rancimat tests, see above) was introduced after 15 s of analysis through a capillary using an HPLC pump. The chemiluminometer was operated in the SPC (single photon counting) mode. A Hamamatsu R1527p photomultiplier tube was used. The device was operated at −10 °C; the gate time was 1 s. All analyses were carried out in triplicate.

Conjugable Oxidation Products (COP Index)

The COP index was measured in relation to HPLC-grade hexane as a blank. Oil samples were diluted to 1:600 with hexane [26]. Absorbance at 233 nm was measured [26, 27]. All analyses were carried out in triplicate.

Data Computation

Results were processed using standard tools of the Microsoft Office 2007 suite. The time to the chemiluminescence peak and peak area were calculated using the MATLAB v.7.0 software package. EPR spectra for particular spin traps were simulated using the EPR Spectra Simulation for MS-Windows v.0.98 software package (David A. O'Brien, David R. Duling and Yang C. Fann of National Institute of Environmental Health Sciences/National Institutes of Health, USA). Statistical analysis was done using the StatSoft Statistica PL software package.

Results

EPR signals proportional to the mean concentrations of radicals in non-irradiated/UV-irradiated oils are shown in Fig. 1 (radicals trapped with DMPO) and in Fig. 2 (POBN). Curves a–e in Fig. 3a, b show typical EPR spectra recorded for the particular spin trap used.

DMPO Spin Trapping Agent

The concentration of radicals in non-irradiated CLSO oil was significantly lower than in other examined oils (Fig. 1). UV irradiation increased the initial concentration

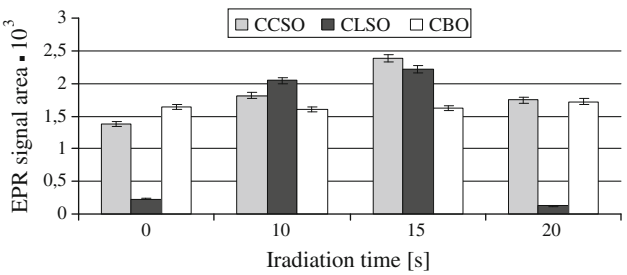

Fig. 1 Time evolution of EPR signals (total radicals) during 20 s UV irradiation of CCSO, SCSO and SCO oils with the DMPO spin trapping agent added

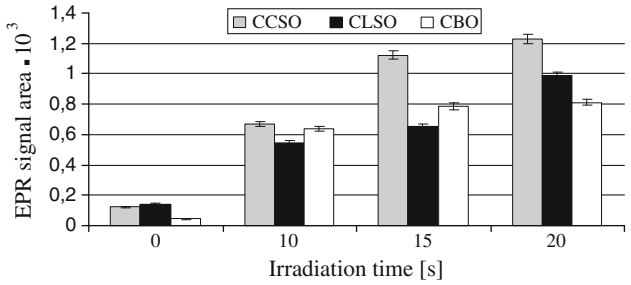

Fig. 2 Time evolution of EPR signals (total radicals) during 20 s UV irradiation of CCSO, SCSO and SCO oils with the POBN spin trapping agent added

of radicals in CLSO and CCSO oils; the increase was much more dynamic in CLSO. The majority of radicals in CLSO and CCSO turned out to be trapped after 15 s irradiation: more irradiation did not increase the concentration. On the other hand, the concentration of radicals in CBO; although initially the highest among all oils; was practically not affected by the irradiation process.

POBN Spin Trapping Agent

Results obtained with the POBN trapping agent were significantly different in comparison to the DMPO case. A very low concentration of radicals was observed in all non-irradiated oils (Fig. 2). The concentration increased with the irradiation time for all samples and apparently did not saturate even for 20 s irradiation time. The highest increase was observed in CCSO, the lowest in CBO.

The curve a in Fig. 3a shows a sample EPR spectrum obtained experimentally for UV-irradiated vegetable oil with POBN addition and respective computer simulation. As the simulations show, the spectrum is produced mainly by two trapped radicals: the superoxide anion radical, and the hydroxyl radical. Splitting constants were $a_N = 1.378$ mT, $a_H = 0.18$ mT, $g = 2.0059$ and $a_N = 1.554$ mT, $a_H = 0.200$ mT, $g = 2.0059$, respectively. It was found that the superoxide anion radical contributed about 80% to the overall trapped radicals. This result was confirmed by EPR spectra obtained for superoxide anion radical generated in reaction of decomposition of hydrogen peroxide in basic solvating media (c) and in the Fenton reaction (d).

