Autoxidation of Linoleic Acid Methyl Ester Studied by Spin Trapping *

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2-Methyl-2-nitrosopropane (MNP) dimer has been used both to initiate autoxidation of linoleic acid methyl ester (LAME) and to trap pentadienyl radicals formed during autoxidation. The EPR spectrum of trapped pentadienyl radical is a triplet of doublets. The growth of spin-trapped pentadienyl radicals was linear with time and the growth rate was studied as a function of concentrations of MNP and LAME using cyclohexane as the solvent. A mechanism is put forward to explain the experimental results, and by comparing the proposed reactions with experiments, rate constants for self-trapping (identical with that of thermal decomposition of the spin trap) and initiation of pentadienyl radicals were found to be: 2×10^{-6} s^{-1} and 6.5×10^{5} dm^3 mol^{-1} s^{-1}, respectively, and the peroxidation rate was: 1.3×10^{5} s^{-1} under laboratory conditions.

Autoxidation of polyunsaturated fatty acids take place in various cases such as fat and oil degradation, cellular membrane damage, the defence system in plants and physiological regulation mechanisms.[1-6] The mechanism of autoxidation processes was originally proposed by Uri,[7,8] and the reactions involved are often divided into three steps: (1) initiation, (2) the chain reactions consisting of peroxidation and propagation, and (3) termination.

In lipid autoxidation experiments under (quasi)stationary conditions, oxygen (O_2) uptake or yield of hydroperoxides (ROOH) are most frequently measured (using a pressure transducer or gas chromatography). However, in most autoxidation systems the rate and nature of initiation are unknown. Thermally unstable azo compounds[9,10] are often used to promote controlled initiation. In steady-state experiments of this type the so-called oxidizability ($k_p/2k_t^{\frac{1}{2}}$; where k_p and k_t are the rate constants of propagation and termination, respectively) can be measured.

The intermediates in autoxidation, which determine the reaction mechanisms, are free radicals. Free radicals are most directly studied by EPR spectroscopy.[11,12] In steady-state experiments, however, concentrations of the free radical intermediates (R$^{\bullet}$, ROO$^{\bullet}$; dienyl and peroxyl radicals) are in general too low to be measured. Recently, spin traps have been introduced[13,14] to overcome this problem. Spin traps are small organic molecules that react with free radicals by which stable free radicals are formed. Moreover, some of the spin traps are thermally unstable and might hence be used as initiators of autoxidation. In this paper we describe the use made of these two different properties of MNP in the autoxidation of LAME: firstly, its thermal instability to initiate autoxidation, and secondly, its ability to trap radicals produced in autoxidation reactions.

Experimental

Chemicals.—Linoleic acid methyl ester (LAME, purity 99%) and 2-methyl-2-nitrosopropane dimer (MNP, the spin trap) were purchased from Aldrich. Cyclohexane (spectroscopic grade, Aldrich) was used as the solvent.

Instrumentation.—A Bruker 100D ESR instrument was used. The centre field was 3485 and the scan width was 50 G. The modulation width and modulation-frequency were 0.5 G and 100 kHz, respectively. The gain of the phase-sensitive amplifier was 5×10^5. The recorded EPR spectra were digitized and stored in an EG&G Signal Averager (Model 4001) and the digitized EDR spectra were transferred to a VAX computer, *via* an IBM PC (type 8555). The spectra were plotted on a laser printer (LN-03 PLUS) by means of graphical programs in the VAX computer library.

Absorption spectra were measured with a Shimadzu UV-160A spectrophotometer.

Experimental Procedure.—Before each experiment, a stock solution of MNP in cyclohexane was prepared (1–5 mg cm^{-3}). In solution, MNP is in both its dimeric and monomeric forms. In order to obtain the monomer–dimer equilibrium, the stock solution was stored in darkness for 30 min prior to each experiment. Small bottles containing specific volume fractions of LAME in cyclohexane were also prepared. Autoxidation and spin trapping was started by addition of MNP, by means of graded pipettes from stock solutions, to the LAME solutions. In all cases the overall volume of the mixture was 300 μl of which 200 μl was transferred to an EPR tube. The tube was placed in the cavity of the EPR instrument and care was taken each time that the tube position was the same and that no light penetrated the cavity (the spin trap is light sensitive). The EPR spectrum from the mixture was measured simply as a function of time after addition of MNP. In these experiments the rate of growth of the EPR signal was observed as [LAME] and [MNP] were systematically charged. The experiments were carried out at room temperature (21 °C).

