Free Rad. Res., Vol. 22, No. 5, pp. 441–482
Reprints available directly from the publisher
Photocopying permitted by license only

DETECTION OF ALDEHYDES AND THEIR CONJUGATED HYDROPEROXYDIENE PRECURSORS IN THERMALLY-STRESSED CULINARY OILS AND FATS: INVESTIGATIONS USING HIGH RESOLUTION PROTON NMR SPECTROSCOPY

RACHEL M. HAYWOOD[1,2], ANDREW W. D. CLAXSON[1], GEOFFREY E. HAWKES[2], DAVID P. RICHARDSON[3], DECLAN P. NAUGHTON[1], GREGORY COUMBARIDES[2], JANE HAWKES[4], EDWARD J. LYNCH[1], and MARTIN C. GROOTVELD[1]

[1]*The Inflammation Research Group, The London Hospital Medical College, London E1 2AD, UK*
[2]*Department of Chemistry, Queen Mary and Westfield College, Mile End Road, London E1 4NS, UK*
[3]*Group Scientific and Quality Services, Nestlé UK Ltd, St George's House, Croydon, Surrey*
[4]*Department of Chemistry, King's College London, Strand Campus, Strand, London WC2R 2LS, U.K.*

(Received August 18th, 1994, in revised form September 21st, 1994)

High field (400 and 600 MHz) proton NMR spectroscopy has been employed to investigate the thermally-induced autoxidation of glycerol-bound polyunsaturated fatty acids present in intact culinary frying oils and fats. Heating of these materials at 180°C for periods of 30, 60 and 90 min. generated a variety of peroxidation products, notably aldehydes (alkanals, trans-2-alkenals and alka-2,4-dienals) and their conjugated hydroperoxydiene precursors. Since such aldehydes appear to be absorbed into the systemic circulation from the gut in vivo, the toxicological significance of their production during standard frying practices is discussed.

INTRODUCTION

A high percentage of humans are continually exposed to oxidised oils and fats in the diet which arise from either shallow or deep-fat frying processes, and the possibility that regular consumption of such materials may be deleterious to human health has evoked much interest (reviewed in[1]). The most important reaction involved in the oxidative degradation of lipids is the autoxidation of polyunsaturated fatty acids (PUFAs), a process which occurs during the heating of culinary oils and fats. PUFAs are particularly susceptible to oxidative damage by virtue of the facile abstraction of one of their *bis*-allylic methylene group hydrogen atoms on exposure to light or radical species of sufficient reactivity, a process facilitated by the low bond dissociation energy

Correspondence to: Dr M C Grootveld, The Inflammation Research Group, London Hospital Medical College, ARC Building, 25–29 Ashfield Street, London E1 2AD, UK.

of their methylene group C–H bonds. Subsequently, one major reaction pathway for the resulting resonance-stabilised carbon-centred pentadienyl lipid radical generated in this manner involves its interaction with molecular oxygen to produce a peroxyl radical which in turn can abstract a hydrogen atom from an adjacent PUFA to form a conjugated lipid hydroperoxydiene and a further pentadienyl lipid radical species. In the absence of sufficient quantities of chain-terminating, lipid-soluble antioxidants such as vitamin E (α-tocopherol, α-TOH), the process is repeated many times and represents an autocatalytic, self-perpetuating chain reaction. Conjugated diene lipid hydroperoxides are particularly unstable at standard frying temperatures (ca. 180°C) and are degraded to a wide variety of secondary peroxidation products which include saturated and unsaturated aldehydes, *di-* and epoxyaldehydes, lactones, furans, ketones, oxo- and hydroxy-acids, and saturated and unsaturated hydrocarbons. Moreover, during the later stages of the peroxidation process, many polymerisation reactions occur.

The aldehydes generated have the capacity to exert a range of toxicological effects in view of their high reactivities with critical biomolecules *in vivo*[2] (e.g., endogenous thiols such as glutathione, free amino acids, proteins such as low-density-lipoprotein and DNA).

In view of the above observations, the detection and quantification of specific products arising from the oxidation of culinary oils and fats during normal frying procedures is of paramount importance. Previous investigations of this nature have been hampered by a requirement for the concentrations of each component with putative toxicological properties to be individually measured by labour-intensive, time-consuming laboratory methods,[3] some of which lack specificity [e.g., spectrophotometric determination of malondialdehyde (MDA) involving chromophore development on its reaction with thiobarbituric acid (TBA)[4]]. The multicomponent analytical ability of high field nuclear magnetic resonance (NMR) spectroscopy, however, now serves to overcome these problems. Indeed, this technique offers major advantages over alternative methods since it permits the rapid, non-invasive simultaneous study of complex mixtures of components present in biological samples (e.g., biofluids such as human blood plasma and urine[5–7]), and foodstuffs[8–10], and generally requires no knowledge of sample composition prior to analysis. Moreover, chemical shifts, coupling patterns and coupling constants of signals present in NMR spectra of such samples offer much valuable information regarding the structures of molecules present.

In this communication we report for the first time the applications of high field proton (^{1}H) NMR spectroscopy in determining the nature and levels of potentially toxic PUFA autoxidation products (notably aldehydes and their precursors such as isomeric conjugated hydroperoxydienes) which arise during the heating of PUFA-containing culinary oils and fats according to standard frying practices. The dietary, physiological and toxicological significance of the results obtained are discussed.

MATERIALS AND METHODS

Thermal treatment of commercially-available culinary oils and fats.

Corn (maize) oil [containing 57% (w/w) polyunsaturates, 30% (w/w) monounsaturates and 13% (w/w) saturates], sunflower seed oil [64% (w/w) polyunsaturates, 22% (w/w) monounsaturates, 14% (w/w) saturates and an unspecified concentration of added α-TOH], soyabean oil [52% (w/w) polyunsaturates, 21% (w/w) monounsaturates and

13% (w/w) saturates], rapeseed oil [31% (w/w) polyunsaturates, 59% (w/w) monounsaturates and 6% (w/w) saturates], groundnut (peanut) oil [34% (w/w) polyunsaturates, 45% (w/w) monounsaturates and 21% (w/w) saturates] and extra virgin olive oil [9% (w/w) polyunsaturates, 73% (w/w) monounsaturates and 14% (w/w) saturates] were commercially-available samples purchased from local supermarkets. Two commonly-utilised commercial brands of lard (coded L-I and -II) containing 4–11% (w/w) polyunsaturates, an unspecified monounsaturate content and 45–57% (w/w) saturates (L-I), and 10% (w/w) polyunsaturates, 45% (w/w) monounsaturates, 45% (w/w) saturates and an unspecified content of the synthetic dietary antioxidant butylated hydroxytoluene (L-II) were similarly obtained. Samples of control (unheated) and repeatedly-used culinary frying oils were kindly supplied by a fast-food/take-away establishment.

Pentanal, hexanal, *trans*-2-heptenal and *trans*-2-nonenal were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and the methyl, etyhyl, and propyl esters of linoleic and oleic acids were obtained from Sigma (Poole, Dorset, U.K.). Deuterated NMR solvents (C^2HCl_3, $C^2H_3O^2H$ and ^2H_2O) were purchased from Goss Scientific Ltd. (Great Baddow, Essex, U.K.).

Samples of each of the above culinary oils (20 or 25g) and fats (10g) were placed in glass vessels (details of which are given below) and heated at a temperature of 180°C on an electronically-controlled hot-plate in the presence of atmospheric O_2 for periods of up to 90 min. Aliquots (2.0 ml) of the oils and fats were removed at time-points of 30, 60 and 90 min., and then cooled to ambient temperature prior to storage as described below and ^{1}H NMR analysis. Selected culinary oils and fats were subjected to a further 30 min. heating episode at an elevated temperature (250°C). The temperature of these samples was continuously maintained at 180 ± 3°C (or 250 ± 5°C, where appropriate) throughout the heating process using a calibrated thermometer.

Since the nature and size (capacity) of the glassware employed to heat the above materials was found to exert a marked influence on the concentrations of thermally-induced autoxidation products generated, the same size and type of vessel was utilised to heat the same class of culinary oil or fat for the purpose of comparative, quantitative ^{1}H NMR evaluations. The glass vessels employed for each series of heating episodes comprised 25–250 ml volume beakers (corn, sunflower seed and rapeseed oils, and lard sample L-I), 25–100 ml volume conical flasks (corn, groundnut and extra virgin olive oils, and lard sample L-II), and 2–10 ml volume sample tubes (soyabean oil, and the methyl, ethyl and propyl esters of oleic and linoleic acids). The class and size of the glassware employed, together with the surface area of the culinary oil or fat heated therein (10,20 or 25g quantities) are clearly specified in the text (Section 3).

Beef and lamb fat samples (40g) were heated in a conventional domestic fan-assisted oven at a temperature of 180°C for periods of 74 and 90 min. respectively.

Samples were stored in 2 ml capacity glass sample tubes in the dark at ambient temperature for durations of up to 2,112 hr. The storage time under these conditions was also found to influence the concentrations of ^{1}H NMR-detectable, PUFA-derived peroxidation products in thermally-stressed culinary oils and fats, and hence each series of samples investigated were stored for exactly the same time periods prior to ^{1}H NMR analysis. For each group of samples, the period of storage time was carefully noted and is also specified in the text.

Model PUFA compounds (methyl, ethyl and proplyl esters of linoleic and oleic acids) were heated and stored in a similar manner prior to ^{1}H NMR analysis.

Proton NMR measurements

Proton (^{1}H) NMR measurements on the above samples were conducted on Bruker AMX-600 [University of London Intercolleigate Research Services (ULIRS), Queen Mary and Westfield College Facility, University of London, U.K.] or Bruker AMX-400 (ULIRS, King's College Facility, University of London, U.K.) spectrometers operating at frequencies of 600.13 and 400.13 MHz respectively and a probe temperature of 298 K. Typically, a 0.30 ml aliquot of each culinary oil and *mono*- or *di*-unsaturated fatty acid alkyl esters was diluted to a volume of 0.90 ml with deuterated chloroform (C^2HCl_3) which provided a field frequency lock, and these samples were then placed in 5-mm diameter NMR tubes. For culinary fats (lard and dripping), accurately weighed samples (ca. 50 mg.) were directly dissolved in 0.60 ml of C^2HCl_3, the solutions thoroughly rotamixed and then transferred to 5-mm diameter NMR tubes. For experiments which involved the quantification of NMR-detectable autoxidation products, samples were treated with 1,3,5-trichlorobenzene (final concentration 1.10×10^{-3} mol.dm^{-3}) which served as an internal quantitative standard ($\delta = 7.227$ ppm in C^2HCl_3 solution).

Further 0.30 ml aliquots of control and heated (60 min. at 180°C) samples of ethyl linoleate were also diluted to a final volume of 0.90 ml with C^2HCl_3 and then treated with 0.05 or 0.03 ml volumes of ^2H_2O respectively. The mixtures were thoroughly rotamixed, equilibrated at ambient temperature for a 45 min. period, centrifuged and the predominant, lower C^2HCl_3 phase removed for ^{1}H NMR analysis as described above.

Typical pulsing conditions were: 128 or 256 free induction decays (FIDS) using 32,768 or 65,536 data points, 72° pulses, a relaxation delay of 2.00 s and an acquisition time of 1.28 s. The spectral width was 7,246 Hz. Two-dimensional shift-correlated ^{1}H NMR (COSY) spectra were recorded using the standard sequence of Aue *et. al.*,[11] with 2,048 data points in the t_2 dimension, 512 increments of t_1, a relaxation delay of 2.00 s, and 48 transients. Exponential line-broadening functions of 0.20 Hz were employed for purposes of processing.

Chemical shifts were referenced to tetramethylsilane ($\delta = 0.00$ ppm, internal) and/or residual chloroform ($\delta = 7.262$ ppm). Resonances present in spectra of culinary oils and fats were assigned by a consideration of chemical shift values, coupling patterns and coupling constants. The molecular nature of particular classes of aldehydes detectable in spectra of thermally-stressed culinary oils was confirmed by standard additions of authentic, commercially-available compounds (alkanals and *trans*-2-alkenals). The relative intensities of selected signals were determined by electronic integration, and the concentrations of aldehydes present were computed by comparing their resonance areas with that of the added 1,3,5-trichlorobenzene.

Selective estimation of the bi-functional aldehyde MDA in culinary oil samples.

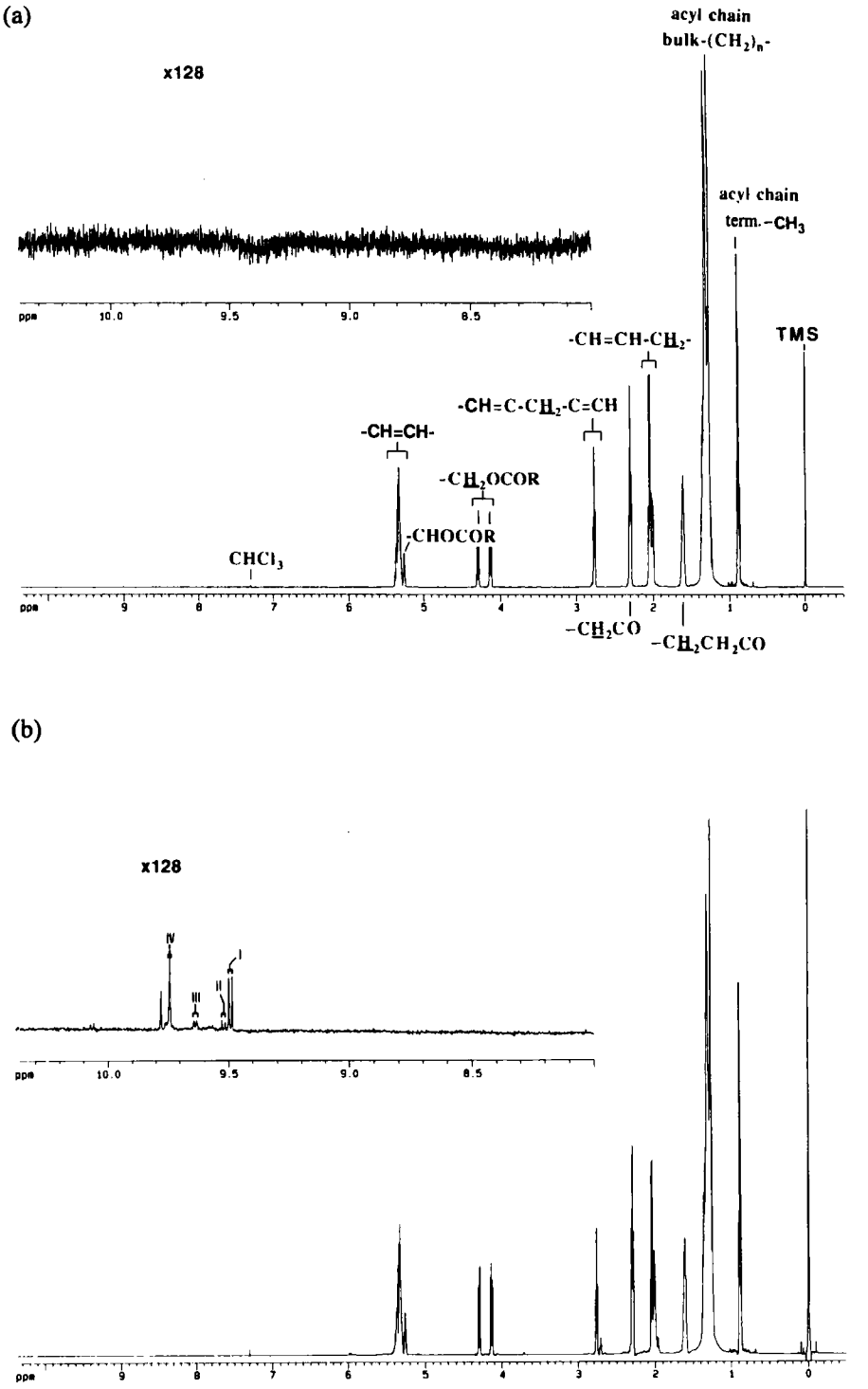
Typically, 0.75 ml aliquots of a 1.00g dm^{-3} solution of thiobarbituric acid in doubly-distilled water was added to 2.00 ml volumes of control and thermally-stressed culinary oil samples, the mixture thoroughly rotamixed, centrifuged (20 min., 500g), the lower (aqueous) phase removed and then heated at a temperature of 96°C for 20 min. A 0.30 ml aliquot of the resulting pink-coloured solution was then diluted to a final volume of 4.00 ml with doubly-distilled water prior to spectrophotometric analysis. Zero-order and corresponding second-derivative electronic absorption spectra of these samples were obtained on a Unicam UV-2 spectrophotometer, and MDA concentrations were determined using an ε_{532} value for the 2:1 TBA:MDA adduct of 1.56×10^5 M^{-1}.cm^{-1}.[22]