Curve b shows a sample EPR spectrum recorded for cold-pressed vegetable oil irradiated with a UV lamp. Curve e shows a sample EPR spectrum recorded for trapped hydroxyl radicals generated in the Fenton reaction (e). Splitting constants for DMPO-OH adduct were $a_N = 1.500$ mT and $a_H = 1.500$ mT, $g = 2.0059$. EPR spectra of the oil sample were significantly more complicated since various radicals had been produced by irradiation. Computer simulation of three DMPO-trapped radicals are shown (DMPO-OH: $a_N = 1.490$ mT, $a_H = 1.490$ mT, $g = 2.0059$; DMPO-OOH: $a_N = 1.430$ mT, $a_H = 1.170$ mT, $g = 2.0059$; and DMPO-CH_3: $a_N = 1.640$ mT, $a_H = 2.240$ mT, $g = 2.0057$). These simulations agreed well with EPR signals experimentally obtained for photo-oxidized vegetable oil.

Chemiluminescence

Figures 4, 5 and 6 shows chemiluminograms of oil samples heated to 90 °C with/without the POBN spin trapping agent added. The same samples were also analyzed with addition of DMPO as a trapping agent. DMPO did not influence the observed results (data not shown), while POBN very significantly increased the oxidative stability of the oils.

Chemiluminescence signal peaked for CCSO without/with PONB at $\tau_{CL\ max}$ (SD = standard deviation):

- 230/360 min (SD = 11.7/15.2), respectively. Similar shifts were observed for other oils
- 90/250 min (SD = 7.1/9.9), respectively, for CLSO
- 310/>500 min (more than the preset analysis time) (SD = 9.8/NA), respectively, for CBO

Rancimat Test

Results of the Rancimat test are shown in Fig. 7.

DMPO did not change the induction time determined by the Rancimat test (data not shown). On the other hand, POBN influenced the oxidative stability of the oils (just like in the experiment reported above). The Rancimat test induction time without/with PONB added:

- 7.78/9.5 h (SD = 0.088/0.064), respectively, for CCSO
- 6.00/6.61 h (SD = 0.085/0.049), respectively, for CLSO
- 15.3/20.9 h (SD = 0.141/0.354), respectively, for CBO

The increase is most spectacular in the case of the CBO oil sample, which anyway had the highest initial oxidative stability among all the oil samples examined.

COP Determination

Figure 8 shows the time evolution of absorbance in oil samples oxidized at an accelerated rate (at 90 °C). We may conclude that conjugated dienes are forming as the oil oxidation process proceeds. The highest formation rate was observed in CCSO and CLSO, whereas the lowest one in CBO, which turned out to be the most stable oil in this test. Effects of POBN/DMPO additives on formation of conjugated dienes could not be determined due to fact that both these agents show absorption at $\lambda = 233$ nm and even after oil dilution gave unacceptably high absorbance values.

