Use of EPR and ENDOR spectroscopy in conjunction with the spin trapping technique to study the high-temperature oxidative degradation of fatty acid methyl esters †

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Free radicals produced during the autoxidation of unsaturated edible oils are extremely short-lived, but are able to react with spin traps to produce adducts with sufficient stability for spectroscopic characterisation at (near) cooking temperatures (353–443 K). EPR spectra have shown that the model esters methyl oleate, linoleate and linolenate each formed three distinct radical adducts with *N-tert*-butyl-α-phenylnitrone (PBN). These adducts have been further characterised by obtaining spectra under conditions of limited oxygen availability and in the presence of α-tocopherol; two of these adducts corresponded to peroxyl and alkyl radical adducts of PBN, whereas the other was an alkyl adduct of 2-methyl-2-nitrosopropane (MNP), which was formed as a result of decomposition of the PBN peroxyl radical adduct. The origins of the various ^{1}H hyperfine splittings have been determined by using selectively and fully deuterated PBN and selectively deuterated oleate and some of their magnitudes have been confirmed by ENDOR spectroscopy. The results obtained clearly confirm the high temperature oxidation of fatty acid esters to proceed *via* a different mechanism from that observed at low temperature, and point to significant differences in oxidation mechanisms of monounsaturated fatty acid esters relative to polyunsaturated.

Introduction

Oils and fats are used extensively in the cooking of food and in recent years there has been an increase in the consumption of vegetable oils with appreciable levels of unsaturation at the expense of the more highly saturated animal products. Polyunsaturated lipids are more labile with respect to oxidation than saturated and monounsaturated products and readily undergo free radical reactions[1] to produce a variety of products including hydroperoxides and both cyclic and polymeric fatty acid derivatives,[2,3] all of which may have health implications for the consumer. The major components of vegetable oils are palmitic, oleic, linoleic and linolenic triglycerides, containing 0, 1, 2 and 3 double bonds per fatty acid group, respectively.[4] Palmitates are relatively stable to oxidation and the methyl esters of oleic, linoleic and linolenic acids are generally used as model compounds to characterise lipid decomposition reactions.

The temperature dependence of the reaction kinetics and product distribution of heated cooking oils[5] indicate that the oxidation processes are temperature dependent. Free radicals formed during oil autoxidation, however, have short lifespans and their direct detection by EPR spectroscopy has so far proved to be impossible. Chain-breaking antioxidants, that are either present naturally in oils or added deliberately, inhibit oxidation and themselves yield relatively stable free radicals that are detectable by EPR spectroscopy.[6] Similarly, addition of *N-tert*-butylphenylnitrone (PBN) to grape-seed oil[7] resulted in the production of spin-trap-derived radicals that could be studied at temperatures up to 453 K. In a preliminary study of methyl esters heated in the presence of PBN, analysis of the ^{1}H and ^{14}N hyperfine coupling data obtained as a function of time demonstrated the formation of a succession of adducts[8] and attempts were made to characterise these by comparison with published data for PBN adducts.[9]

In the present paper we present a more detailed picture of the reactions of spin traps in the initial stages of oxidation of fatty acid methyl esters at high temperatures using PBN, [$^2H_{14}$]PBN, [$^2H_{15}$]PBN and 2-methyl-2-nitrosopropane (MNP) spin traps, selectively deuterated methyl oleates, as well as esters with natural isotopic abundances in conjunction with EPR and ENDOR spectroscopies. The effects of added α-tocopherol and variations in oxygen availability have also been investigated in order to provide additional supportive evidence for the proposed reaction pathways. Evidence is provided to show that the high temperature oxidation pathways for the lipid esters are different from those inferred for low-temperature oxidation.[10]

Experimental

Fatty acid methyl esters (>99% pure) were acquired from Nu Chek Prep, Inc. (Elysian, Minnesota) and stored under nitrogen at −15 °C. PBN, MNP and α-tocopherol were purchased from the Sigma Chemical Company (Poole, UK) and used without further purification, as was [$^2H_{14}$]PBN (*i.e.* with fully deuterated *tert*-butyl and phenyl groups), obtained from Oklahoma Medical Research Foundation Spin Trap Source (Oklahoma, USA), 9,10-dideuterooleic acid methyl ester, 8,8,11,11-tetradeuterooleic acid methyl ester (purity >99%) and [$^2H_{15}$]PBN (*i.e.* deuterated at the α-carbon as well as the *tert*-butyl and phenyl groups), which were obtained from Unilever Research (Vlaardingen, The Netherlands).