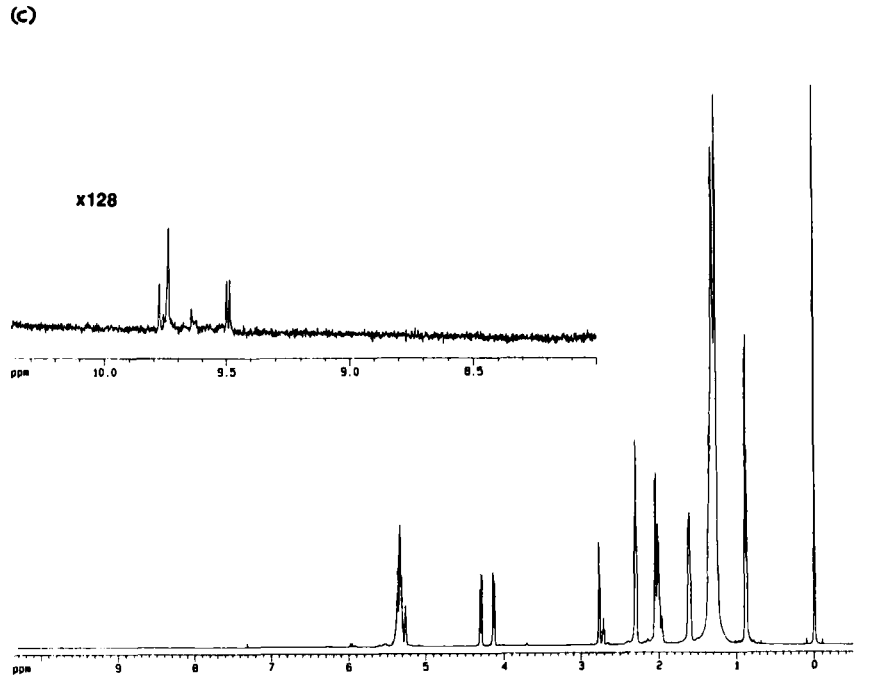
RESULTS

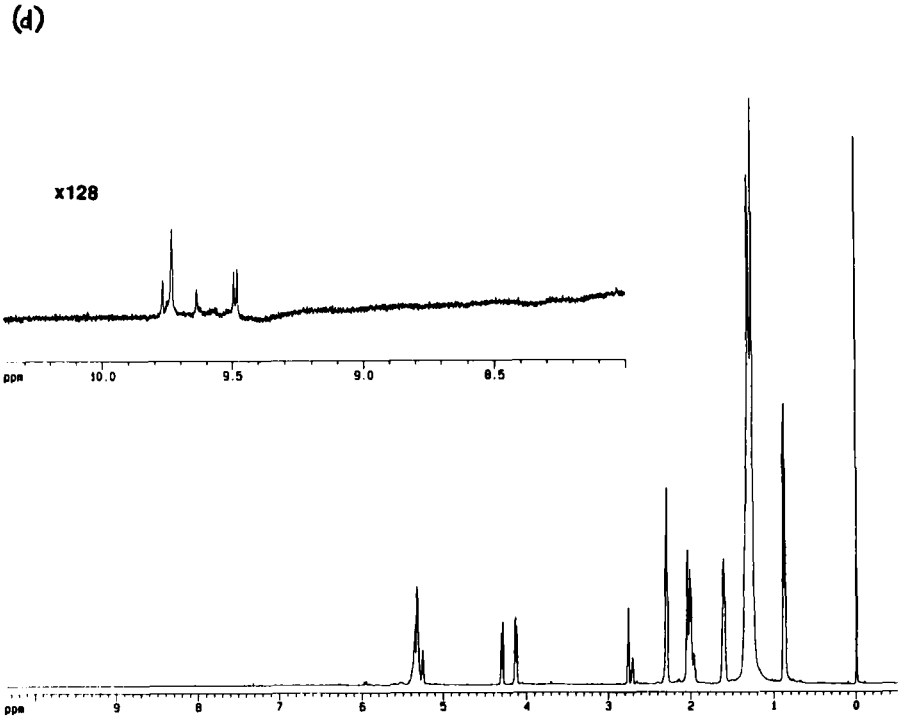
High field proton (^{1}H) NMR analysis demonstrated the thermally-induced consumption of glycerol-bound PUFAs in a variety of commonly utilised culinary oil samples [corn (maize) oil, soyabean oil, sunflower seed oil, rapeseed oil and groundnut oil], and also provided much detailed information regarding the precise molecular nature of autoxidation products generated. Figure 1 shows the complete, and expanded 8.00–10.40 ppm regions of 600 MHz ^{1}H NMR spectra of a commercially-available sample of corn oil acquired before, and after heating at a temperature of 180°C for periods of 30, 60 and 90 min. The spectra obtained show clear and selective reductions in the intensities of the *mono*- and *bis*-allylic-CH_2 group resonances ($\delta = 2.03$ and 2.76 ppm respectively), and the olefinic proton signal ($\delta = 5.38$ ppm) after subjecting this sample to episodes of heating in the above manner. The intensity of the PUFA *bis*-allylic-CH_2 group resonance diminished to approximately 50% of its control value after thermal stressing at 180°C for 90 min.

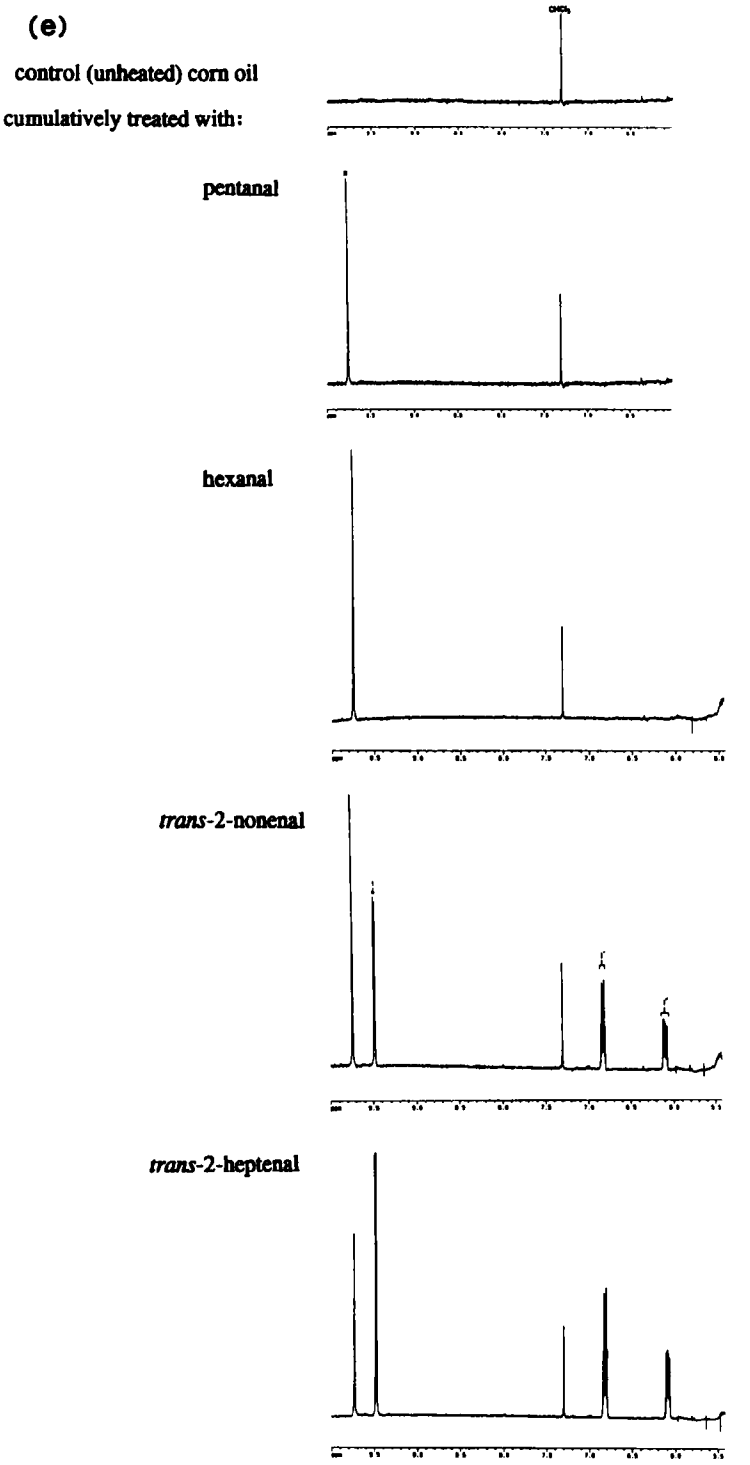
The aldehydic (9.30–9.80 ppm) regions of these spectra contain prominent doublets located at 9.48, 9.52 and 9.63 ppm ($j = 8.2$ Hz in each case) and triplets at 9.74 and 9.78 ppm ($^3j = 1.7$ and 1.6 Hz respectively). Two-dimensional COSY ^{1}H NMR spectra of thermally-stressed corn oil samples showed clear connectivities between the 9.48 ppm doublet and resonances centred at 6.85 (doublet of triplets, j 6.9, 15.3 Hz) and 6.10 ppm (doublet of doublets, j 15.3, 7.7 Hz), the 9.52 ppm doublet and signals at 7.07 (doublet of doublets, j 15.5, 10.1 Hz) and 6.04 ppm (multiplet), and the 9.74 ppm triplet and a multiplet located at 2.44 ppm (data not shown), demonstrating that the 9.48 and 9.52 ppm doublets arise from unsaturated aldehydes (the former from an unsubstituted *mono*-α,β-unsaturated adduct such as *trans*-2-heptenal, -octenal and/or -nonenal), and that the 9.74 ppm triplet with a small ^3j value is assignable to a saturated aldehyde such as pentanal or hexanal [the ^{1}H NMR spectrum of pentanal in C^2HCl_3 solution contained an α-CH_2 group resonance (dt) centred at 2.43 ppm]. The aldehydic doublet located at 9.52 ppm, together with its linked olefinic multiplet resonances ($\delta = 7.07$ and 6.04 ppm) are assignable to an alka-2,4-dienal adduct since spectra of *trans*, *trans*-nona-2,4-dienal and -hepta-2, 4-dienal in C^2HCl_3 solution also contain this series of signals with identical chemical shift values, coupling patterns and coupling constants.[12] However, in ^{1}H spectra of thermally-stressed culinary oils these resonances are assignable to *trans,trans*-nona- and/or *trans,trans*-deca-2,4-dienals since these aldehydic fragments are the predominant alka-2,4-dienals arising from the peroxidation of glycerol-bound linoleate,[2] the latter via cleavage of the alkoxyl radical derived from its 9-hydroperoxide. The two-dimensional COSY ^{1}H NMR spectra acquired also revealed that the relatively weak 9.63 ppm aldehydic proton doublet had connectivities with olefinic proton multiplets centred at 7.39 (apparent doublet of doublets) and 6.15 ppm. In view of the chemical shift values and coupling patterns observed, this series of linked resonances are tentatively assigned to 4-hydroxy-*trans*-2-nonenal (HNE).