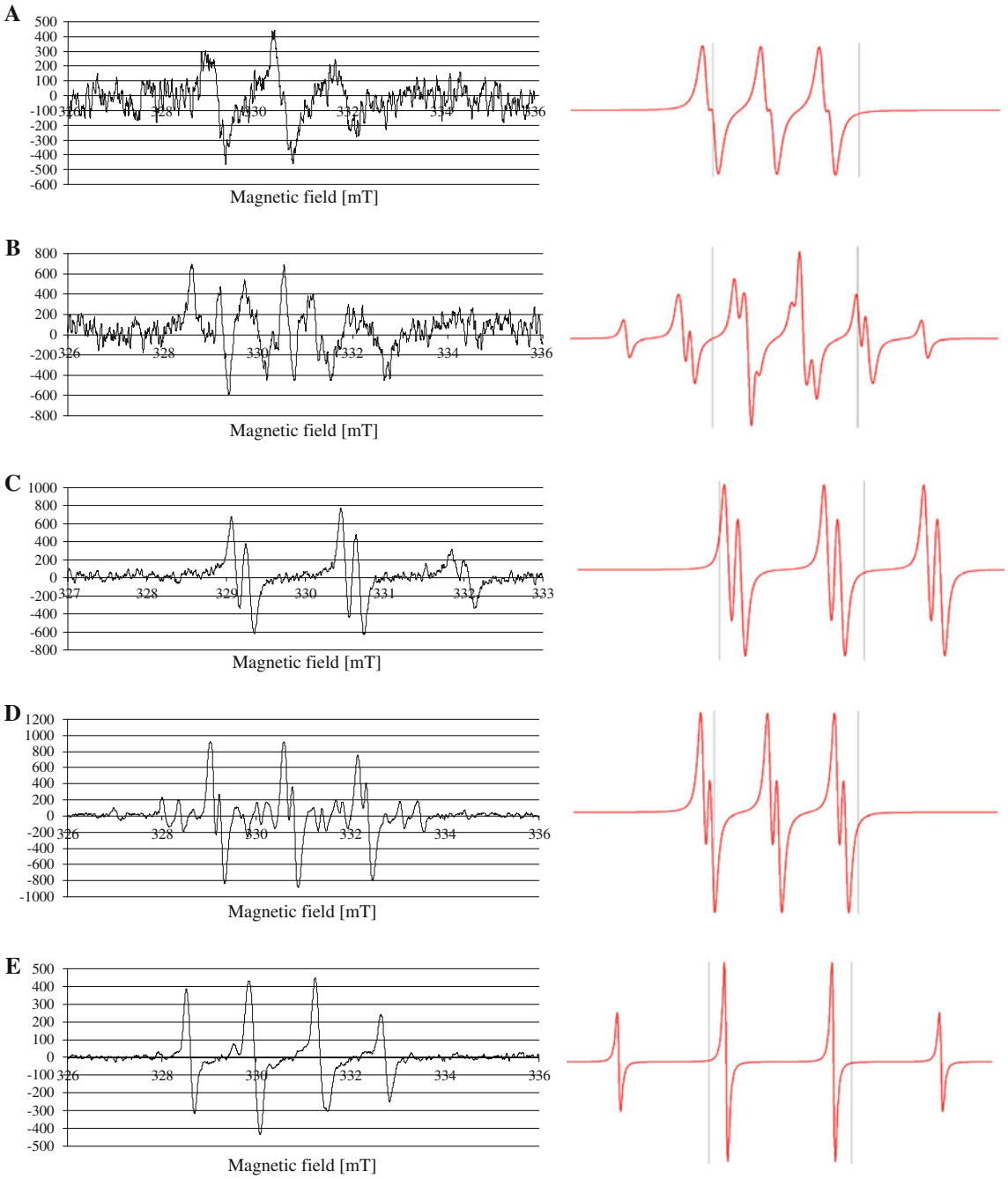

Fig. 3 **a** Experimental and simulated EPR spectra of oil samples measured after UV irradiation in the presence of POBN (*a*) and DMPO (*b*) spin trapping agents. Superoxide anion trapping by POBN, generation in KOH/H_2O_2/DMSO systems (*c*). **b** Experimental and simulated EPR spectra of POBN-OH (*d*) and DMPO-OH (*e*) spin adducts generated in Fenton reaction $CuSO_4/H_2O_2$. Spectrometer settings: center field 331 mT, sweep width 10 mT, gain 0.5 × 10^6, modulation 0 1 mT, scan time 65 s, microwave power 20 m W, scan count 3

Discussion

The EPR spectrum of DMPO-trapped radicals (curve b in Fig. 3a) is composed of many lines most probably representing many different radicals formed during lipid photooxidation. Similar results were obtained by Qian et al. (2000) [12], who suggested that the spectrum was a compilation of trapped hydroxyl radicals formed during decomposition of lipid hydroperoxides, superoxide anion radicals, and lipid radicals (of a particular fatty acid). Yoshimura et al. (1999) [17] studied the trapping of hydroxyl/superoxide anion/carbon (methylene) radicals by

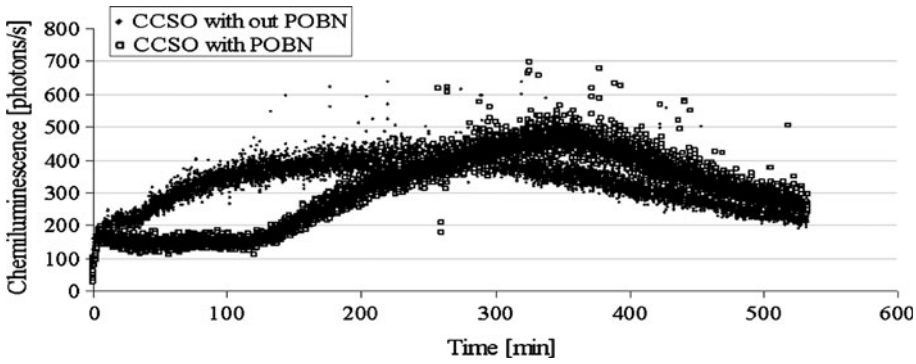

Fig. 4 Chemiluminescence of CCSO at 90 °C with/without POBN spin trapping agent added