Samples for EPR measurements were prepared by dissolving the spin trap (10 mg g^{-1}, except where stated otherwise) in the lipid methyl esters at ambient temperature (approximately 10 min) at a variety of concentrations specified in the text. With MNP it was necessary to avoid exposure to light when making up these solutions, which were used immediately. All experi-

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ments used 0.09 g of ester in a 4.2 mm (ID) quartz EPR tube, either aerated and kept open to ensure constant atmospheric oxygen supply, sealed after partially degassing with helium for 20 min, or under reduced pressure. In an attempt to obtain spectra of each of the adducts from single specimens of the deuterated oleates (only small quantities were available), samples were heated with PBN according to a regime which involved progressive increases in temperature: 20 min at 353 K, followed by 30 min at 363 K, 60 min at 373 K, 4 h at 378 K, 30 min at 423 K, and for the tetradeuterated sample, 10 min at 443 K.

X-Band (*ca.* 9.5 GHz) EPR spectra were obtained on a Bruker EPS300E computer controlled spectrometer, in which the microwave cavity was preheated to the desired temperature (353–443 K) before inserting samples, and the temperature was maintained using a Bruker VT accessory. First and second derivative spectra were acquired over extended periods of time, commencing immediately after tuning (*ca.* 45 s after insertion of the sample into the microwave cavity) using the following instrumental parameters: microwave power 10 mW, modulation frequency 100 KHz, modulation amplitude 0.03 mT and a scan range of 6 mT. In the early stages of experiments (0–10 min), spectra were recorded using the Bruker fast digitiser (scan time 1 s), whereas longer runs were controlled by an automation program in which spectra were acquired with a scan time of 355 s.

For ENDOR measurements the samples were diluted with toluene (50%) and outgassed on a vacuum line to *vacua* < 10^{-5} Torr prior to recording spectra. ENDOR measurements were made using a Bruker ESP300E interfaced to a Bruker digital DICE ENDOR ESP360 spectrometer. Spectra were recorded by saturating individual EPR transitions at temperatures of 220 or 230 K using a microwave power of between 50 and 80 mW and 200 W radiofrequency power. In the presence of mixed spectra the ENDOR spectrum of the individual components could be obtained by saturating selective EPR transitions. The hyperfine splittings reported for all of the spectra in this paper were confirmed by simulation using the Bruker 'Simfonia' software package.

Results

EPR spectroscopy

PBN adducts. Formation of adducts was rapid at 378 K with each of the esters, and first derivative EPR spectra were characterised by a sextet hyperfine structure from the ^{14}N and one ^{1}H, that is very similar to that reported previously for grape-seed oil.[7] In aerated samples the adduct spectral intensities were similar for each of the esters in the early stages of oxidation at the highest PBN concentration used (56.5 mM), but appreciable differences were seen with longer periods of heating and also with lower PBN concentrations (Fig. 1), though similar alterations were apparent for each ester, even if at different rates. Integrating the spectral intensities in Fig. 1 over time shows a linear response to PBN concentration and an overall increase in the order linolenate < linoleate < oleate (Fig. 2), demonstrating that adduct stability mirrors the stabilities of the parent esters. In samples that were degassed with helium, curves with shapes similar to those in Fig. 1 were obtained, but with lower rates of generation and lower overall spectral intensity, confirming that oxygen availability has a critical influence on the free radical pathways in the heated lipids and that oxygen-derived radicals are of fundamental importance in the generation of the PBN adducts.

Although all spectra were superficially similar, there were small progressive variations in ^{1}H hyperfine splittings as a function of time in the spectra responsible for Fig. 1. Additional ^{1}H hyperfine structure, that was identical for methyl linolenate and linoleate, was seen in second derivative acquisitions and this was investigated further by use of [$^{2}H_{14}$]PBN, which gave

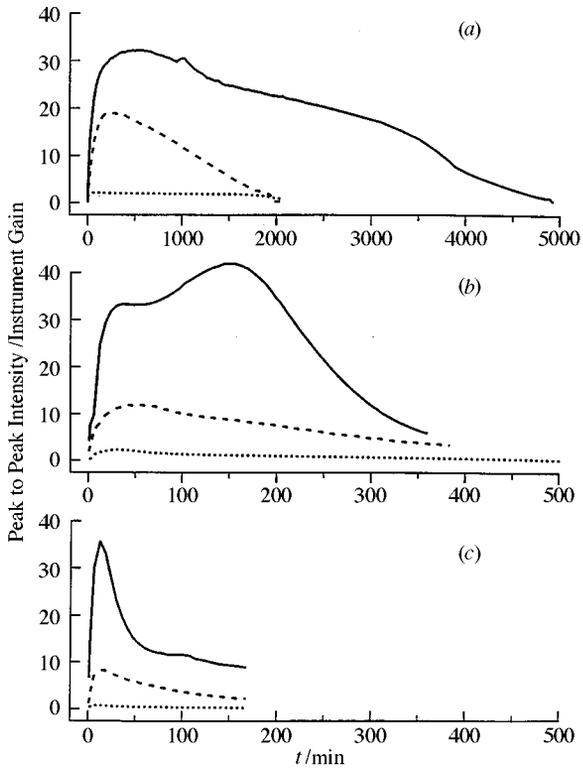


Fig. 1 Relative intensities as a function of time of the EPR spectra of adducts of PBN with radicals produced by heating (*a*) oleic, (*b*) linoleic and (*c*) linolenic methyl esters at 378 K in the presence of 10 mg (——), 2.5 mg (– – –), 0.25 mg (· · · · · ·) PBN g^{-1} lipid