Results

In Fig. 1 is shown the EPR spectra measured as a function of time after addition of MNP to solutions of LAME. The intensity of the EPR signal increases linearly with time. The EPR spectrum itself is a triplet of doublets. The triplet splitting constant, due to the N atom in MNP, is 14.6 G, while the doublet splitting is 1.9 G. The same spectrum was originally observed by de Groot *et al.*,[15] in order to prove that radical intermediates are involved in enzymatic production of hydroperoxides from PUFA. In Fig. 2 is shown the peak to peak height of the low-field line as a function of time when [LAME] was constant and [MNP] varied. In all cases the height of the EPR signal increased linearly with time.

In Fig. 3 the growth rate (relative) of the EPR signal is plotted as a function of [MNP]. For low [MNP] the growth rate

* Abbreviations: MNP: 2-methyl-2-nitrosopropane; LAME: Linoleic acid methyl ester; EPR: Electron paramagnetic resonance; PUFA: Polyunsaturated fatty acids.

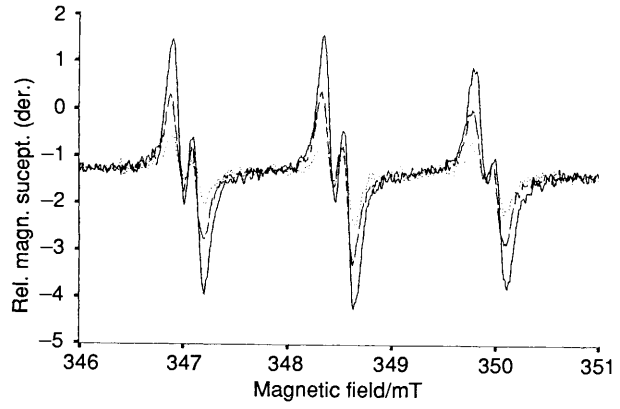

Fig. 1 2-Methyl-2-nitrosopropane (the spin trap) was added to a solution of linoleic acid methyl ester in cyclohexane and the resulting EPR spectrum was recorded at different times after mixing: ——, 9 min; – – – 5 min; ····, 1 min. In all cases the concentration of the spin trap was 0.5 mg cm^{-3} and the relative volume proportions to solvent were 1 : 3. The EPR spectrum is a triplet of doublets.

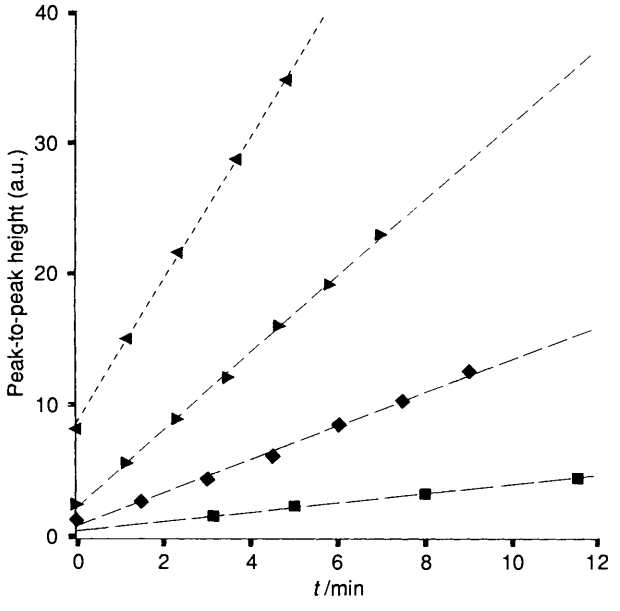

Fig. 2 The peak-to-peak height of the low-field EPR doublet plotted *versus* time after mixing of the spin trap into a solution of linoleic acid methyl ester in cyclohexane: [MNP]/mg cm^{-3} 4 (◀); 2 (▶); 1 (◆); 0.5 (■). The volume ratio of linoleic acid methyl ester to cyclohexane was 1 : 3 in all cases.

increases superlinearly with [MNP], while for relatively higher [MNP], the growth rate is linear. The growth rate was measured as a function of [MNP] for four different [LAME].