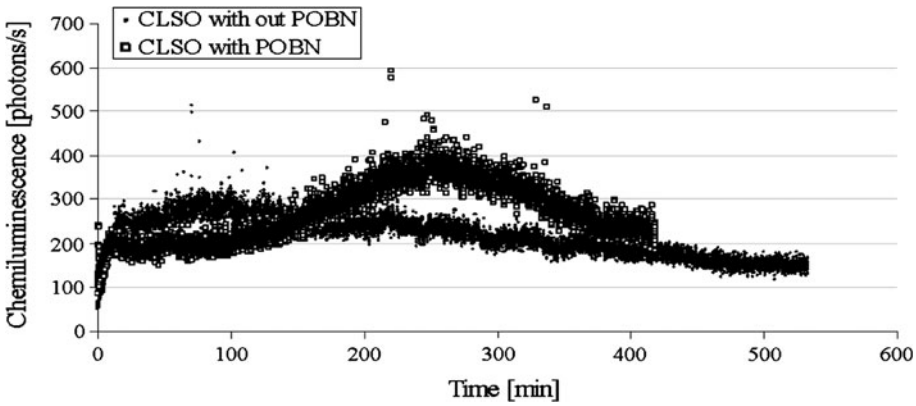

Fig. 5 Chemiluminescence of CLSO at 90 °C with/without POBN spin trapping agent added

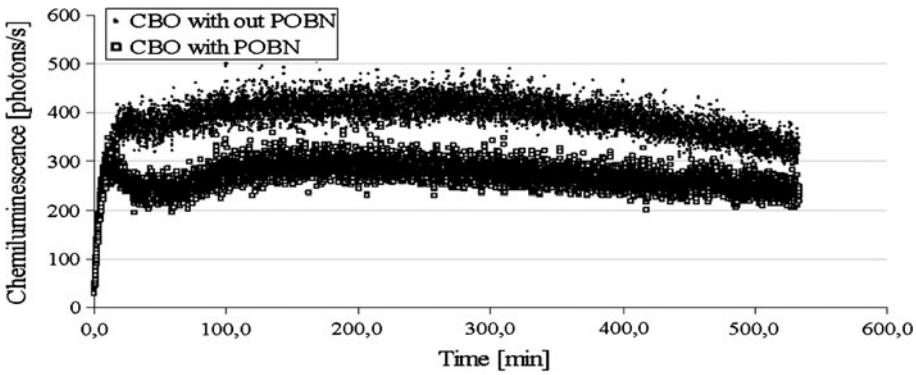

Fig. 6 Chemiluminescence of CBO at 90 °C without/with POBN spin trapping agent added

DMPO and found that OH· was trapped exceptionally well (DMPO-OH adduct), much better than CH_3· and $O_2^{\cdot-}$. Low-stability DMPO-$O_2^{\cdot-}$ adducts decompose relatively fast into DMPO-OH ones [21].

Much simpler EPR spectra observed in the case of POBN are most probably dominated by the superoxide anion radicals. An argument in favor of that supposition is that EPR spectra obtained for non-Fenton reactions of hydrogen peroxide decomposition ($NaOH/H_2O_2$/DMSO) in laboratory conditions shown in the literature [15, 17, 18] exhibit identical splitting constants. However, POBN is also known to trap several radicals other than $O_2^{\cdot-}$, like, for example, hydroxyl radicals.

The EPR spectra obtained showed that large amounts of the $O_2^{\cdot-}$ radicals and various radicals produced in subsequent oxidation reactions may form during the oil photo-oxidation process. Hydroxyl radicals and lipid radicals formed in subsequent reactions during the auto-oxidation process are trapped by DMPO. Most probably the hydroxyl anion radicals (efficiently trapped by POBN) are the first to form during fresh vegetable oil oxidation. Superoxide anion radicals originate from reactions of lipids with atmospheric oxygen. Single electron reduction of triplet oxygen $^3O_2$ must take place, what leads to formation of $O_2^{\cdot-}$ [5].

This study showed that POBN is a better choice than DMPO from the point of view of the oxidative stability determination with EPR spectroscopy. POBN has selectivity against $O_2^{\cdot-}$ and allows us to quickly (within a few min) find out which oil is less susceptible to oxidation by atmospheric oxygen when UV irradiation is used to catalyze the reactions.