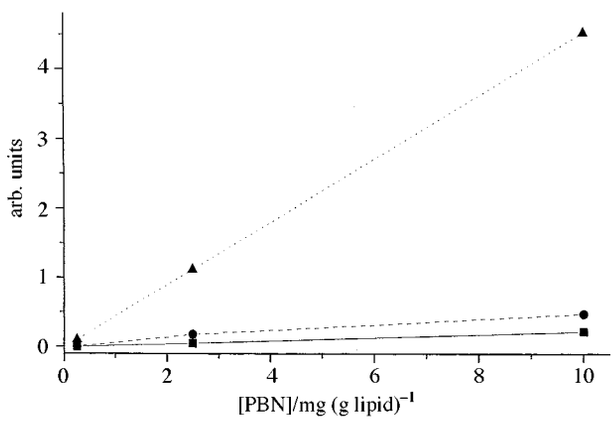


Fig. 2 Integrations over time of EPR spectral intensities of PBN adducts of lipids (· · ·▲· · · methyl oleate, —●— methyl linoleate, —■— methyl linolenate) heated at 378 K

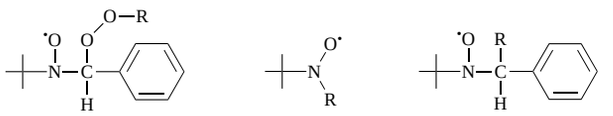
improved resolution by removing line broadening from unresolved ^{1}H hyperfine structure associated with the *tert*-butyl and phenyl groups and ENDOR spectroscopy.

This methodology does not discriminate between positionally different allylic additions which are supposed to happen concurrently. Thus, for instance, whenever addition to oleate is described for simplicity as yielding an adduct at position 9 (with a double bond shifted to position 10, see Table 2), this means one is expected to obtain quasi-equimolar mixtures of this adduct with the one at position 10 (with the double bond shifted to position 8). The various adducts believed to be formed are all represented in Scheme 2.

EPR of [$^{2}H_{14}$]PBN adducts. Three distinct EPR signals were observed when samples of the methyl esters were heated with [$^{2}H_{14}$]PBN (Fig. 3). Most spectra consisted of a mixture of more than one component and, of the individual spectra that are shown in Fig. 3, both (*a*) and (*c*) were obtained by selective subtraction of small amounts of spectrum (*b*), because although

Table 1 EPR hyperfine coupling constants and proposed structures for [$^2H_{14}$]PBN adducts of heated methyl esters of oleic, linoleic and linolenic acids[a] (R represents the fatty ester group bound by an allylic position)

Adduct	$a(^{14}N)$/mT	$a(^1H)$/mT
1	1.37	0.20(1)
2	1.49	0.21(1)
		0.056(1)
		0.035(2)
3	1.47	0.326(1)
		0.0405(2)



Adduct **1** Adduct **2** Adduct **3**

[a] The larger couplings are due to a lipid proton in adduct **2** and to the PBN proton in the other cases. Smaller couplings are attributed to unspecified β-protons (see text).

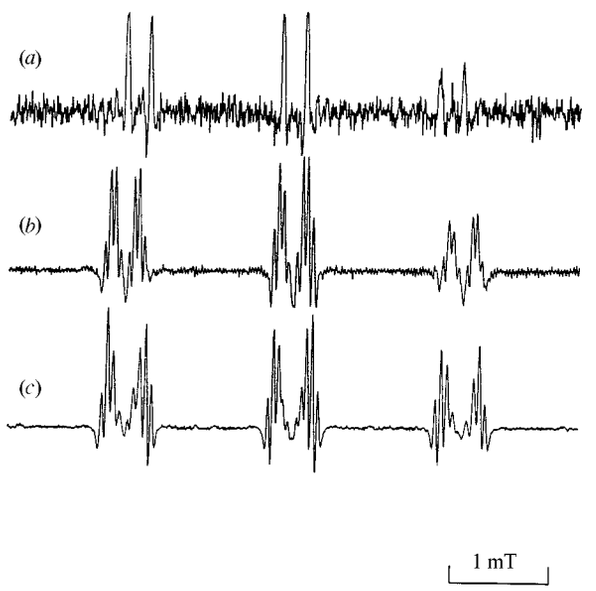


Fig. 3 Second derivative EPR spectra of (*a*) adduct **1**, (*b*) adduct **2** and (*c*) adduct **3** from lipid methyl esters heated in the presence of [$^2H_{14}$]-PBN

'pure' (*a*) and (*c*) spectra were obtained, these were of much poorer signal-to-noise ratio. The relevant EPR measured hyperfine parameters and the chemical structures proposed for these are presented in Table 1. The time to the appearance of the radical adducts in aerated samples was always in the order **1** < **2** < **3**, although adduct **3** was only seen in the presence of adduct **2**.