In Fig. 4 is shown the growth rate of spin-trapped radicals as a function of [LAME] for constant [MNP]. In this case the rate increases linearly with [LAME]. At higher LAME concentrations there is some deviation from linearity, which may be due to line-broadening caused by the increasing viscosity of the solution. Fig. 5 shows two successively recorded EPR spectra from pure MNP in cyclohexane. The concentration of spins in the sample is much lower in this case compared with the above cases. The EPR spectrum is a triplet and the line-width is larger than in the case of binary solution. The increase in the EPR signal height with time indicated that thermal decomposition and self-trapping was occurring.

Absolute spin concentrations can be determined by EPR spectroscopy. In order to find absolute growth rates, areas under the integrated EPR curves were compared with those of a standard. The standard chosen was di-*tert*-butylaminoxyl

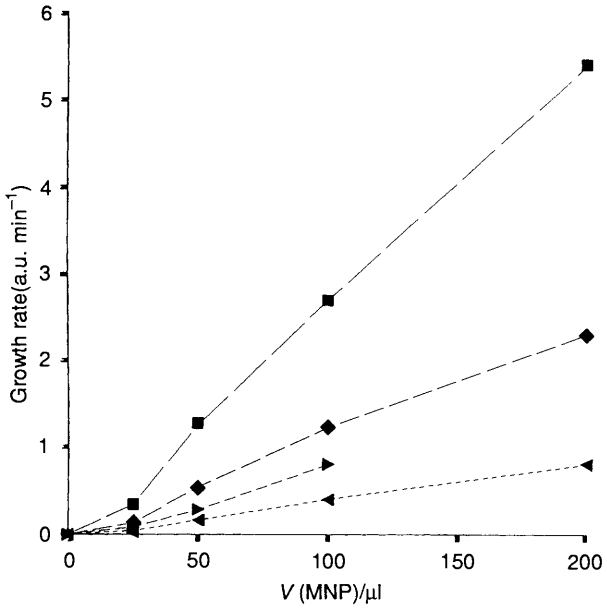

Fig. 3 The relative growth rate of spin-trapped molecules, derived from Fig. 2 and similar measurements, as a function of spin-trap concentration: [MNP]/mg cm^{-3} (volume added from stock solution) = 1.5 (····) (50 μl, ◀); 1.5 (– – –) (100 μl, ▶); 3.2 (– – –) (100 μl, ◆); 6.5 (— — —) (100 μl, ■). In three cases the volume ratio of LAME to cyclohexane was 1 : 3 and in one case 1 : 6.

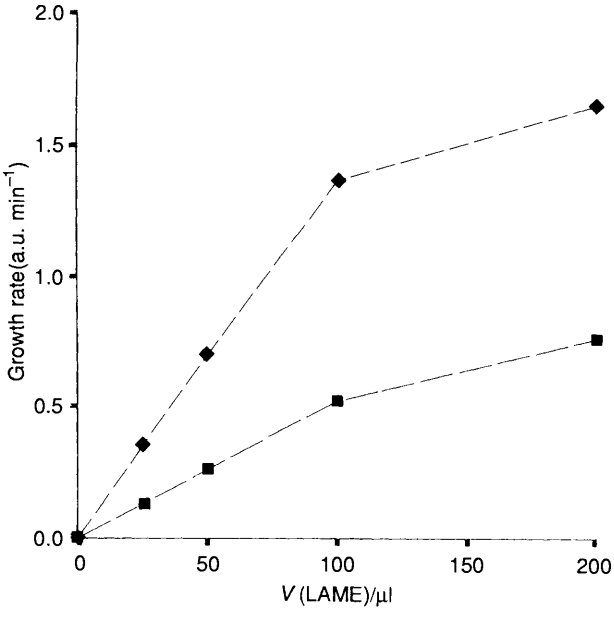

Fig. 4 The growth rate of radicals as a function of volume fraction of linoleic acid methyl ester, for two different concentrations of the spin trap. In all cases the total volume was 300 μl. 100 μl LAME in 300 μl corresponds to 1.05 mol dm^{-3}. [MNP]/mg cm^{-3} = 0.8 (◆); 1.6 (■).

($[C(CH_3)_3]_2NO$), a spin label chemically related to the spin trap used in this work. By this procedure, it was found that unity along the *y*-axis in Figs. 3 and 4 corresponded to an absolute growth rate of 1.2 (±0.5) × 10^{-6} dm^3 mol^{-1} min^{-1} (of spins). In Fig.6 is shown the steady-state absorption spectrum of MNP in cyclohexane. The absorption band at 671 nm [A^M(671)] is due to monomeric MNP and the peak at 292 is due to dimeric MNP [A^D(292)].[14] In Fig. 7 A^D(292) and A^M(671) are plotted as a function of total [MNP] in solution. In this concentration range, it is seen that dimer concentration increases with the [MNP]^2 and monomers linearly with total [MNP].