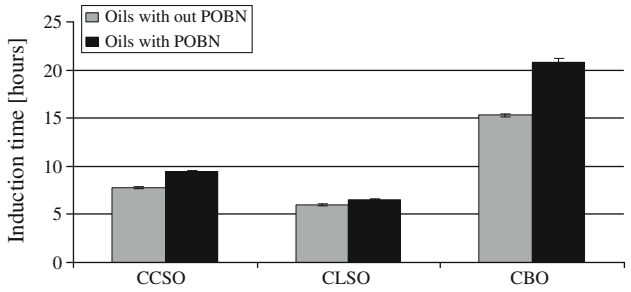

Fig. 7 Rancimat test for CCSO, SLSO and CBO without/with POBN spin trapping agent added. Experimental conditions: temperature 90 °C, air flow rate 10 L h^{-1}, 2.5 g samples

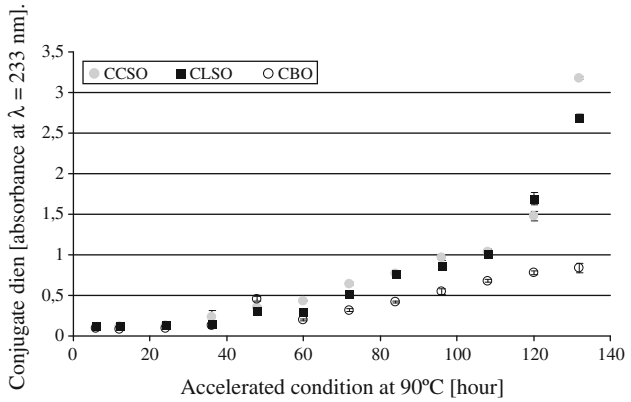

Fig. 8 Absorbance at $\lambda = 233$ nm in CCSO, CLSO and CBO oils during accelerated oxidation at 90 °C

The proposed EPR-based lipid oxidative stability evaluation technique might find its application as a fast method to determine freshness and quality of food products containing large amounts of fats. This issue may be of great importance to public health since the negative effects of oxidized lipids in human diet on health are well-known. EPR results agree with the results obtained using other methods applied in this work (Rancimat tests, chemiluminescence, conjugated diens).

POBN additives significantly increased the oxidative stability of the analyzed oils. The anti-oxidative effect was not observed for DMPO, most probably because DMPO adducts have low-stability at room temperature, and they reacted with the oil, while DMPO-trapped radicals interacted mutually. POBN and/or PBN adducts are significantly more stable [5].

On the other hand, the POBN additive significantly increased the oxidative stability of the analyzed oils at a temperature elevated to 90 °C. Most probably, this may be attributed to the inhibition of the lipid oxidation initiation stage by trapping $O_2^{\cdot-}$ radicals formed during lipid thermo-oxidation. As soon as all the spin trap molecules had been occupied by the radicals, a high increase in the chemiluminescence and conductivity in the Rancimat test was observed.

Spin traps have never been used as functional additives i.e. to improve anti-oxidative properties of oils. Not much is known about their toxicological profiles or their influence on living organisms. However, they may significantly improve lipids oxidative stability, especially in very labile oils such as linseed oil. It seems that even if spin traps are not used as food additives, they may be effectively used in laboratory practice to prevent oils from oxidative degradation.

Determination of the conjugated dienes contents showed that borage oil has the highest oxidative stability among all analyzed oils. *Camelina sativa* oil and linseed oil were less stable. Results of this study seem to confirm findings of our previous study [28]. EPR spectroscopy may be used to quickly determine oxidative stability of vegetable oils. POBN and DMPO spin trapping compounds may act as effective antioxidants. Literature data suggest that some spin trapping compounds have a toxic impact on living organisms [29]; therefore, they must not be used as food additives. However, they might be used in the laboratory environment to protect oxidatively non-stable oils/fat tissue samples during prolonged periods of storage. The superoxide anion radical is the main factor responsible for the photo-oxidation of vegetable oils. Thermo-oxidation is a much slower/less effective process than photo-oxidation (long times are necessary to reach the CL peak, and a long time of induction in the Rancimat test). The thermo-oxidation process of lipids at elevated temperatures should be investigated using various spin traps to determine what sorts of radicals are generated at the first stage of the process, and which of them plays the most important role in the kinetics of the process. Results of such studies might give a clue to the proper selection of natural or synthetic anti-oxidants capable of inhibiting vegetable oil oxidation processes.

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