Further useful information regarding the identities of the above signals was provided by 'spiking' control (unheated) and thermally-stressed culinary oils with aldehydes known to be generated from the autoxidation of PUFAs, and Figure 1(e) shows the 5.50–10.00 ppm regions of ^{1}H NMR spectra acquired on an unheated corn oil sample cumulatively treated with pentanal, hexanal, *trans*-2-nonenal and *trans*-2-heptenal in the given order. Data obtained are fully consistent with the assignments made using two-dimensional COSY ^{1}H NMR spectroscopy described above. Although distinction between the two classes of aldehydes (α,β-unsaturated and saturated) was facile, we were unable to resolve the aldehydic and olefinic signals of *trans*-2-nonenal









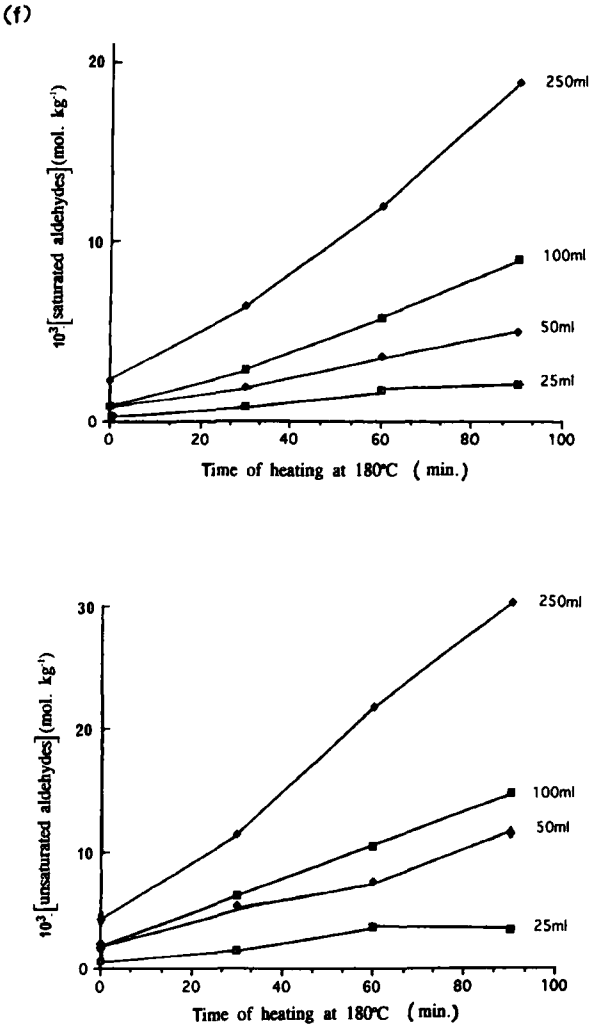


FIGURE 1. 600 MHz ^{1}H NMR spectra of a commercially-available sample of corn (maize) oil obtained (a) before, and at (b) 30, (c) 60 and (d) 90 min. after heating at a temperature of 180°C in the presence of atmospheric 0_2. The insets show the expanded 8.00–10.40 ppm (aldehydic) regions of the spectra shown. The culinary oil (25g) was heated in a 50 ml volume conical flask (oil surface area 15.91 cm^2) and samples removed at the above time-points were stored in the dark at ambient temperature (22°C) for a period of 72 hr. prior to ^{1}H NMR analysis (the unheated control sample was stored in the same manner for an equivalent length of time). (e), 5.50–10.00 ppm regions of 600 MHz ^{1}H NMR spectra of a control (unheated) corn oil sample cumulatively treated with pentanal, hexanal, *trans*-2-nonenal and *trans*-2-heptenal (1.00×10^{-1} mol.kg^{-1}) in the given order. Typical spectra are shown. (f), Plots of the total concentrations of saturated and unsaturated aldehydes (mol. kg^{-1}) generated versus time of heating at 180°C for 18.48g quantities of the above commercially-available corn oil sample heated in 25, 50, 100 and 250 ml volume beakers (these samples were subjected to ^{1}H NMR analysis immediately after each period of heating). The concentrations of aldehydes present at a heating time of 0 min. represent those generated during the elevation of the oil temperature from 22 (ambient) to 180°C. Abbreviations: $CHCl_3$ residual chloroform; TMS, tetramethylsilane (internal chemical shift reference). I, II, III and IV represent the aldehydic (–CHO) group protons of *trans*-2-alkenals, alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals and alkanals respectively. I′ denotes the olefinic proton resonances of the added *trans*-2-alkenals.

from those arising from *trans*-2-heptenal, and similarly, the aldehydic triplet resonance of pentanal was irresolvable from that of hexanal, even at an operating frequency of 600 MHz. However, the 9.48 ppm doublet present in ^{1}H spectra of thermally-stressed culinary oils is probably largely attributable to a mixture of *trans*-2-heptenal and -octenal, and the 9.74 ppm signal to hexanal since these species are the predominant α,β-unsaturated and saturated aldehydes, respectively, generated from the peroxidation of linoleate.[2]

Fixed volumes of corn oil were subjected to 30, 60 and 90 min. periods of heating at 180°C in vessels of increasing size in order to investigate the influence of the effective surface area of this culinary frying oil (i.e., surface level exposed to atmospheric O_2) on the concentrations of aldehydes generated during episodes of thermal stressing in this manner (samples were subjected to ^{1}H NMR analysis immediately after the heating process). Figure 1(f) shows plots of the total levels of both saturated, and unsaturated (*trans*-2-alkenals and alka-2,4-dienals) aldehydes generated against time of heating at 180°C for 20.0 ml (18.48 g) aliquots of a commercially-available corn oil sample heated in 25, 50, 100 and 250 ml beakers (the surface areas of this volume of culinary oil placed in these vessels were 7.75, 12.20, 18.41 and 34.64 cm^2 respectively). Clearly, the concentrations of both classes of aldehyde generated increases with increasing vessel size, the sample collected after heating in a 250 ml beaker for a 90 min. period containing extremely high levels of these peroxidation products (1.88 and 3.01×10^{-2} $mol.kg^{-1}$ for saturated and unsaturated aldehydes respectively). These data demonstrate that the surface area of the culinary oil, and therefore the quantity of it exposed to atmospheric O_2 during episodes of heating in the above manner, has a substantial influence on the concentrations of both saturated and unsaturated aldehydes generated therein, an observation of much relevance to common frying practices (domestic or otherwise). Hence, it is anticipated that shallow frying procedures employing PUFA-rich culinary oils will give rise to a high level of aldehyde production. Purging of corn oil samples with air during periods of heating in the manner described above was found to elevate the concentrations of both classes of aldehyde generated approximately 3-fold (data not shown), an observation consistent with the above results.

As expected, the concentration of both saturated and unsaturated aldehydes generated in corn oil was approximately proportional to the length of heating time at 180°C.

The total level of aldehydes present in heated samples of culinary oils was found to increase with increasing storage time, an observation consistent with the autocatalytic, self-propagating PUFA autoxidation process. Indeed, storage of thermally-stressed corn oil samples in the dark at ambient temperature for a 240 hr. period effectively elevated the concentrations of NMR-detectable aldehydes approximately two-fold (data not shown). Hence, much care was taken to note the period of storage time prior to analysis for each group of culinary oil and fat samples examined in this investigation.

Extraction of thermally-stressed culinary oil samples with deuterated methanol ($C^2H_3O^2H$) gave rise to an effective partition of the aldehydic components present into the lower, $C^2H_3O^2H$ phase, a phenomenon of much analytical pertinence since it facilitated the identification and quantification of these species by ^{1}H NMR spectroscopy (data not shown).

The above aldehydic components arise from the fragmentation of conjugated hydroperoxydienes (i.e., hydroperoxides of linoleoylglycerols) via β-scission of preformed alkoxyl radicals (equations 1 and 2)[13] and high field ^{1}H NMR spectra of thermally-stressed culinary oils contained signals arising from these adducts. The 5.00–9.00 ppm regions of 400 MHz ^{1}H NMR spectra of heated corn oil samples [Figure 2(b)] contained multiplet resonances centred at 6.56, 6.00, 5.57 and 5.45 ppm for the

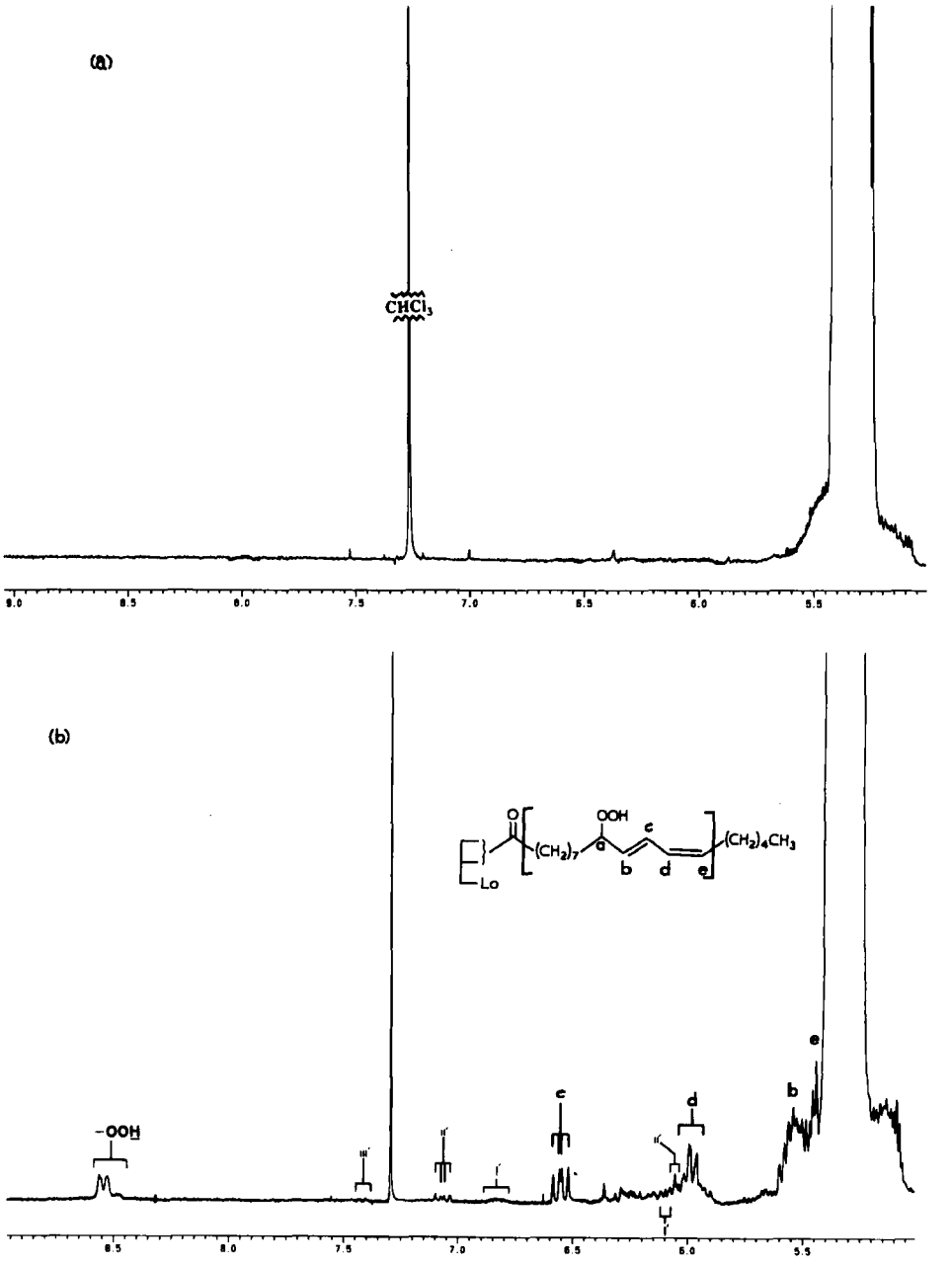
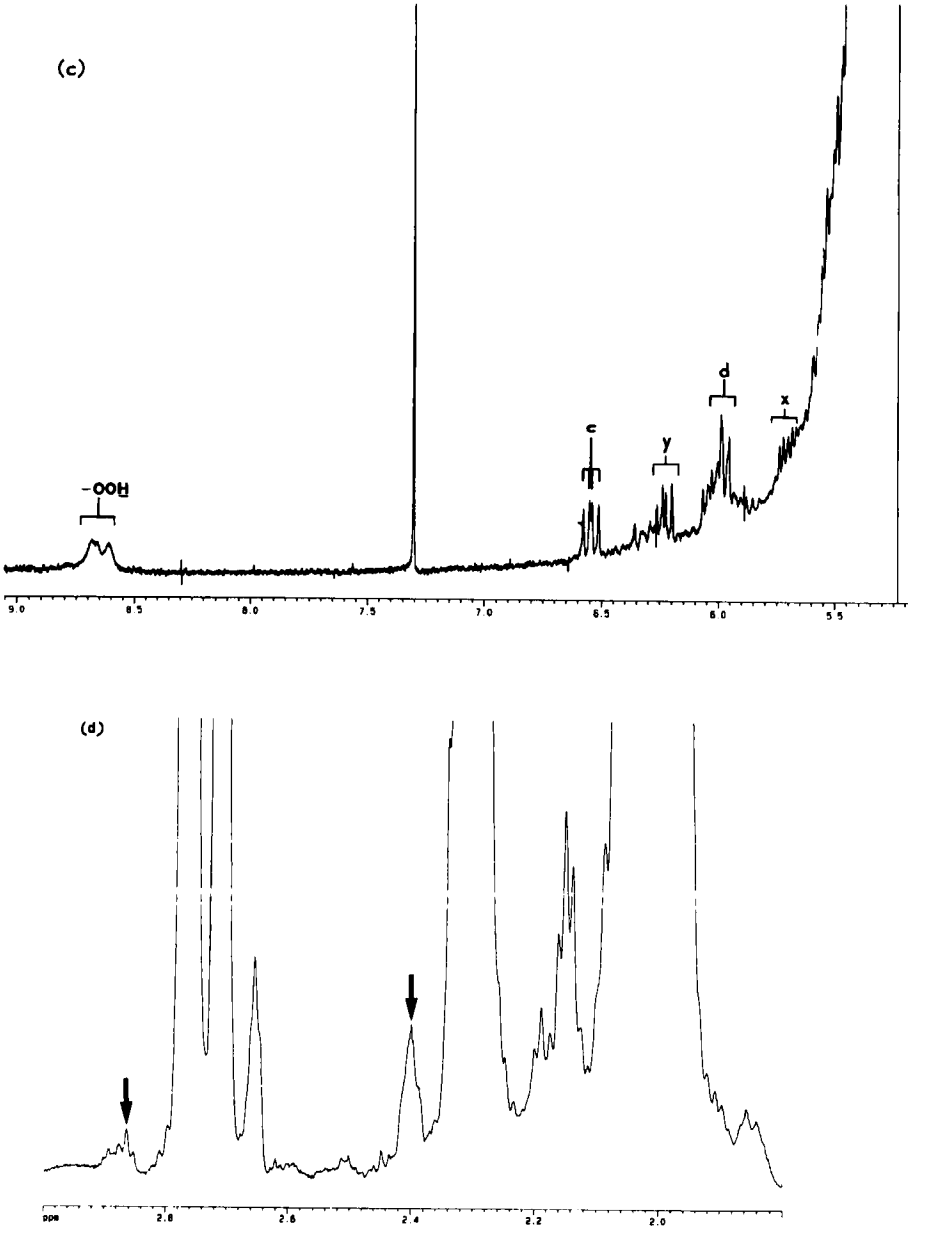


FIGURE 2. Partial (5.00–9.00 ppm regions of) 400 MHz ^{1}H NMR spectra of a commercially-available sample of corn oil obtained (a), prior and (b), subsequent to heating at a temperature of 180°C for a 90 min. period. A 25g quantity of this culinary oil was heated in a 100 ml volume conical flask (oil surface area 24.64 cm^2) and an aliquot removed at the 90 min. time-point was stored in the dark at ambient temperature for a period of 2,112 hr. prior to the acquisition of spectra. The unheated control sample was stored in the same manner for an equivalent period of time. (c), 5.00–7.20 ppm region of a corresponding spectrum of soyabean oil heated at pasteurisation temperature (72°C) in the presence of atmospheric O_2 for 69 hr. [this sample (3.0g) was heated in a 13.5-mm diameter sample tube (oil surface area 1.43 cm^2) and was stored in the dark at ambient temperature for 768 hr. prior to NMR analysis (the unheated control sample was stored in the same



manner for an equivalent time period)]. (d), Expanded 1.80–3.00 ppm region of the ^{1}H NMR spectrum of a corn oil sample subjected to thermal stressing at a temperature of 180°C for a period of 90 min. [Figure 1(d)]. Typical spectra are shown. Abbreviations: as Figure 1, with b, c, d and e, olefinic proton resources of the conjugated diene systems of 13- and/or 9- hydroperoxy-substituted octadecadienoylglycerol species (the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers respectively) as denoted in (b). x and y represent resonances assignable to the olefinic protons of *trans,trans*-conjugated hydroperoxydiene species. HOO–, hydroperoxy group proton resonances of conjugated hydroperoxydienes; II′ and III′, olefinic proton resonances of alka-2,4-dienals and 4-hydroxy-*trans*-2-alkenals respectively. The arrows in spectrum (d) indicate further multiplet resonances detectable in spectra of corn oil thermally stressed in the above manner.