The absorption spectrum from freshly dissolved MNP is exclusively due to dimeric MNP. The initial absorption at 292

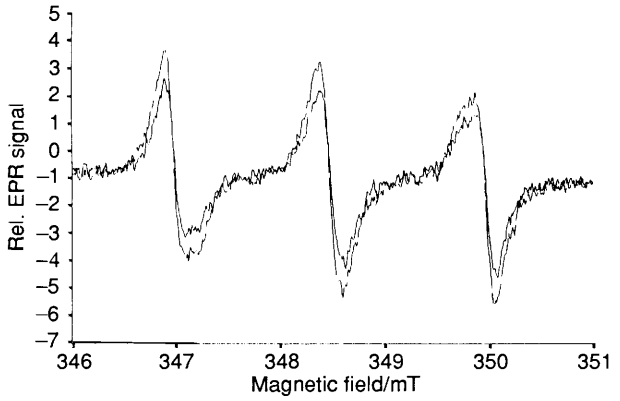

Fig. 5 EPR spectra of 2-methyl-2-nitrosopropane in cyclohexane (concentration 6 mg cm^{-3}) at different times after mixing (– – –, 25 min; ——, 10 min). The amplifier gain and the modulation width were twice as large as usual.

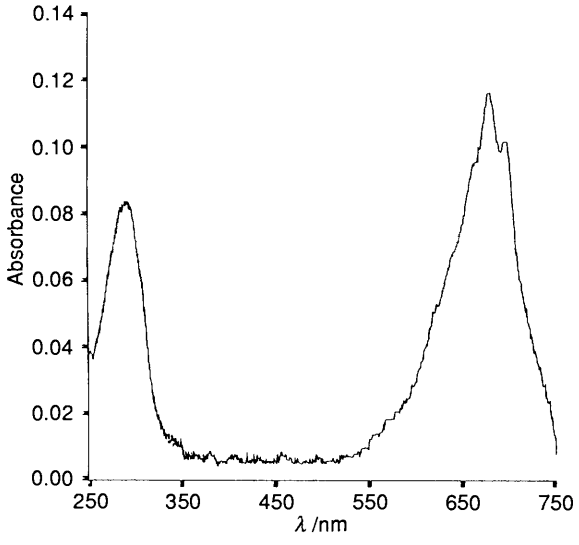

Fig. 6 The absorption spectrum of 2-methyl-2-nitrosopropane (the spin trap) in cyclohexane. The concentration was 0.4 mg cm^{-3} and the spectrum was recorded 30 min after solvation. The absorption band peaking at 671 nm is due to the spin trap in the monomeric form, while the peak at 292 nm is due to the dimeric form.

nm from 0.5 mg MNP in 3 cm^3 cyclohexane is 2.5, and hence the dimer extinction is $\varepsilon_D(292) = 2300\ dm^3\ mol^{-1}\ cm^{-1}$. The decay of $A^D(292)$ to the equilibrium value is exponential with time with a rate constant of 0.05 min^{-1}; a value somewhat different from that given by Riesz and Rosenthal.[14] At equilibrium, in the applied concentration range, MNP is mainly in monomeric form, and hence from Fig. 7 a monomer extinction of $\varepsilon_M(671) = 21\ dm^3\ mol^{-1}\ cm^{-1}$ is derived. Monomer MNP is the active form of the spin trap.

Discussion

The equilibrium reaction between monomeric and dimeric MNP in organic solvents is written as follows.[14]

$$Bu^t\text{–}\underset{\displaystyle O}{\overset{\displaystyle O}{\underset{|}{\overset{|}{N}}}}\text{=N–}Bu^t \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2\ Bu^t\text{–N=O},$$

(dimer, denoted D) (monomer S, for spin trap)

Equilibrium concentrations are determined by the mass action law where K is the equilibrium constant. Concentrations