In aerated samples, the maximum intensity of the signal from adduct **2** was always higher than those from the other adducts and was, therefore, the major contributor to the intensity plots in Fig. 1. Adduct stability, also not surprisingly, decreased with increasing temperature. The adduct **1** signal was more intense for oleate than for the other esters, and its spectrum was most significant for samples oxidised at the lower end of the temperature range studied. Adduct **3** was most important for linoleate and linolenate oxidation and at the higher temperatures used. Insofar as EPR analysis is concerned, no other differences were apparent between the spectra of oleate, linoleate and linolenate.

Partial degassing of samples decreased the contribution of adduct **2** to the spectra and adduct **3** increased in importance for each of the esters. Opening the EPR tube to air after the formation of adduct **3**, however, led to the formation of adduct **2**, which increased in intensity rapidly, and became the main

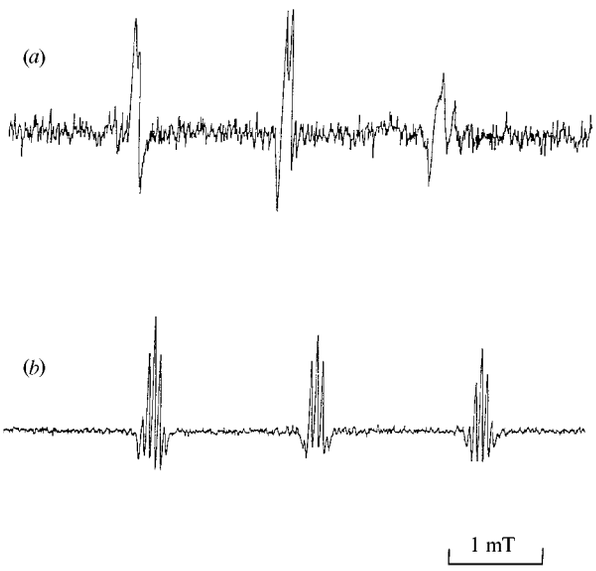


Fig. 4 Second derivative EPR spectra of (*a*) oleate adduct **1** and (*b*) linoleate adduct **3** formed with [$^2H_{15}$]PBN

spectral component, as was the case with samples that were exposed to air throughout their measurements.

When methyl oleate, linoleate and linolenate were heated with [$^2H_{14}$]PBN at high temperature under vacuum (*i.e.* very limited oxygen availability), only the spectrum of adduct **3** was observed.

Effects of added α-tocopherol. EPR spectra were also recorded for lipid esters heated with 5000 ppm α-tocopherol and [$^2H_{14}$]PBN. In partially-degassed samples of linoleate and linolenate methyl esters, only adduct **3** was observed after about 5 min at 373 K and there was no evidence for the spectra of either adducts **1** or **2**. Partially degassed samples of oleate gave no signal over a period of several hours heating in the temperature range 353–423 K.

[$^2H_{15}$]PBN adducts. Oleate was heated with [$^2H_{15}$]PBN using conditions appropriate to optimise the spectra of adducts **1** and **2** and linoleate was heated with [$^2H_{15}$]PBN and α-tocopherol (5000 ppm) in order to obtain predominantly adduct **3**. The spectrum of adduct **2** using [$^2H_{15}$]PBN was identical to that obtained with [$^2H_{14}$]PBN [Fig. 3(*b*)], whereas those of adducts **1** and **3** showed a loss of the large ^1H coupling (Fig. 4). In addition, multiplet structures were observed with splittings of *ca.* 0.030 and 0.045 mT, respectively, which are about one seventh of the $a(^1H)$ values obtained with [$^2H_{14}$]PBN, as expected for exchange of deuterium for hydrogen.[11]

PBN adducts of selectively deuterated oleate samples. Selected EPR spectra of methyl 9,10-dideuterooleate and 8,8,11,11-tetradeuterooleate samples (deuterated at the vinylic and allylic carbons, respectively) heated at temperatures between 353 and 443 K in the presence of [$^2H_{14}$]PBN are shown in Fig. 5.

Both esters produced weak spectra from adduct **1** at 353 K immediately after insertion of the samples into the microwave cavity along with small amounts of the other adducts, as was seen with the non-deuterated specimen; the 0.20 mT ^1H coupling was retained in this spectrum. With the degassed dideuterated ester there was a progressive increase in spectral intensity over the 20 min at 353 K and a noticeable growth of the adduct **3** component relative to that of adduct **1**. This continued at 363 K and the spectrum obtained after 30 min at this temperature consisted of a mixture of adducts **1** and **3** along with a small amount of adduct **2** [Fig. 5(*a*)]. Adduct **2** grew progressively in importance through the 373 and 378 K cycles and dominated the spectrum obtained at the end of the latter [Fig. 5(*b*)] and higher temperature. The spectrum of adduct **3** was virtually