protons *c,d,b* and *e* of the conjugated diene system of the *cis,trans*-isomers of 9- and 13-hydroperoxy-octadecadienoylglycerol species.[14-16] Multiplet resonances of a lower intensity located at 5.75 and 6.25 ppm, which arise from the *trans,trans*-isomers of 9- and 13-hydroperoxy-octadecadienoylglycerols,[15] are also present in these spectra. A signal attributable to the methine proton *a* of the hydroperoxide-bearing carbon (multiplet, $\delta = 4.35$ ppm) was also detectable in spectra of heated corn oil (data not shown). The spectrum of the thermally-stressed sample also contains relatively broad hydroperoxide group proton (–OOH) signals at 8.47, 8.525 and 8.56 ppm, demonstrating the presence of three or more conjugated hydroperoxydiene adducts.[16]

$$\begin{array}{c} \text{OOH} \\ | \\ \text{CH}_3(\text{CH}_2)_4\text{CH–R} \end{array} \rightarrow \begin{array}{c} \text{O}^{\cdot} \\ | \\ \text{CH}_3(\text{CH}_2)_4\text{CH–R} \end{array} + {}^{\cdot}\text{OH} \qquad (1)$$

$$\begin{array}{c} \text{O}^{\cdot} \\ | \\ \text{CH}_3(\text{CH}_2)_4\text{CH–R} \end{array} \xrightarrow{\beta\text{–scission}} \text{CH}_3(\text{CH}_2)_3\text{CH}_2{\cdot} + \text{RCHO} \qquad (2)$$

Interestingly, heating of a sample of soyabean oil at pasteurisation temperature (72°C) for a period of 69 hr. generated characteristic conjugated hydroperoxydiene resonances in its ^{1}H NMR spectrum, including broad hydroperoxy group proton resonances located at 8.58, 8.63 and 8.645 ppm [Figure 2(c)].

The 1.80–3.00 ppm regions of the heated corn oil spectra shown in Figures 1(b)–(d) contain resonances arising from additional thermally-induced PUFA peroxidation products, the most intense being multiplets centred at.96 and 2.71 ppm which are slightly upfield and have similar coupling patterns to the unheated oil PUFA *mono*-and *bis*-allylic-CH_2 group signals, the latter predominantly arising from linoleoylglycerol species. These resonances are tentatively assigned to cross-linked fatty acid dimers or alternative lipid aggregates, the former representing an important 'sink' for the consumption of PUFAs during toxidation reactions.[17] Expansion of the 1.80–3.00 ppm region of Figure 1(d) revealed further new resonances generated from the thermal stressing of corn oil [Figure 2(d)], *i.e.*, multiplets located at 2.40 and 2.863 ppm. However, these signals are not assignable to non-volatile products arising from the homolytic decomposition of an isomeric mixture of linoleic acid-derived conjugated hydroperoxydienes [i.e. 13(*S*)-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid (79%) and 9(*R*/*S*)-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (21%)], e.g., conjugated oxodiene and hydroxydiene species, and *mono*-unsaturated oxoepoxides and hydroxyepoxides, as previously reported by Gardner *et al.*[18] Furthermore, these resonances do not correspond to those of epoxide adducts generated from the Cu(II)ion-mediated oxidation of low-density-lipoprotein-associated PUFAs,[19] nor those arising from 6-membered hydroperoxy-epidioxides[20] which are important secondary products derived from the autoxidation of linoleate. However, the signals located at 2.40 and 2.863 ppm have chemical shift values and coupling patterns similar to those previously reported for 9- and 16-hydroperoxy-substituted 5-membered cyclic epidioxides derived from the autoxidation of trilinolenoylglycerol[21] (i.e., multiplets centred at 2.44 and 2.65

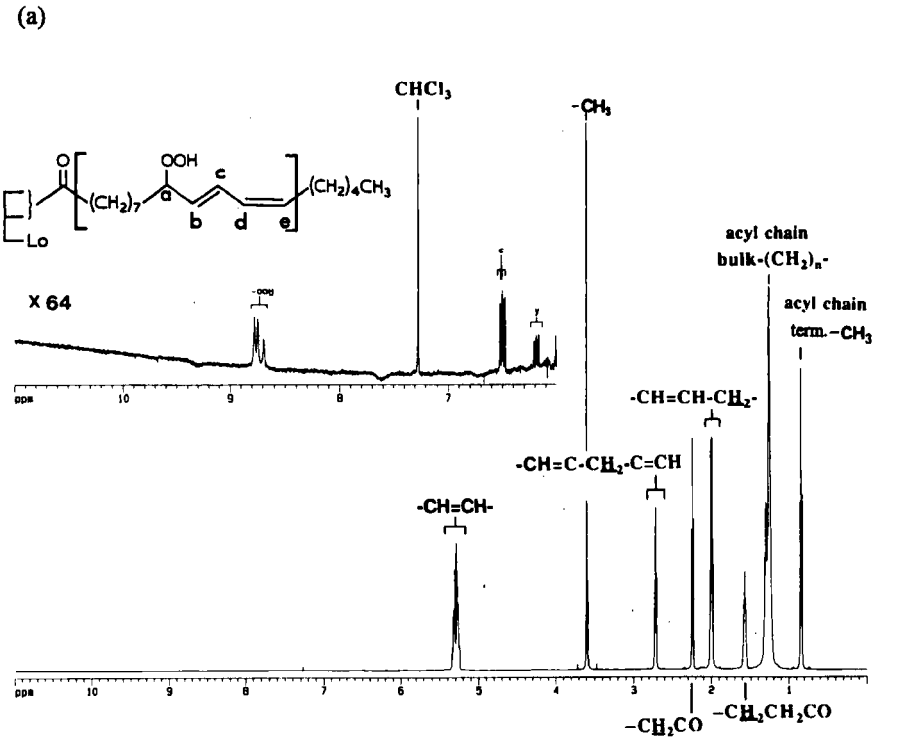
ppm for the 11-position protons of the epidioxide ring). Further experiments to determine the precise molecular nature of the adducts giving rise to the additional thermally-induced resonances shown in Figure 2(c) are currently in progress.

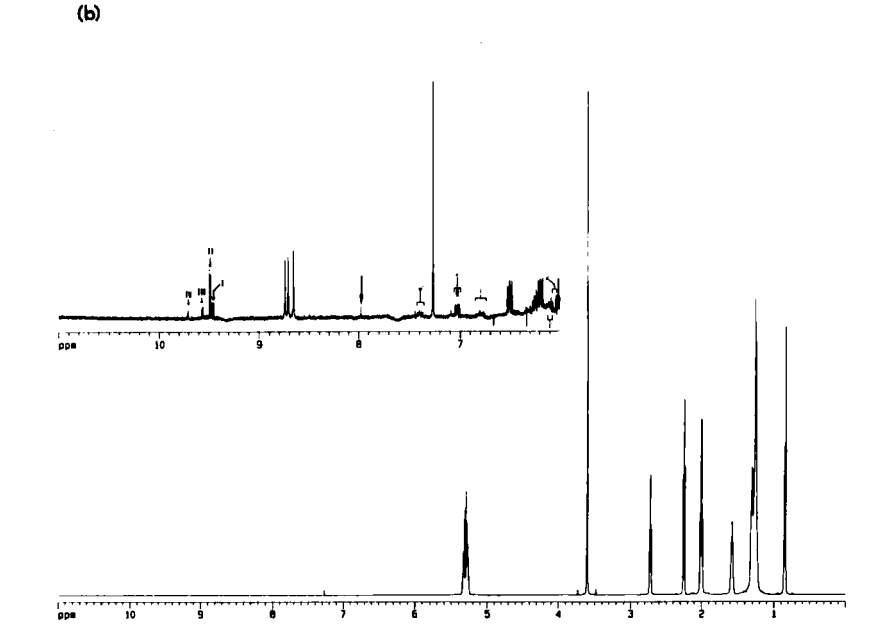
Similar results were obtained when sunflower seed oil, soyabean oil and rapeseed oil were subjected to episodes of heating in the above manner, but the relative levels of *cis,trans*- and *trans,trans*-conjugated hydroperoxydiene isomers, aldehydes and further NMR-detectable autoxidation products generated were found to differ with the identity of the oil utilised for these investigations. These data will be reported in detail elsewhere.

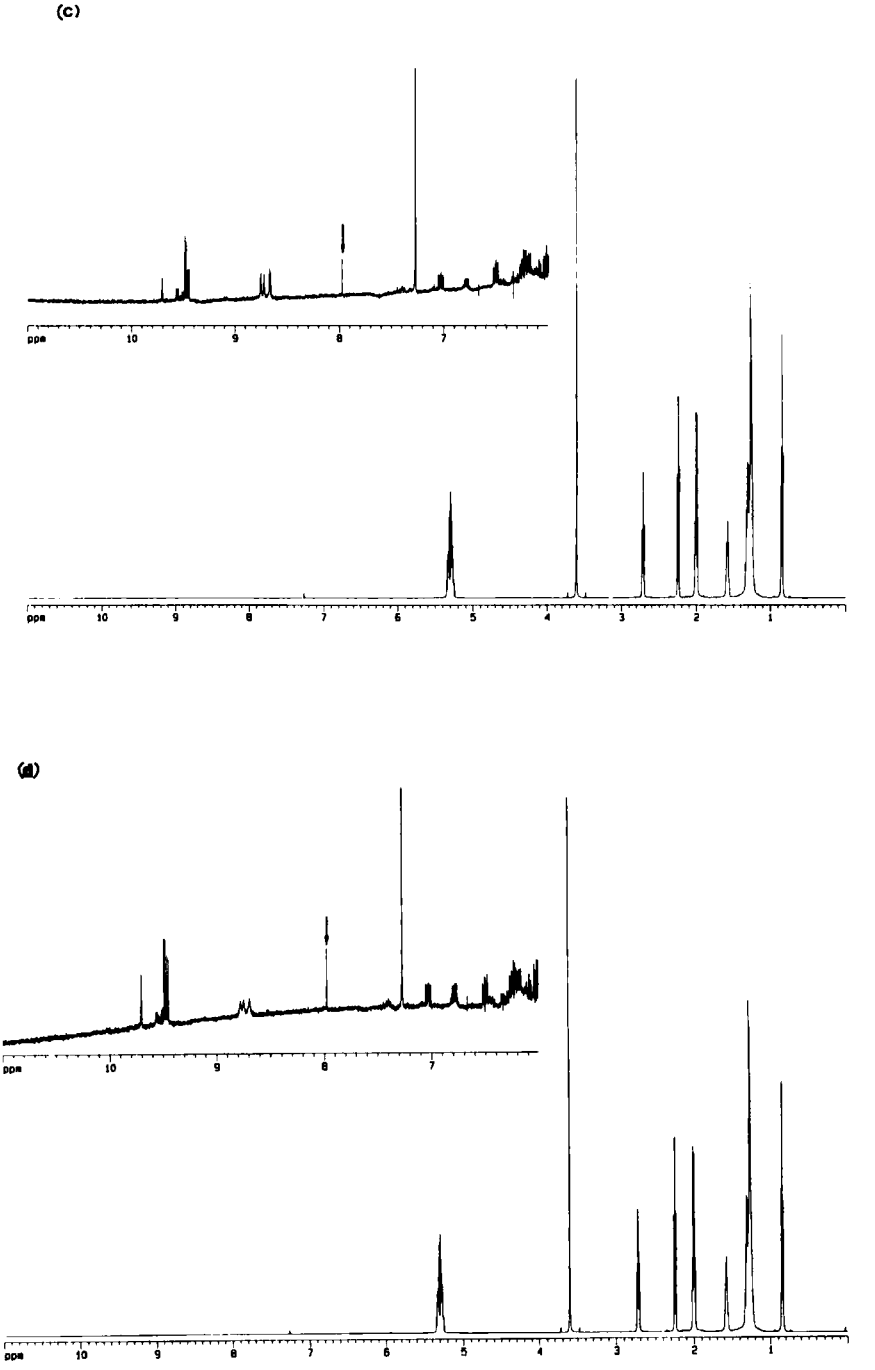
Electronic absorption spectroscopic analysis of aqueous extracts of control and thermally-stressed samples of culinary oils permitted a relatively selective estimation of the water-soluble *bi*-functional aldehyde MDA via reaction with TBA to form a pink-coloured chromogen (the 2:1 TBA:MDA adduct) as detailed in section 2. Indeed, an extract obtained from a typical sample of soyabean oil heated at 180°C for a period of 90 min. had an absorbance value at a wavelength of 532 nm corresponding to an MDA level of 1.23×10^{-5} mol.kg^{-1} in the thermally-stressed oil, assuming a 100% recovery of this aldehyde into the lower, aqueous phase. The selectivity of this method was confirmed by second-derivative spectrophotometric analysis (i.e., no interfering absorption bands arising from the chromophoric TBA adducts of alternative aldehydic components were detectable). As expected, direct dissolution of solid TBA in heated culinary oils immediately after they attained a temperature of 180°C generated an intense red colouration therein within 15 min. for each material investigated (corn, sunflower seed, soyabean, groundnut and rapeseed oils).

The *di*-unsaturated fatty acid ester methyl linoleate was also subjected to episodes of heating at 180°C in the above manner and Figure 3 shows the complete, and expanded 6.00–11.00 regions of 600 MHz ^{1}H NMR spectra acquired on these samples. The spectrum of unheated (control) methyl linoleate [Figure 3(a)] contains olefinic resonances arising from the conjugated diene systems of both *cis,trans*- and *trans,trans*-isomers of its conjugated hydroperoxydienes, demonstrating the ready autoxidation of this PUFA when stored at ambient temperature in the presence of atmospheric O_2. The relatively broad but distinct singlet resonances located at 8.70, 8.75 and 8.785 ppm are assignable to hydroperoxide group –OO**H** protons,[16] and treatment of a solution of autoxidised methyl linoleate in C^2HCl_3 (1:3 v/v) with ^2H_2O resulted in their removal from the spectrum. The presence of three separate hydroperoxy group proton signals in this spectrum presumably reflects the number of ^{1}H NMR-distinguishable conjugated hydroperoxydiene species present in this sample.