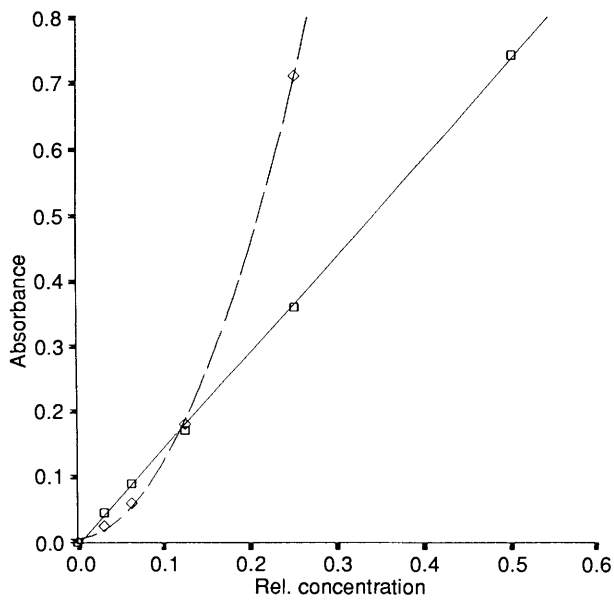

Fig. 7 Steady-state absorbances at 292 λ = (◇, RSQ = 1,000) and 671 nm (□, RSQ = 0.999) from the spin trap in cyclohexane as a function of concentration (rel. units). Initially, 5 mg of the spin trap were dissolved in 1 cm^3 cyclohexane (corresponding to a relative concentration of 1), and the spectra were recorded for successive dilutions. The amount of monomer present increases linearly and that of dimer with the square of the concentration.

(*c*) are in proportion to absorbances A ($A = \varepsilon cl$), and from the ratio between the monomer and dimer extinction coefficients, an equilibrium constant of $K = 0.6\ (\pm 0.2)\ mol\ dm^{-3}$ is obtained (see Fig. 7).

$$[S]^2/[D] = k_1/k_{-1} \equiv K$$

In this case autoxidation is initiated either by some internal process running at constant speed or by the spin trap itself. If initiation is internal, a limit in the growth rate of trapped radicals would be reached for increasing [MNP]. Since this is not found initiation must be induced by the spin trap itself. The thermal decomposition of nitroso-compounds might be visualized as follows:[16]

$$Bu^t\text{–N=O} \longrightarrow Bu^{t\bullet} + O\text{=}N^\bullet$$
(denoted S) (denoted $S_1^\bullet$, and $S_2^\bullet$)

or in an abbreviated form, eqn (1). EPR measurements on MNP

$$S \xrightarrow{k_d} S_1^\bullet + S_2^\bullet \qquad (1)$$

in solutions indicate the accumulation of small amounts of radicals in solution, which might originate from the trapping of $S_1^\bullet$ (or $S_2^\bullet$) with S itself (see Fig. 5). In a mixture of LAME and

$$S_1^\bullet + S \xrightarrow{k_t} S_1\text{–}S^\bullet \text{ (self-trapping)} \qquad (2)$$

MNP, the thermal fragments of S ($S_1^\bullet$ or $S_2^\bullet$) might abstract a methylene hydrogen in LAME, which is the initiation step of autoxidation:

$$\ldots\text{–}CH_2\text{–}CH\text{=}CH\text{–}CH_2\text{–}CH\text{=}CH\text{–}CH_2\text{–}\ldots + S_1^\bullet \longrightarrow$$
(denoted RH)
$$\cdots\text{–}CH_2\text{–}CH\text{=}CH\text{–}CH^\bullet\text{–}CH\text{=}CH\text{–}CH_2\text{–}\ldots + S_1H$$
(denoted $R^\bullet$)

In abbreviated for this is written as in eqn. (3).

$$RH + S_1^{\bullet} \xrightarrow{k_i} R^{\bullet} + S_1H \quad (3)$$

The pentadienyl radical ($R^{\bullet}$) is subsequently trapped by MNP:

$$Bu^t\text{–}N{=}O + R^{\bullet} \longrightarrow \ldots\text{–}CH_2\text{–}CH{=}CH\text{–}CH\text{–}CH{=}CH\text{–}CH_2\text{–}\ldots$$
$$\big|$$
$$Bu^t\text{–}N\text{–}O^{\bullet}$$

(denoted R–S$^{\bullet}$)

This is abbreviated as eqn. (4).

$$S + R^{\bullet} \xrightarrow{k_t} R\text{–}S^{\bullet} \quad (4)$$

The triplet splitting in the observed spectrum (see Fig. 1) is due to the N atom and the doublet splitting to the nearest H atom in the backbone of the fatty acid residue (in the γ position). The pentadienyl radical is also subject to peroxidation [eqn. (5)] and hence trapping of the pentadienyl radical is competing with the peroxidation step in autoxidation.