Table 2 ENDOR hyperfine coupling constants for methyl esters of oleic, linoleic and linolenic acids 'MNP' radical adducts[a]

Methyl ester	Coupling (MHz)	Assignment
Oleate	4.5	H9
	1.5, 1.1	H8 (2[b])
Linoleate	4.6	H10 (non-conjugate adducts)
	4.2	H9 (conjugate adducts)
	1.6, 1.0	H8 (2[b]), (conjugate adducts); H11 (2[b]) (non-conjugate adducts)
Linolenate	4.6	H10 (non-conjugate adducts)
	4.2	H9 (conjugate adducts)
	1.6, 1.0	H8 (2[b]), (conjugate adducts); H11 (2[b]) (non-conjugate adducts)

[a] In each case it is assumed that the mixtures depicted in Scheme 2 are formed and for simplicity assignments are shown only for one position isomer. [b] Tentative assignments for smaller couplings compatible with the ENDOR spectra (and similar to those observed under EPR conditions), involve two non-equivalent protons in this position.

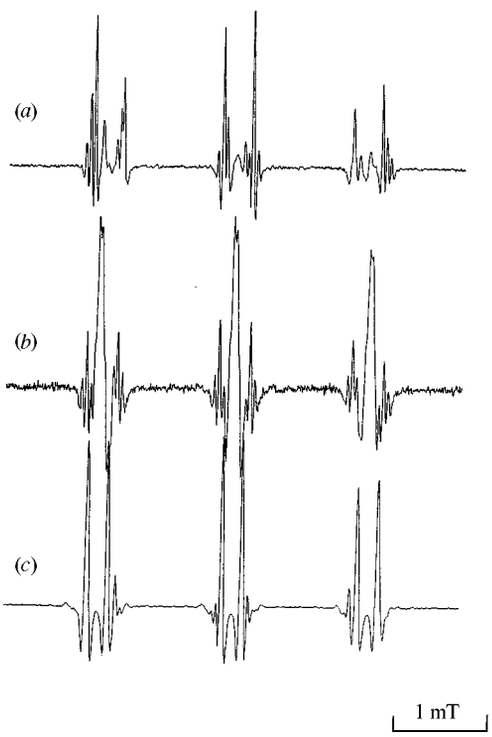


Fig. 5 Selected second derivative EPR spectra of deuterated oleate samples heated with [$^2H_{14}$]PBN (*a*) dideuterooleate after 20 min at 353 K and 30 min at 363 K, (*b*) dideuterooleate after 20 min at 353 K, 30 min at 363 K, 60 min at 373 K and 4 h at 378 K and (*c*) tetradeuterooleate after 20 min at 353 K, 30 min at 363 K, 4 h at 378 K, 30 min at 423 K and 10 min at 443 K

identical to that obtained with the non-deuterated ester apart from a small increase in the magnitude of the ^1H hyperfine splittings to 0.339 and 0.050 mT, whereas the major 0.21 mT ^1H hyperfine splitting was absent from adduct **2**. Thus the major ^1H coupling in adduct **2** must arise from the vinylic group of the lipid moiety and not the PBN proton, as is the case with adducts **1** and **3** and is generally assumed for all PBN adducts in spin trapping studies.

The contribution to the EPR spectra from adduct **1** persisted longer with the tetradeuterooleate ester and was identical to that obtained with the dideuterated and non-deuterated esters, confirming that the hyperfine structure originates in the spin trap moiety. As with the dideuterooleate, adduct **2** became important during the 378 K phase of the heating cycle and remained the dominant component in all of the subsequent spectra; the largest ^1H splitting in adduct **2** is the same as that seen with the non-deuterated esters, but the smaller splittings are absent, showing that they originate from the allylic carbons of the lipid. The spectrum of adduct **3** was only seen at the highest temperatures in the heating cycle and was never the major component in the spectra [Fig. 5(*c*)]. Although its ^{14}N hyperfine splitting at 1.47 mT remained unchanged from the value obtained with the non-deuterated and deuterated esters, the larger of the ^1H splittings showed a further increase from that obtained with the dideuterooleate to 0.352 mT, whereas the smaller splitting of 0.050 mT was the same as that with the dideuterooleate. The decrease in the reaction rate with tetradeuterooleate compared with the non-deuterated and dideuterated esters suggests that a bond involving an allylic proton is broken during the slowest step in the reaction sequence.