As expected, heating of this model PUFA at 180°C for periods of 30, 60 and 90 min. gave rise to a series of prominent aldehydic resonances in the 9.3–9.8 ppm chemical shift range, i.e., those attributable to *trans*-2-alkenals, alka-2,4-dienals, alkanals and possibly 4-hydroxy-*trans*-2-alkenals [Figures 3(b)–(d)]. The thermally-induced generation of aldehydes in this sample was accompanied by a corresponding reduction in the intensities of the *cis,trans*- and *trans,trans*-hydroperoxydiene signals. The intensity of each aldehydic proton resonance increased with increasing heating time and particularly noteworthy is the observation that the amplitude of the alka-2,4-dienal doublet is greater than that of the *trans*-2-alkenal one after heating for a period of 30 min; however, the intensity of the latter signal steadily increases with increasing time of heating when expressed relative to that of the former. A similar phenomenon was observed for the 9.74 ppm alkanal aldehydic proton resonance. These data suggest that the α,β-unsaturated and saturated aldehydic components giving rise to the resonances located at 9.48 and 9.74 ppm respectively in ^{1}H NMR spectra of heated oils are







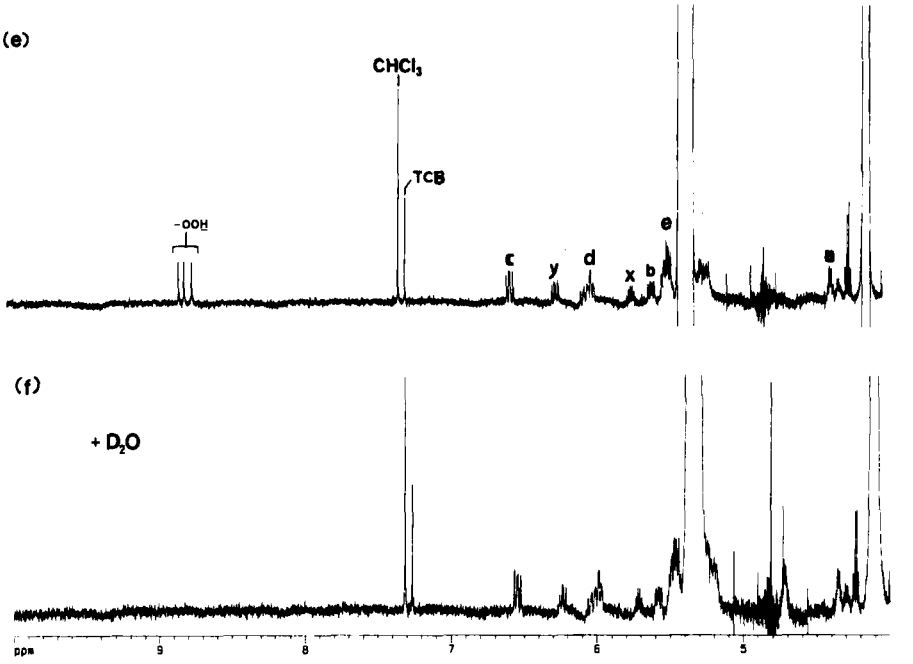


FIGURE 3. Complete and expanded 6.00–11.00 ppm regions of 600 MHz ^{1}H NMR spectra of methyl linoleate obtained (a) before, and at (b) 30, (c) 60 and (d) 90 min. after heating at a temperature of 180°C in the presence of atmospheric O_2. This model PUFA compound (2.0g) was heated in a 10-mm diameter sample tube (sample surface area 0.79 cm^2) and samples removed at the above time-points were stored in the dark at ambient temperature for a period of 96 hr. prior to analysis. The unheated control sample was stored in the same manner for an equivalent period of time. (e) and (f), Expanded 4.00–10.00 ppm regions of the 600 MHz ^{1}H NMR spectra of autoxidised ethyl linoleate acquired prior and subsequent, respectively, to shaking with a 0.03 ml volume of ^2H_2O (section 2). This model PUFA compound (2.0g) was placed in 10-mm diameter sample tubes (sample surface area 0.79 cm^2) and allowed to autoxidise at ambient temperature in the presence of atmospheric O_2 for a period of 2,112 hr. prior to analysis. Typical spectra are shown. Abbreviations: as Figures 1 and 2, with *a* representing the methine proton of the hydroperoxyl group-bearing carbon of *cis,trans*-conjugated hydroperoxydiene species, as indicated in Figure 2(b). The arrow in spectra (b), (c) and (d) denotes a sharp singlet resonance (δ = 7.97 ppm) generated during episodes of heating at 180°C. $-CH_3$, methyl ester functional group protons ($-COOCH_3$) of methyl linoleate; TCB, 1,3,5-trichlorobenzene (internal quantitative ^{1}H NMR standard).

generated as secondary products, i.e., subsequent to the alka-2,4-dienal responsible for the 9.52 ppm doublet. However, further contributory factors here include (1) the susceptibility of these species to thermally-induced oxidation, producing corresponding carboxylic acids, and (2) the relative volatilities of these species (e.g., hexanal, *trans*-2-octenal and *trans,trans*-hepta-2,4-dienal have boiling points of 131, 85 and 84°C respectively[12]). The increase in the intensities of the *trans*-2-alkenal, alka-2,4-dienal and putative 4-hydroxy-*trans*-2-alkenal signals paralleled elevations in those of olefinic proton multiplet resonances located at 6.85 (*dt*), 7.07 (*dd*) and 7.39 ppm (apparent *dd*) respectively, consistent with the ^{1}H NMR data obtained on thermally-stressed culinary oils (e.g., Figure 1).

In the spectra acquired on methyl linoleate samples heated at 180°C for periods of 30 and 60 min., the hydroperoxide group (–OOH) proton resonances were narrower than those present in the unheated (control) sample spectrum. However, it should be

noted that the chemical shift values and line-shapes of these signals are expected to be critically dependent upon experimental conditions such as hydroperoxide concentration and spectral acquisition temperature. Moreover, intermolecular exchange of H^+ between hydroperoxy groups will also influence the $\Delta\nu_{1/2}$ values of these resonances. Interestingly, thermal-stressing of this model PUFA compound also gave rise to the production of a sharp singlet resonance of unknown identity located at 7.97 ppm. The intensity of this signal increased with increasing time of heating at 180°C.

Similar ^{1}H NMR data were obtained on control and thermally-stressed samples of ethyl and propyl linoleate.

Figures 3(e) and (f) show the expanded 4.00–10.00 ppm regions of a typical ^{1}H NMR spectrum of a commercially-available sample of control (unheated) ethyl linoleate in C^2HCl_3 solution obtained before and after shaking with a small quantity of ^2H_2O.

Clearly, the singlet resonances located at 8.72, 8.775 and 8.81 ppm disappear from the spectrum following ^2H_2O treatment, providing further evidence for their assignment to –OOH group protons. The 4.20–6.00 ppm regions of each spectrum contain multiplet ^{1}H resonances characteristic of conjugated hydroperoxydiene species (6.56, 6.00, 5.57 and 5.45 ppm for protons *c, d, b* and *e* of the conjugated diene systems of the *cis,trans*-9-and 13-hydroperoxides of linoleate, 4.35 ppm for the methine proton *a* of the hydroperoxide group-bearing carbon, and 6.23 and 5.72 ppm for conjugated diene system protons of the corresponding *trans,trans*-isomers of the 9- and 13-hydroperoxides).

Although the chemical shift values of the 8.72, 8.775 and 8.81 ppm singlet resonances are very similar to those of the hydroperoxide group protons of 6-membered hydroperoxy-substituted products derived from the autoxidation of linoleate,[20] further signals ascribable to species such as the 12- and 9-position methine proton multiplets of 13-hydroperoxy-9, 12-epidioxy-10-octadecenoates (4.61 and 4.53–4.54 ppm respectively), and the 10- and 13-position methine proton multiplets of 9-hydroperoxy-10,13-epidioxy-11-octadecenoate (4.66 and 4.48–4.49 ppm respectively) were absent from the spectra acquired.

The 7.97 ppm singlet resonance noted above in ^{1}H NMR spectra of thermally-stressed methyl linoleate samples was also present in corresponding spectra of ethyl linoleate heated at 180°C for periods of 30–90 min, and this signal was removed following treatment with ^2H_2O, demonstrating its assignment to an ^2H_2O-exchangable proton.

The thermally-induced fragmentation of *cis,trans*-linoleate hydroperoxides gives rise to corresponding 9- and 13-substituted hydroxydienes (equations 3 and 4), and the ^{1}H NMR spectrum of 13-hydroxy-*cis*-9, *trans*-11-octadecadienoate contains multiplet resonances centred at 4.05, 5.56, ~6.35, ~5.90 and 5.30 ppm for the 13-. 12-, 11-, 10- and 9-position protons, respectively, of its conjugated diene system.[14] However, although single-pulse, one-dimensional spectra acquired on control and heated model alkyl-substituted PUFA compounds provided little or no evidence for the generation of these species, a two-dimensional COSY ^{1}H NMR spectrum of ethyl linoleate heated at 180°C for a period of 90 min. revealed a conjugated diene system multiplet signal located at 5.56 ppm that was clearly linked to one at 4.09 ppm (data not shown). These resonances correspond to the 12- (5.56 ppm) and 13- (4.09 ppm) position protons of the conjugated *cis,trans*-13-hydroxydiene isomer derived from the corresponding hydroperoxydiene of linoleate, the latter representing the methine proton of the hydroxy group-bearing carbon. A similar pattern of connected multiplet resonances is expected for the *cis, trans*-9-hydroxydiene adduct. This two-dimensional spectrum also showed connectivities between conjugated diene system olefinic proton signals centred

at 5.53 and 5.60 ppm (of greater intensity than the 5.56 ppm resonance noted above) and those at 4.27 and 4.34 ppm respectively. The lower (5.53, 5.60 ppm) and higher field (4.27, 4.34 ppm) resonances are ascribable to the olefinic proton directly adjacent to (i.e., α-to) hydroperoxide group-substituted carbons (e.g., proton *b* in Figures 2 and 3) and the single methine proton of the hydroperoxide-bearing carbon (e.g., proton *a* in Figures 2 and 3), respectively, of conjugated hydroperoxydiene species.

$$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH \quad (3)$$

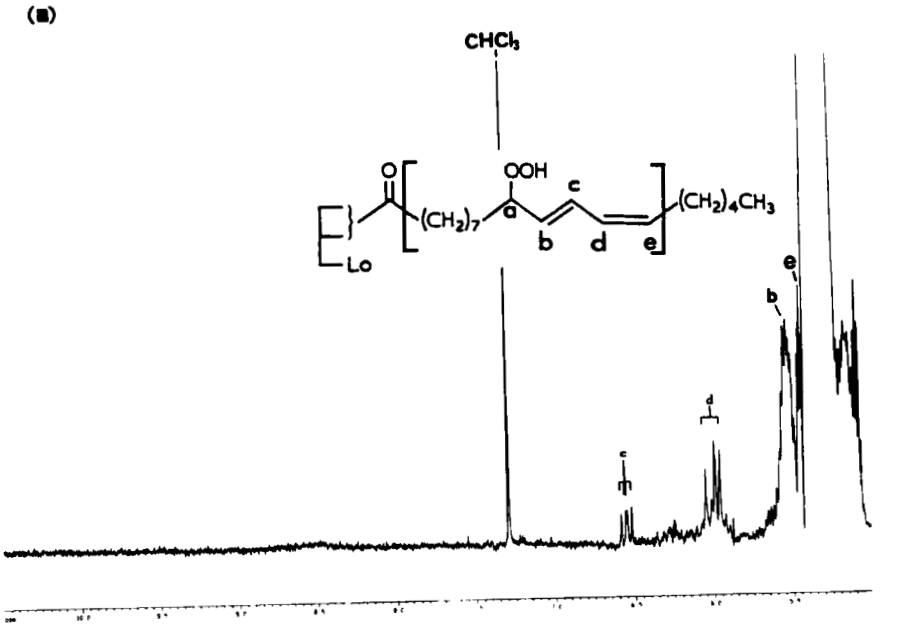
$$RO^{\bullet} + RH \rightarrow ROH + R \quad (4)$$

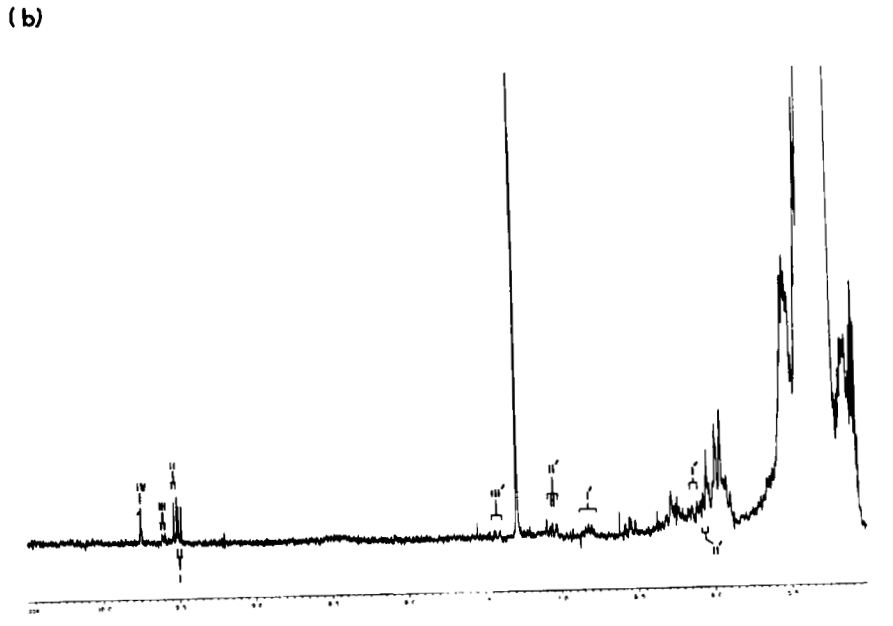
As expected, electronic absorption spectra of thermally-stressed samples of methyl, ethyl and propyl linoleates contained intense conjugated diene system maxima located at 232 and 242 nm (resolved by second-derivative spectrophotometry), which are ascribable to *trans,trans*- and *cis,trans*- isomers, respectively, of PUFA-derived hydroperoxy and/or hydroxydienes.[24] However, these spectra also contained a weaker absorption band at ca. 270 nm attributable to conjugated oxodiene species which represent secondary PUFA autoxidation products.[25]