$$R^{\bullet} + O_2 \xrightarrow{k_o} ROO^{\bullet} \quad \text{(peroxidation)} \quad (5)$$

From these five equations the rates of formation for the intermediates, $S_1^{\bullet}$ and $R^{\bullet}$, can be obtained:

$$d[S_1^{\bullet}]/dt = k_d[S] - k_i[S_1^{\bullet}][RH] - k_t[S_1^{\bullet}][S]$$

$$d[R^{\bullet}]/dt = k_i[S_1^{\bullet}][RH] - k_o[R^{\bullet}][O_2] - k_t[R^{\bullet}][S]$$

On applying the steady-state condition, that is that the concentrations of intermediates are constant ($d[S_1^{\bullet}]]/dt = 0$ and $d[R^{\bullet}]/dt = 0$), $[R^{\bullet}]$ is obtained [eqn. (6)] and hence the

$$[R^{\bullet}] = (k_d k_i[RH][S])/\{(k_i[RH] + k_t[S])(k_o[O_2] + k_t[S])\} \quad (6)$$

rate of formation of the spin adduct S–R$^{\bullet}$ can finally be derived [eqn. (7)].

$$d[R\text{–}S^{\bullet}]/dt = k_t[R^{\bullet}][S] = k_d k_i k_t[RH][S]^2/\{(k_i[RH] + k_t[S])(k_o[O_2] + k_t[S])\} \quad (7)$$

The rate of self-trapping, when LAME is not present, will be (again applying the steady-state condition):

$$d[S_1\text{–}S^{\bullet}]/dt = k_d[S]$$

From the observed rates of self-trapping and spin-trapped LAME as a function of [RH] and [S], rate constants for peroxidation and initiation can be deduced as follows. Since the observed growth rate varies linearly with [RH], the rate of trapping is larger than that of initiation ($k_t[S] > k_i[RH]$, see above expression). Apart from an initial range of small [S] the observed trapping rate also varies linearly with [S], which implies that the rate of peroxidation is larger than that of trapping ($k_o[O_2] > k_t[S]$). When the growth rate varies linearly with [S] one obtains:

$$d[S\text{–}R^{\bullet}]/dt = k_d(k_i/k_o[O_2])[RH][S]$$

In the initial range of [S], the growth rate varies wih $[S]^2$:

$$d[S\text{–}R^{\bullet}]/dt = k_d(k_t/k_o[O_2])[S]^2$$

By comparing these theoretical trapping rates (both for small and large [S]) with the observations made in Fig. 5, one obtains:

$$k_d(k_t/k_o[O_2]) = 70 \text{ au dm}^6 \text{ mol}^{-2} \text{ min}^{-1} \text{ (small [S])}$$
$$k_d^{\bullet}(k_i[RH]/k_o[O_2]) = 5 \text{ au dm}^3 \text{ mol}^{-1} \text{ min}^{-1} \text{ (large [S], [RH] = 1 mol dm}^{-3})$$

k_d is found from the self-trapping experiments to be:

$$k_d = 1.0 \text{ au min}^{-1} \text{ (relative value),}$$

or $k_d = 2 \times 10^{-8}$ s^{-1} (absolute value, unity corresponds to 1.2×10^{-6} s^{-1}).

Schmid and Ingold[17] have determined k_t for this particular spin trap to be 90×10^5 dm^3 mol^{-1} s^{-1}, and hence the following value for the rate of peroxidation is obtained.

$$k_o[O_2] = 1.3 \times 10^5 \text{ s}^{-1}. \quad (8)$$

Assuming an oxygen tension[18] of 2 mmol dm^{-3} (found for olive oil) in this system, a peroxidation rate constant of $k_o = 6.5 \times 10^7$ dm^3 mol^{-1} s^{-1} is estimated, which is a value in agreement with others (6.8×10^7, ethyl linoleate).[8] The initiation rate constant can finally be derived: $k_i = 6.5 \times 10^5$ dm^3 mol^{-1} s^{-1}.

From the above expressions it is seen that k_i can be found, when k_t is known, without the knowledge of k_d. However, the peroxidation rate cannot be found without the knowledge of k_d. It turns out that the absolute value of k_d in this nitroso compound (2×10^{-8} s^{-1}, 37 °C) is somewhat smaller than those of azo compounds (0.6×10^{-6} s^{-1}),[9] more commonly used to initiate autoxidation.

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