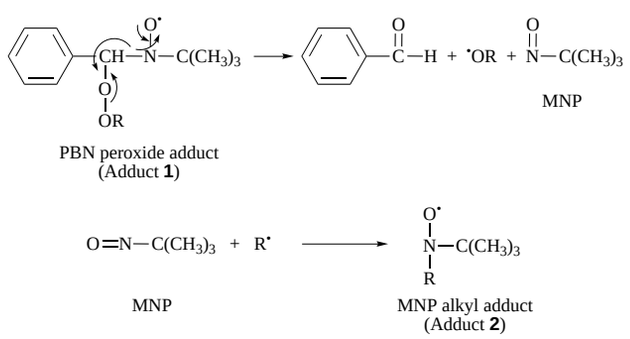
MNP adducts. The EPR spectra (not shown) obtained on heating the methyl esters with MNP at 353–443 K showed only one adduct signal. Hyperfine parameters are identical to those of the [$^2H_{14}$]PBN adduct **2** signal [Fig. 3(*b*)], though linewidths were larger in the MNP experiments. This result strongly suggests that adduct **2** formed in the presence of PBN is in fact an adduct of MNP and that PBN adduct **1** decomposes to MNP, which then reacts with lipid alkyl radicals in solution. A similar reaction has been reported by Janzen *et al.*[12]

ENDOR spectroscopy

ENDOR spectra were obtainable only under slightly different experimental conditions, namely temperature and added solvent. Though the larger couplings remain essentially unchanged relative to those found by EPR analysis, clear assignments of these as well as additional fine structure were evidenced due to the greater resolution of ENDOR and the use of specifically deuterated samples. Details are given below and summarised in Table 2 and Scheme 2.

Methyl oleate. Fig. 6 shows the ENDOR spectra for the BPN adduct of methyl oleate radical adduct **2** (spectrum *a*) together with the 9,10-dideuterooleate (spectrum *c*) and the 8,8,11,11-tetradeuterooleate analogues (spectrum *d*). The MNP adduct is shown in Fig. 6(*b*).

The similarity between spectra in Fig. 6(*a*) and (*b*) indicates that the adducts are identical inferring that the PBN adduct of methyl oleate has rearranged to give an MNP adduct (see Scheme 1). As pointed out earlier a similar rearrangement for



Scheme 1

the PBN peroxide adduct has been proposed by Janzen *et al.*[12] Note, such an adduct would not have the ^1H coupling typical of PBN adducts. Confirmation of such a rearrangement is given by the EPR results, obtained from the use of [$^2H_{15}$]PBN, which

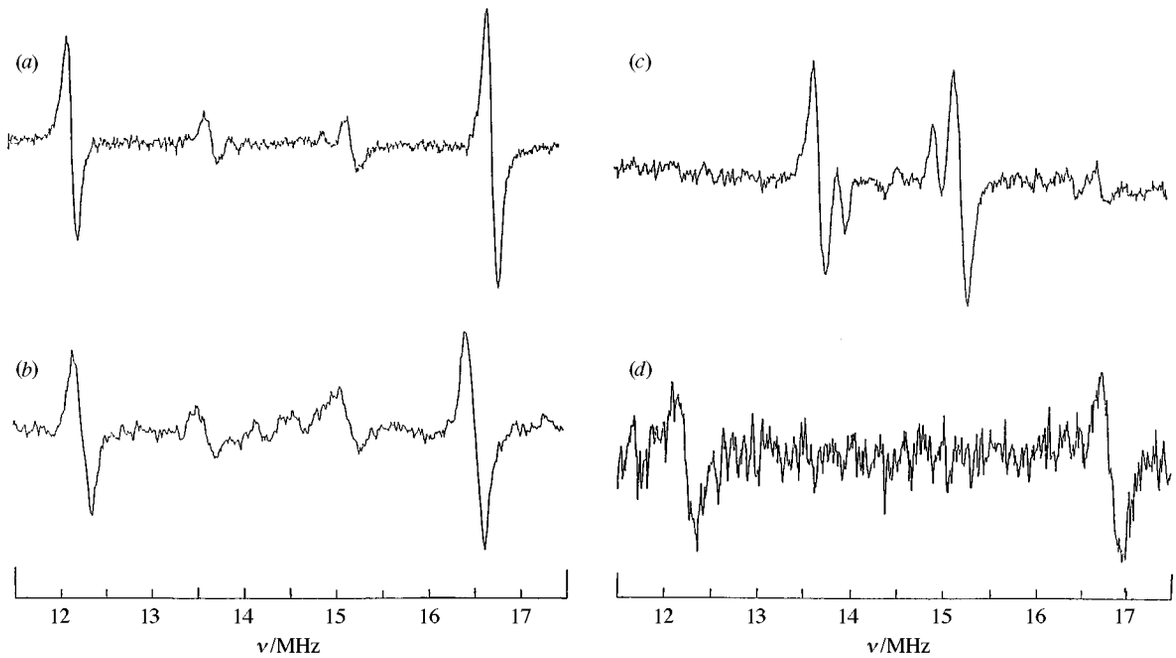


Fig. 6 ENDOR spectra of polyunsaturated methyl esters adduct **2** in (1:1) toluene–ester: (*a*) linoleate with PBN, (*b*) linoleate with MNP, (*c*) linolenate with PBN and (*d*) linolenate with MNP

showed that the large proton coupling did not arise from the nitrone proton.