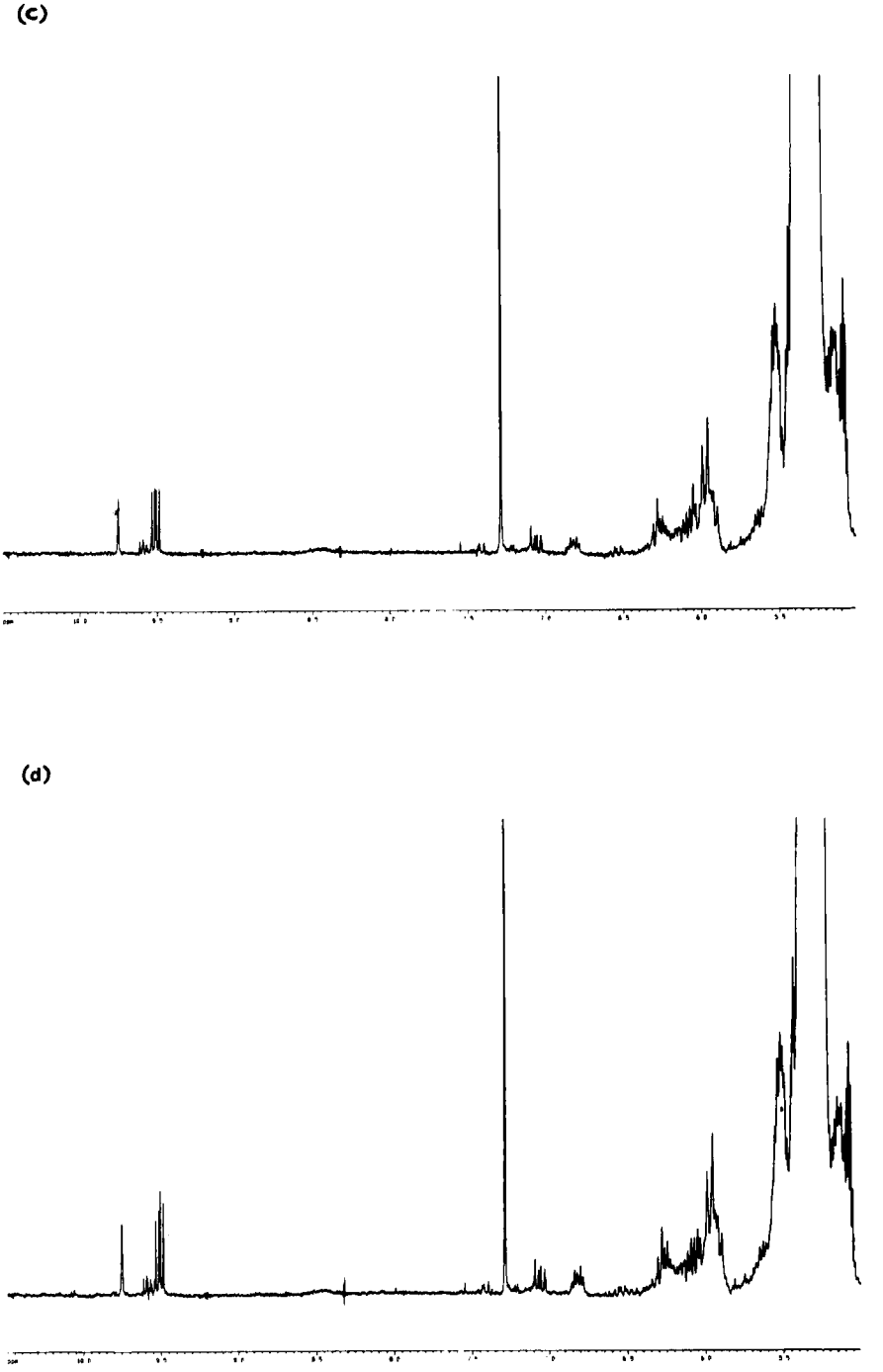
The expanded 5.00–10.00 ppm regions of ^{1}H NMR spectra of control (unheated) and thermally-stressed samples of groundnut oil are shown in Figure 4. Interestingly, the spectrum of the unheated sample [Figure 4(a)] contains resonances characteristic of the conjugated diene systems of 9- and 13-hydroperoxy-octadecadienoylglycerol species (predominantly *cis,trans*-isomers) which presumably arise during isolation, preparation and/or storage of this culinary oil by the manufacturers. Heating of this material at a temperature of 180°C for periods of up to 90 min. gave rise to time-dependent reductions in the intensities of these conjugated hydroperoxydiene signals which were accompanied by the generation of aldehydic proton resonances in the 9.40–9.80 ppm chemical shift range [Figures 4(b)–(d)]. The intensity of each aldehydic signal increased with increasing time of heating at the above specified temperature, and, as expected, those of resonances located at 9.48, 9.52 and 9.63 ppm (doublets in each case) correlate with those of olefinic proton multiplets at 6.85, 7.07 and 7.39 ppm respectively. The intensity of a singlet resonance at 7.545 ppm of unknown identity also increased with increasing time of heating at 180°C.

Subjection of groundnut oil samples to an additional 30 min. period of heating at an elevated temperature (250°C) after thermal stressing at 180°C for periods of 90 min. gave rise to (1) the production of prominent multiplets centred at 5.62, 5.64, 5.81, 5.93, 5.98, 6.32, 7.09 and 7.22 ppm, together with a singlet at 8.04 ppm (the 5.64, 5.93, 5.98, 7.22 and 8.04 ppm resonances developed during heating of this culinary oil at 180°C); (2) the generation of new aldehydic signals at 9.609 (multiplet), 9.78 (apparent triplet) and 10.06 ppm (doublet, $j = 7.8$ Hz); (3) the almost complete disappearance of the 9.52 ppm signal (paralleling major reductions in the intensities of linked multiplets at 7.07 and 6.04 ppm), and a substantial decrease in the amplitude of that at 9.63 ppm (accompanied by a corresponding decrease in its connected 7.39 ppm multiplet). The new conjugated diene signals in the 5.6–7.3 ppm chemical shift range, however, did not correspond to those of products arising from the thermally-induced deterioration of the 9- and 13-hydroperoxides of linoleate (i.e., epoxides, hydroxyepoxides, 5- and 6-membered hydroperoxyepidioxides and conjugated oxodienes[18,20,21,23]), and further experiments to establish their identities are currently in progress.

^{1}H NMR analysis also revealed high levels of potentially toxic conjugated diene species and aldehydic fragments in samples of repeatedly-used frying oils obtained







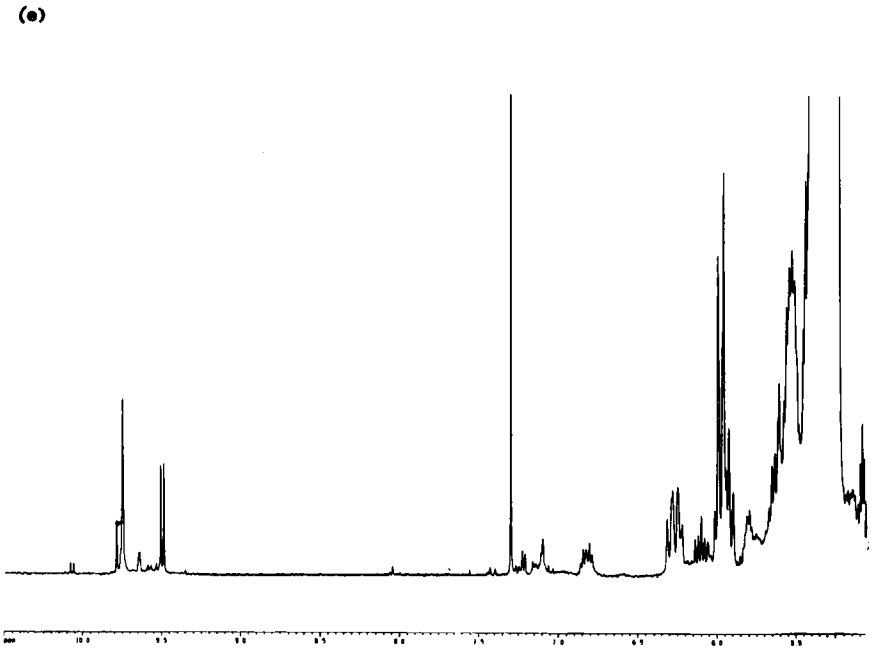
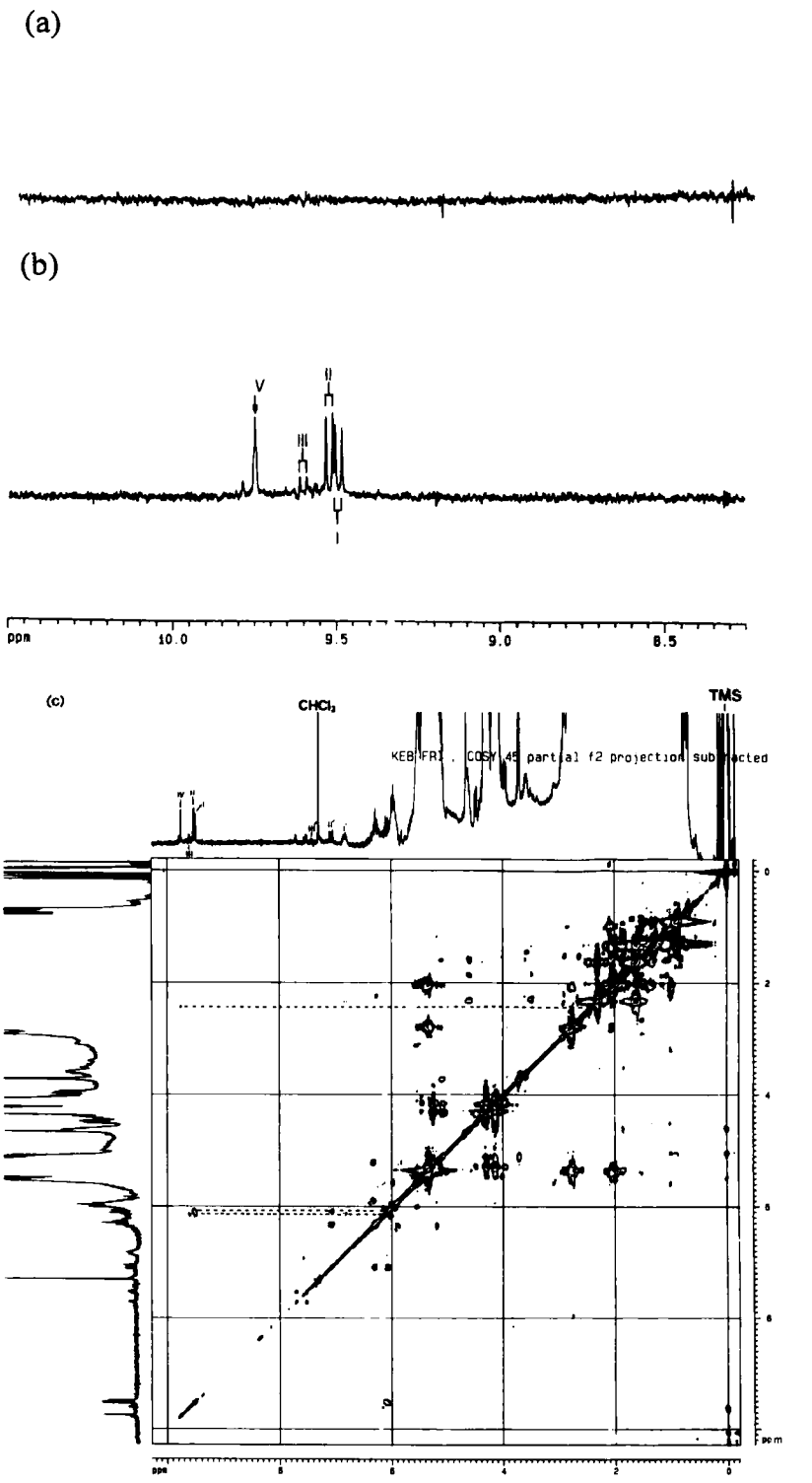


FIGURE 4. Expanded 5.00–11.00 ppm regions of the 400 MHz ^{1}H NMR spectra of a commercially-available sample of groundnut oil obtained (a) before and after subjecting to episodes of thermal stressing at 180°C for periods of (b) 30, (c) 60 and (d) 90 min. in the presence of atmospheric O_2. (e), as (d), but after a further 30 min. period of heating at an elevated temperature (250°C). A 25g quantity of this culinary oil was heated in a 100 ml volume conical flask (oil surface area 24.64 cm^2) and samples removed at the above time-points were stored in the dark at ambient temperature for a 912 hr. period prior to NMR analysis (the unheated control sample was stored in the same manner for an equivalent time period). Typical spectra are shown. Abbreviations: as Figures 1 and 2.

from fast-food/take-away establishments. Figure 5 shows the expanded 8.00–10.50 ppm region of the 400 MHz ^{1}H NMR spectrum of such a sample [(b)], together with that of a control (unheated) sample collected from the same source [(a)]. As expected, the spectrum of the repeatedly-used culinary oil contains intense aldehydic resonances located at 9.48 (doublet), 9.52 (doublet), 9.63 (doublet), and 9.74 ppm (triplet) [together with a further one at 9.775 ppm (apparent triplet)], whereas that of the unheated sample had no ^{1}H NMR-detectable signals in this spectral region. Although the 5.40–6.40 ppm regions of spectra acquired on corresponding unheated frying oils contained a series of complex conjugated diene olefinic proton resonances of weak intensity, this NMR profile was reproducibly modified after the sample was subjected to repeated episodes of thermal stressing (i.e., the frying process employed by the culinary establishment from which it was obtained). For example, the intensity of a multiplet centred at 5.95 ppm was substantially elevated, and a new multiplet at ca. 5.75 ppm was produced (data not shown). The thermally-induced generation of aldehyde group (–CHO) proton signals in the spectra acquired was accompanied by the production of multiplet resonances assignable to the olefinic protons of *trans*-2-alkenal and alka-2,4-dienal adducts [6.10 (*dd*), 6.85 (*dt*) and 6.04 (*dd*), 7.07 (*dd*), respectively].



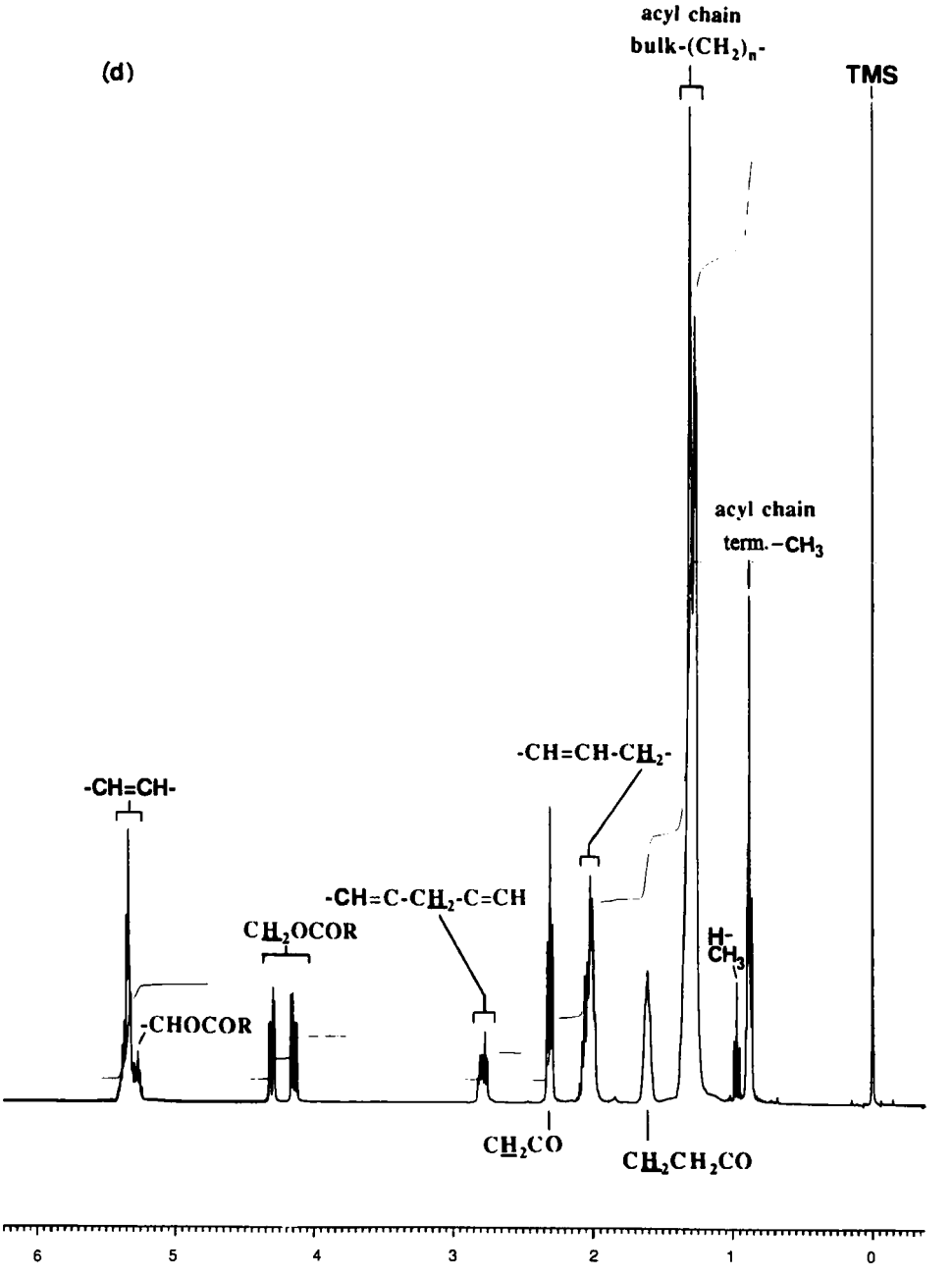
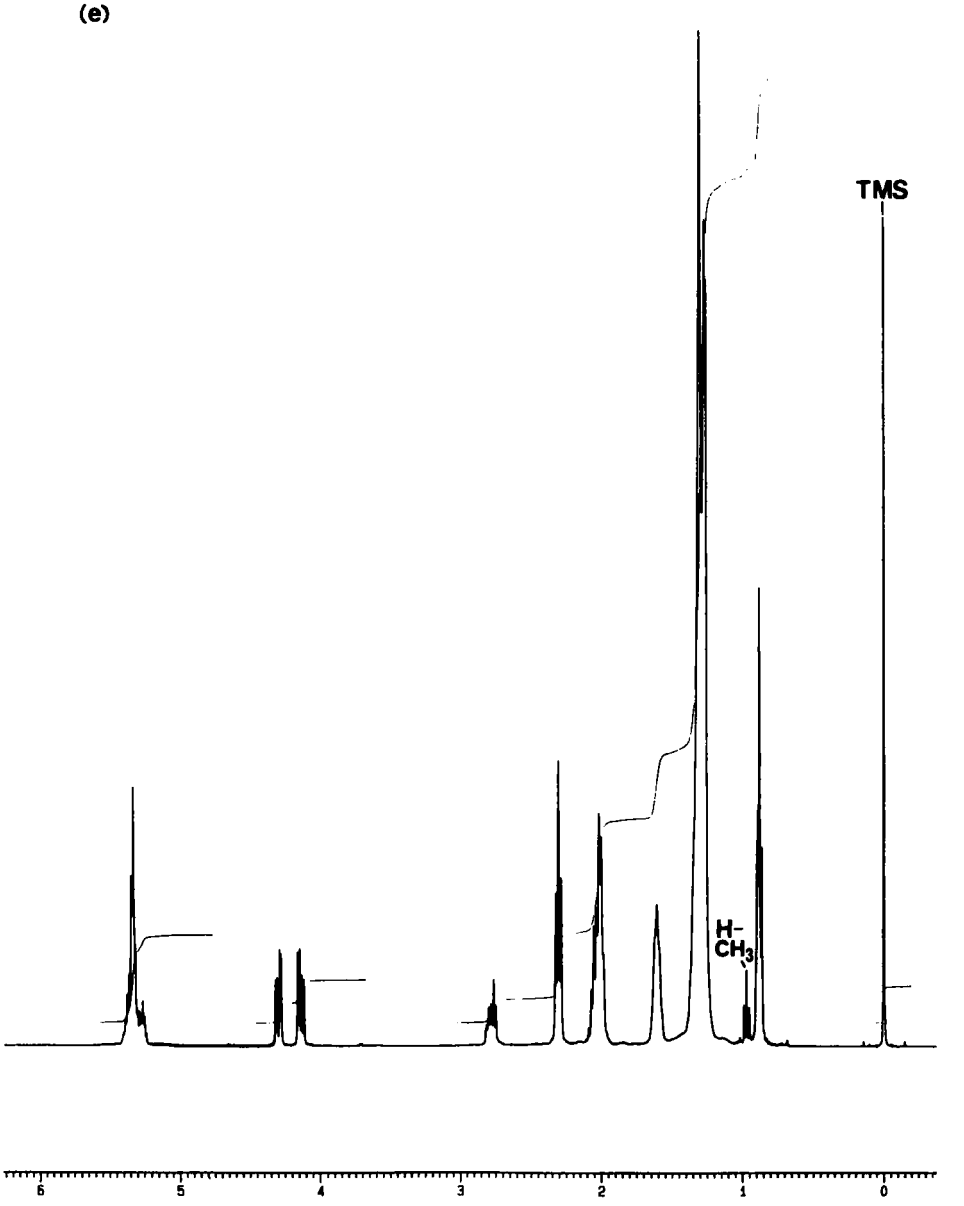


FIGURE 5. Expanded aldehydic (8.00–10.50 ppm) regions of the 400 MHz ^{1}H NMR spectra of (a) control (unheated) and (b) repeatedly-used samples of culinary frying oils obtained from a fast-food/take-away establishment. (c), Two-dimensional COSY ^{1}H NMR spectrum of a typical sample of repeatedly-used frying oil showing connectivities between the aldehydic (–CHO) resonances and those located upfield, i.e., the 9.48 ppm doublet and the 6.85 and 6.10 ppm olefinic proton multiplets; the 9.52 ppm doublet and the olefinic proton doublet of doublets centred at 7.07 and 6.04 ppm; the 9.74 ppm triplet and a multiplet at 2.44 ppm.



The 0.00–6.00 ppm regions of 400 MHz ^{1}H NMR spectra of typical control (unheated) and repeatedly-used culinary frying oil samples obtained from a restaurant are shown in (d) and (e) respectively.

The above samples were stored in the dark at ambient temperature for a period of 18 hr. prior to analysis. Typical spectra are shown. Abbreviations: as Figures 1 and 2, with H-CH_3 representing the acyl chain terminal-CH_3 group signal of highly unsaturated glycerol-bound fatty acids (i.e., those with ≥3 unconjugated double bonds).

Two-dimensional COSY ^{1}H NMR spectra of repeatedly-used frying oils [Figure 5(c)] showed connectivities between the aldehydic proton resonances and those arising from coupled olefinic or alkyl group protons, specifically (1) the 9.48 ppm doublet and the 6.85 and 6.10 ppm multiplets; (2) the 9.52 ppm doublet and the doublet of doublets located at 7.07 and 6.04 ppm; (3) the 9.74 ppm triplet and a 2.44 ppm multiplet (presumably a doublet of triplets). These data are very similar to those obtained from two-dimensional COSY ^{1}H NMR spectra of the culinary oils and model alkyl-substituted PUFAs that were thermally-stressed under laboratory conditions. Clear connectivities between multiplet resonances of the complex conjugated diene spectral pattern were observed, and evidence for the linkage of one or more of these signals to multiplets located further upfield was also provided.

Further new, thermally-induced resonances detectable in ^{1}H NMR spectra of repeatedly-used frying oil samples included those located at 3.55, 3.72, 4.74 and 4.79 ppm, signals that are clearly visible in the corresponding one- and two-dimensional spectra shown in Figure 5(c). The 3.72 ppm signal is a doublet of relatively high intensity.

The above modifications observed in ^{1}H NMR spectra of culinary oils when subjected to episodes of repeated frying in a fast-food/take-away establishment paralleled a marked consumption of glycerol-bound PUFAs therein. Indeed, electronic integration of the resonance attributable to the methylene proton located between two double bonds (2.76 ppm) and that of the protons of carbon-carbon unsaturation (5.38 ppm), and normalisation of their intensities to that of the acyl chain terminal methyl group protons ($\delta = 0.90$ ppm) revealed significant reductions in their intensities (11 and 9% respectively) for the samples investigated here. Figures 5(d) and (e) show the 0.00–6.00 ppm regions of ^{1}H NMR spectra of typical control (unheated) and repeatedly-utilised frying oil samples obtained from a restaurant. In addition to the *bis*-allylic methylene and olefinic group resonances noted above, signals characteristic of triacylglycerols present in the spectrum of the control sample comprise those at 5.27 and 4.25 ppm for protons on the 2- and 1(3)-position carbons, respectively, of the glycerol moiety; 2.29 ppm for methylene protons α- to the ester carbonyl group; 2.06 ppm for *mono*-allylic methylene group protons; 1.65 and 1.27 ppm for acyl chain methylene protons (the former resonance arises from those β- to the ester carbonyl group); 0.95 ppm for the acyl chain terminal methyl group protons of highly unsaturated fatty acids, i.e., those with three or more unconjugated double bonds (predominantly linolenoylglycerol species in this sample), and 0.90 ppm for the corresponding methyl groups of glycerol-bound saturated, *mono*- and *di*-unsaturated fatty acids. Further useful analytical information regarding the composition of these materials included the ratio of total fatty acid chains to total unsaturated fatty acid chains, a value derived from the integrated ratios of the 0.90 ppm *w*-CH_3 group resonances to that of the total olefinic –CH = CH– signals at 5.38 ppm. Such data are also available from spectra of alternative culinary oil samples [e.g., that of corn oil shown in Figure 1(a)].

Interestingly, the low field-shifted acyl chain terminal methyl group signal ($\delta = 0.95$ ppm) was substantially diminished in intensity (i.e., 36% less than its control value) after subjecting the sample to repeated frying episodes, demonstrating the ready thermal consumption of glycerol-bound linolenate, an observation consistent with the fact that this PUFA autoxidises at a faster rate than linoleate (approximately two-fold).[26]

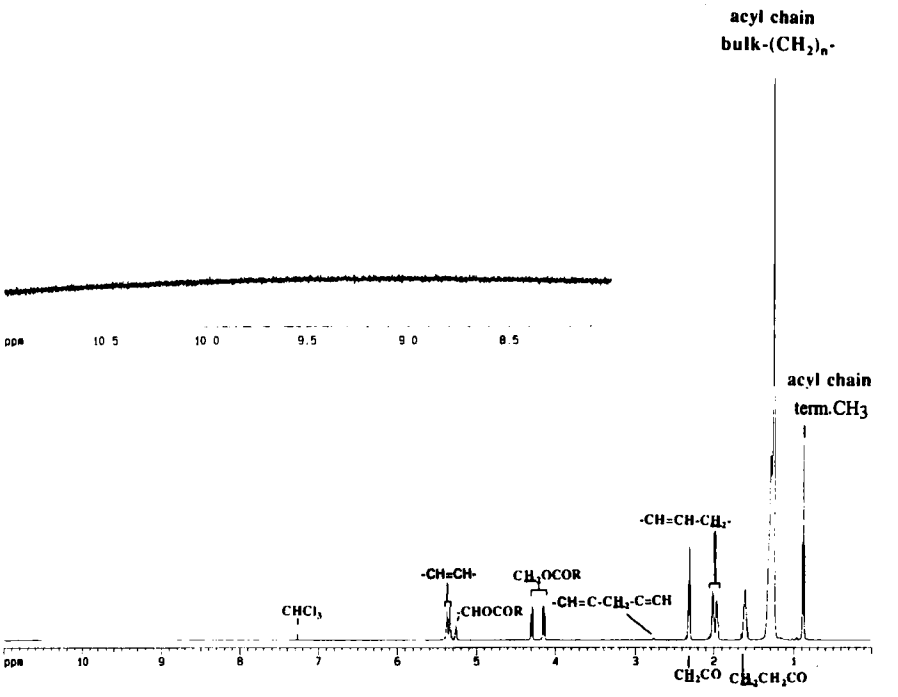
A commercially-available sample of lard generated only low levels of specific aldehydes [i.e., *trans*-2-alkenals and alkanals giving rise to –CHO group resonances at

9.48 (doublet) and 9.74 ppm (triplet) respectively] when subjected to 30, 60 and 90 min. periods of heating at 180°C [Figures 6(a)–(d)]. These results are presumably a reflection of the very low PUFA content of this culinary fat [e.g., Figure 6(a)]. As expected, additional thermal stressing of this sample at a temperature of 250°C for a further 30 min. increased the intensities of the two aldehydic resonances detectable in 600 MHz ^{1}H NMR spectra [Figure 6(e)]. Intriguingly, a further commercially-available brand of lard containing an unspecified butylated hydroxytoluene (BHT) content did not produce any NMR-detectable aldehydes, nor their conjugated hydroperoxydiene precursors when heated at 180°C in the above manner (data not shown), indicating that this synthetic dietary antioxidant exerts a protective effect with regard to the thermally-induced autoxidation of PUFAs therein.