If Fig. 6(*a*) is compared with Fig. 6(*c*) then it is obvious that the largest coupling of 4.5 MHz (0.1607 mT) must arise from the protons at either position 9 and/or 10 from the fatty acyl group. Also in each of them, apart from spectrum (*d*), there is a coupling of 1.5 MHz (0.0536 mT) and one of 1.1 MHz (0.0393 mT) which could be attributed to the protons at positions 8 and/or 11. In the case of the MNP adduct in Fig. 6(*b*) a coupling of 0.5 MHz (0.0179 mT) is also present. This is believed to arise from traces of the normal breakdown product of MNP *i.e.* di-*tert*-butyl-aminoxyl (DTBA).

Methyl linoleate and methyl linolenate. The ENDOR spectra, not shown, obtained with methyl linoleate and linolenate heated with PBN are very similar to those obtained from the same compounds with MNP. The main similarities consisting of couplings at *ca.* 4.6 (0.164), 4.2 (0.15), 1.6 (0.057), 1.0 (0.0357) and 0.5 MHz (0.0179 mT). The latter corresponds to the MNP decomposition product (DTBA) which is only apparent when MNP is used as the spin trap. The coupling of *ca*. 4.2 MHz was not present in oleate, and the rest correspond to features present in oleate moieties. This indicates that adducts obtained from PBN samples could be MNP adducts as was found for the case of the oleate methyl ester.

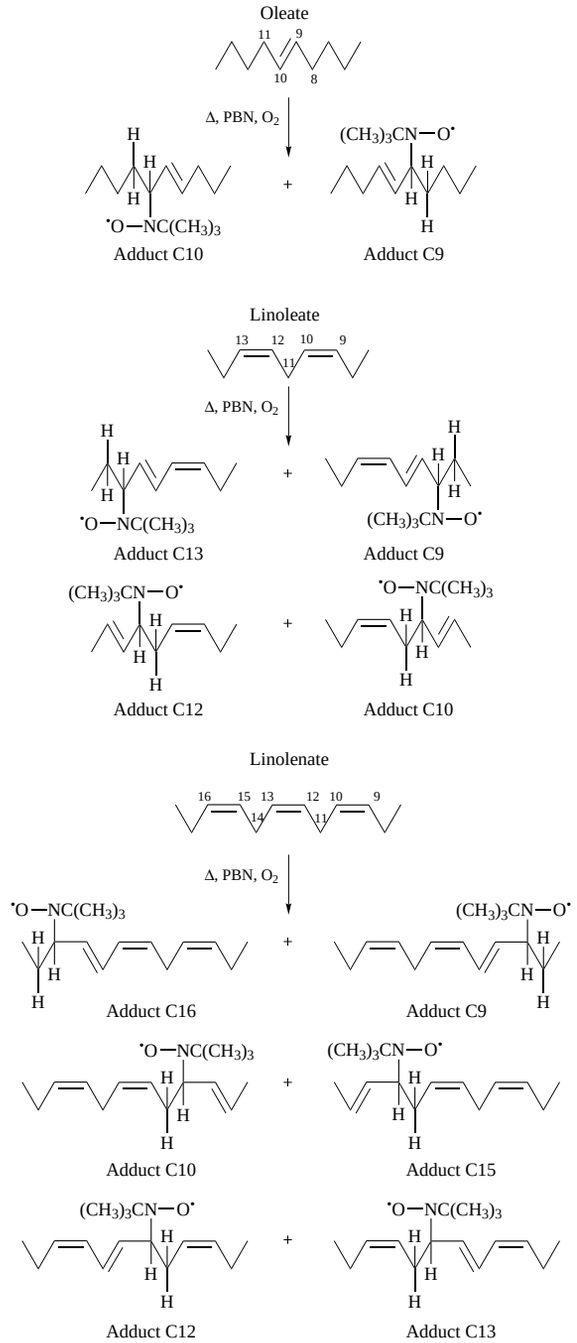
Comparison of these results for methyl linoleate with published ENDOR data[10] of the room temperature oxidation of selectively deuterated methyl linoleates in the presence of MNP means that at a high temperature both conjugated and non-conjugated MNP methyl linoleate adducts seem to be formed.

Since the autoxidation mechanism of methyl linoleate at room temperature would only lead to conjugated hydroperoxides, the ENDOR results indicate that different mechanisms occur or concur at high temperature resulting in the simultaneous formation of conjugated and non-conjugated hydroperoxides. The feature at 4.2 MHz (0.15 mT) originates from a proton whose vicinity is different to that of protons encountered in the oleate moieties. Hence it is part of a longer dienic conjugated system. Exactly the same reasoning applies for linolenate, but for this substrate the appearance of both conjugated and non-conjugated adducts due to oxidation at room temperature had been reported.[10] The smaller coupling constants 1.6 (0.057) and 1.0 MHz (0.0357 mT) of the linoleate and linolenate adduct spectra were generally attributed to β-protons by analogy with the deuterated oleate results. Scheme 1 shows a hypothetical decomposition mechanism of a PBN peroxide adduct into MNP and the subsequent formation of an alkyl MNP adduct based on ref. 12.

Discussion

Although there have been many reports of spin trapping of free radicals from oxidising lipids,[9] most have involved reactions close to ambient temperature and, in addition, the assignment of structures to adducts is not always unambiguous. The present investigation was performed as a result of a preliminary observation that there were small changes with time in the spectral parameters for PBN adducts formed in heated lipids. By using deuterated PBN, we have now shown that three distinct adducts are formed by reaction with pure lipid esters at elevated temperatures, each EPR spectrum being based on sextet structures from ^{14}N and one ^{1}H (although other smaller ^{1}H splittings are also seen with two of the adducts). By selective use of isotopic substitutions and careful control of experimental conditions, we are now able to present a relatively simple explanation of the various reactions involved. (It should be noted that even under conditions when the EPR tubes were open to the air, the samples eventually reached a state of limited oxygen supply because of its low solubility.[13])

By comparing EPR spectra obtained with [$^{2}H_{14}$]PBN and [$^{2}H_{15}$]PBN, and with non-deuterated and selectively deuterated esters, we have shown that the largest ^{1}H hyperfine splitting is derived from the ^{1}H attached to the α-carbon atom of the spin trap in adducts **1** and **3**, whereas it originates from the vinylic group of the lipid moiety in adduct **2** of oleate, and presumably from similar sites in the other esters. Adduct **1** is assigned to a peroxyl–PBN adduct, because of the requirement of oxygen for its formation and the similarity of its parameters to other published PBN–peroxyl radical adducts.[9] Adduct **3** is assigned to an alkyl–PBN adduct, because of its generation under conditions when oxygen availability would be expected to be extremely low (*i.e.* with degassed samples or in the presence of the antioxidant α-tocopherol); its parameters are also similar to those reported for alkyl radical adducts of PBN.[9] The parameters for adduct **2** resemble those reported for alkoxyl adducts of PBN,[9] but the present measurements with [$^{2}H_{15}$]PBN show that this assignment cannot be correct. Indeed, the similarity



Scheme 2 Free radical transformations and proposed structures of the radical adducts

between its parameters and those of the spectrum that was obtained when MNP was used as the spin trap, indicates that it corresponds to an MNP adduct and, as stated above, the ^{1}H splittings originate in the lipid moiety. Adduct **2** is almost certainly an alkyl radical adduct because MNP traps alkyl radicals more efficiently than oxygen-centred radicals,[9] due the inherent instability of MNP–oxygen radical adducts.

The observation that adduct **3** formed in degassed samples was converted rapidly to adduct **2** when the EPR tube was opened to the air is probably the consequence of formation of adduct **1** being slower than its decomposition to MNP and the latter being a more efficient scavenger of alkyl radicals than PBN.

By using relatively large concentrations of PBN (which does not behave as either a pro- or an anti-oxidant[14]), the reagent remains available over a period of many hours and a steady state concentration of adduct **1**, which is just detectable by EPR spectroscopy, was established as the rates of oxygen dissolution into the ester solutions balanced its removal. MNP generated on decomposition of adduct **1** reacts rapidly with alkyl radicals to generate adduct **2**. The observation that for aerated samples the spectral intensity of adduct **2** increased progressively and dominated for long periods in the time sequence indicates that the alkyl–MNP adducts are more stable than the peroxyl–PBN adducts. Adduct **2** was not formed under conditions of severe anoxia because MNP is not generated (because of an absence of the peroxyl–PBN precursor) and alkyl radicals resulting from thermal decomposition of the lipids react with PBN, albeit at a relatively low rate, yielding adduct **3**.

The ^{1}H hyperfine structure of adduct **2** arises entirely from the lipid moiety, with the major splitting originating from the vinylic proton, which presumably forms the α-carbon atom in the MNP adduct. In Scheme 2 we show proposed free radical transformations and the structure of the predominant MNP adducts formed from methyl oleate, linoleate and linolenate, respectively, in experiments where the added spin trap was PBN. Assignments of couplings of 4.2 MHz to the proton attached to the carbon bearing the MNP moiety in adducts containing a dienylic system in polyunsaturated esters is due to its absence in oleate and confirmed by its greater relevance in linolenate than in linoleate (see Table 2 and Scheme 2).

The smaller ^{1}H hyperfine splittings of adducts **2** and **3** arise presumably from protons on the lipid moiety that are more remote from the aminoxyl group of the spin traps.

Finally, the results obtained with samples containing α-tocopherol show that the generation of adduct **2** is greatly inhibited and indicate that this antioxidant scavenges lipid peroxyl radicals far more effectively than PBN thereby inhibiting the formation of the PBN–peroxyl adduct **1** and the generation of MNP through its subsequent decomposition; the resulting EPR spectra are primarily associated with PBN adducts of alkyl radicals.

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