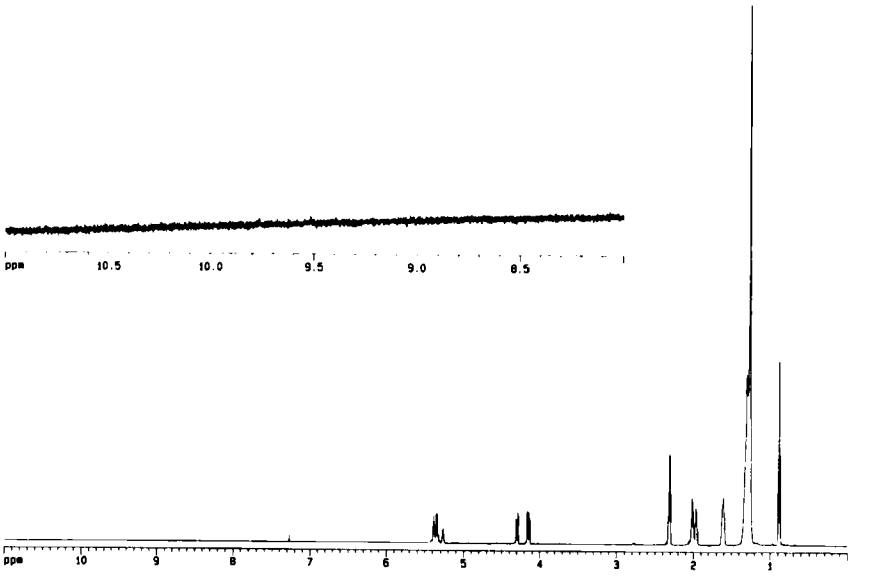
However, although much less susceptible to peroxidation than PUFAs, the glycerol-bound *mono*-unsaturated fatty acids present in this sample may also give rise to selected aldehydes on heating according to standard frying practices, and the ^{1}H NMR data outlined below demonstrate the thermally-induced generation of *trans*-2-alkenals and alkanals from methyl oleate.

The 5.50–7.70 ppm regions of 400 MHz ^{1}H NMR spectra acquired on samples of beef and lamb fat (i.e., dripping) subjected to heating at a temperature of 180°C for periods of 74 and 90 min. respectively in a domestic fan-assisted oven are shown in Figures 6(f) and (g). These spectra contain signals characteristic of conjugated diene systems, specifically those centred at 6.28 (*dd*, j = 13.8, 10.2 Hz) and 5.93 ppm (*dd*, j = 10.9, 10.2 Hz), and two further overlapping multiplets at 5.675 and 5.635 ppm (apparent *dd*s). The conjugated diene resonances detectable, however, were not assignable to *cis,trans-* or *trans,trans*-conjugated hydroperoxydiene species, nor epoxides, hydroxyepoxides, 5-and 6-membered hydroperoxyepidioxides and conjugated oxodienes, i.e. selected products arising from the thermal decomposition of the above

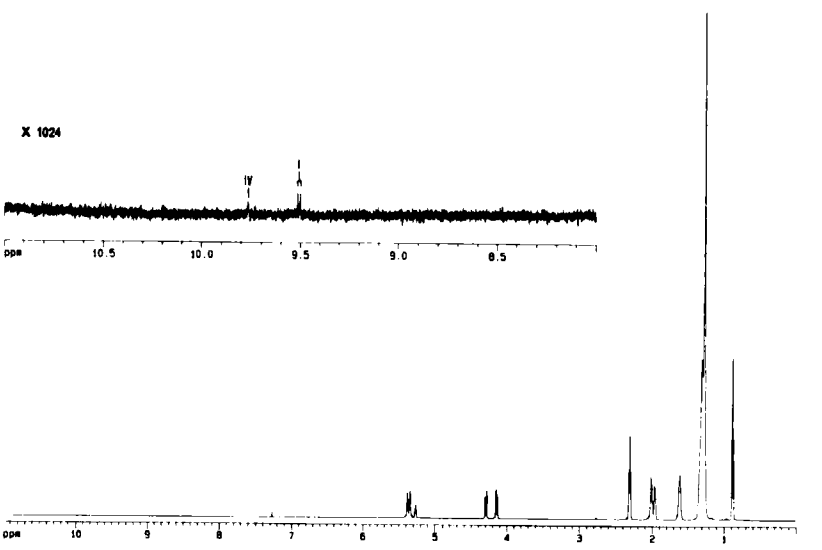
(a)

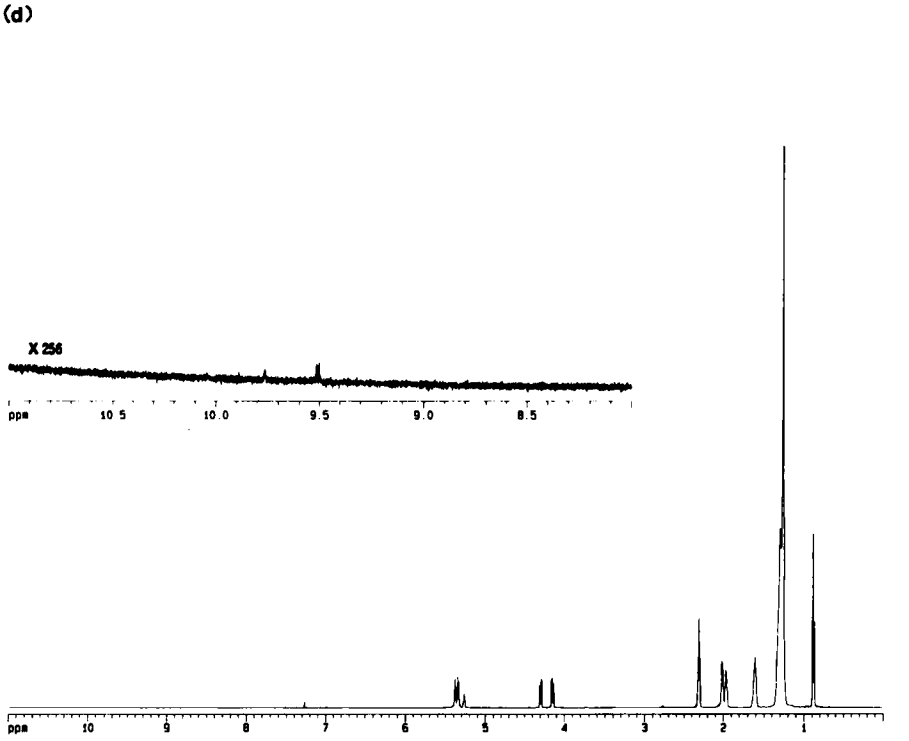


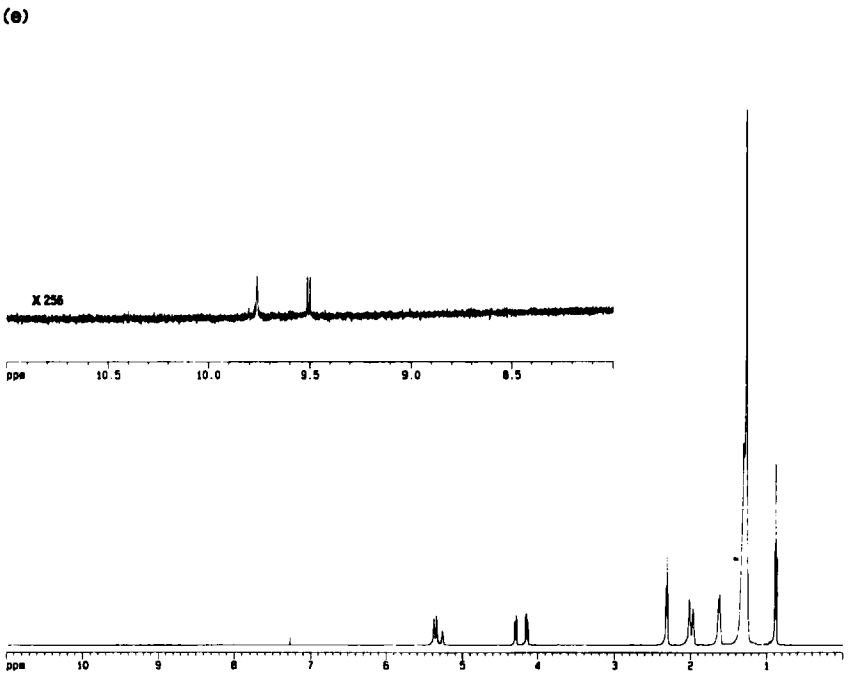
(b)



(c)







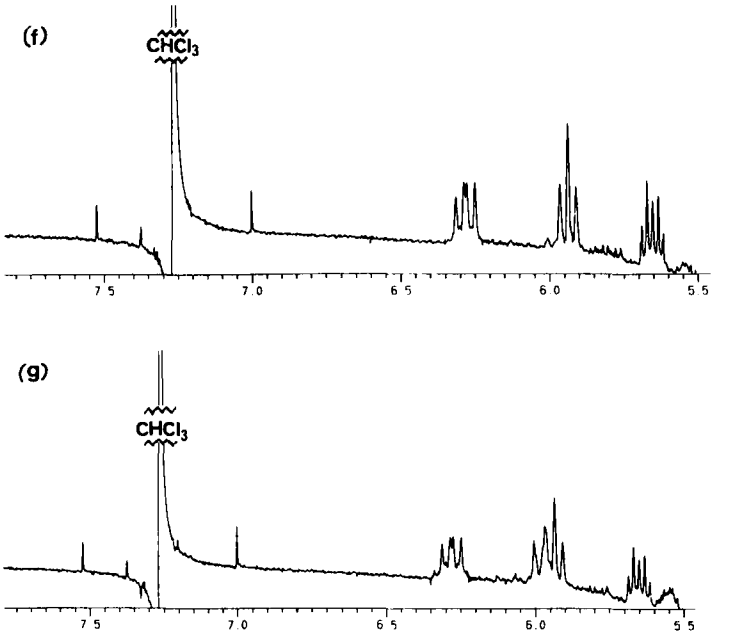
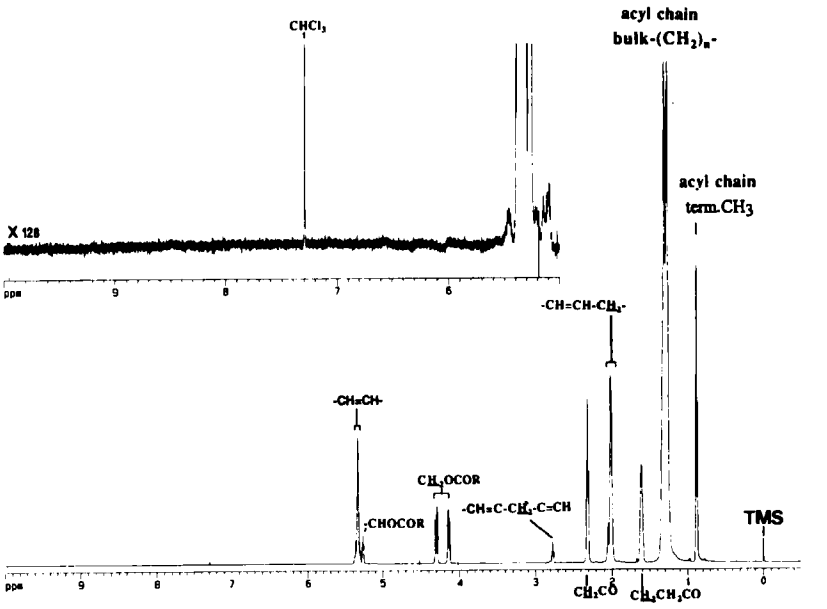


FIGURE 6. 600 MHz ^{1}H NMR spectra of a commercially-available sample of lard (L-1) obtained (a) before, and at (b) 30, (c) 60 and (d) 90 min. after heating at a temperature of 180°C in the presence of atmospheric O_2. The insets show the expanded 8.00–10.40 ppm (aldehydic) regions of these spectra. The 600 MHz spectrum of a sample subjected to an additional period of thermal stressing at an elevated temperature (30 min. at 250°C), together with its expanded 8.00–10.40 ppm region, is shown in (e). 10g of lard was heated in a 100 ml volume beaker (sample surface area 18.41 cm^2) and samples were stored in the dark at ambient temperature for a period of 888 hr. prior to ^{1}H NMR analysis. (f) and (g), Expanded 5.50–7.70 ppm regions of 400 MHz ^{1}H NMR spectra of samples of beef and lamb fat (dripping) subjected to 74 and 90 min. episodes of heating, respectively, at 180°C in a domestic fan-assisted oven (these materials were stored in the dark at ambient temperature for 24 hr. prior to analysis). Typical spectra are shown. Abbreviations: as Figures 1 and 2.

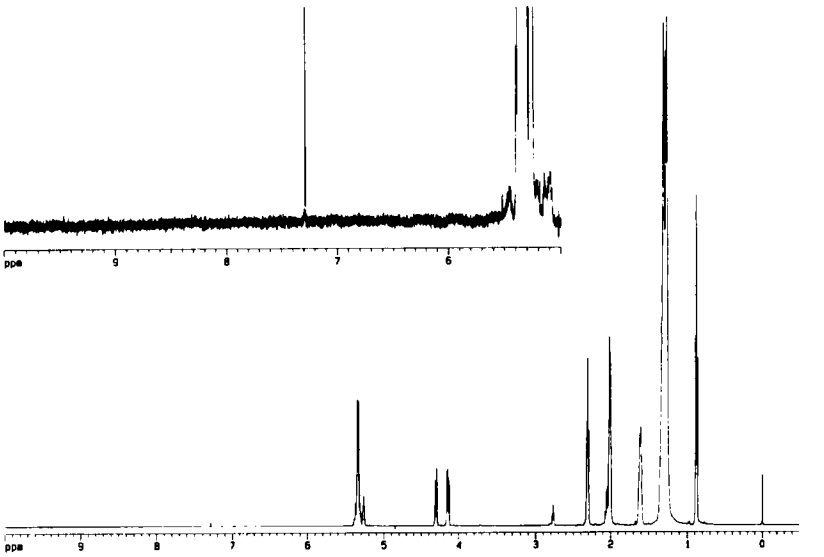
PUFA-derived hydroperoxides. However, the 5.635, 6.28 and 5.93 ppm signals may arise from the 12-, 11- and 10- position olefinic protons, respectively, of 13-hydroxy-*cis*-9,*trans*-11-octadecadienoylglycerol[14,27] (or the corresponding 10-, 11- and 12 position olefinic protons, respectively, of the *cis,trans*-9-hydroxydiene isomer). Moreover, the coupling constants for the 6.28 and 5.93 ppm signals are very similar to those previously reported for the 11- and 10- position protons, respectively, of the *cis,trans*-13-hydroxydiene adduct.[27] Further experiments to establish this are currently underway. Proton NMR spectra of these thermally-stressed fat samples did not contain any detectable aldehydic proton resonances.

The ^{1}H NMR profiles of control (unheated) and heated samples of virgin olive oil were obtained to provide further useful molecular information regarding the thermally-induced autoxidation of a culinary oil which is high in *mono*-unsaturated fatty acid content but low in that of PUFAs. The complete and expanded 5.00–10.00 ppm regions of spectra of control and thermally-stressed virgin olive oil samples are shown in Figures 7(a)–(d). Clearly, heating of this culinary oil according to standard frying procedures yields only very low levels of selected classes of aldehyde, i.e. *trans*-2-alkenals (doublet, $\delta = 9.48$ ppm) and alkanals (triplet, $\delta = 9.74$ ppm), the resonances of

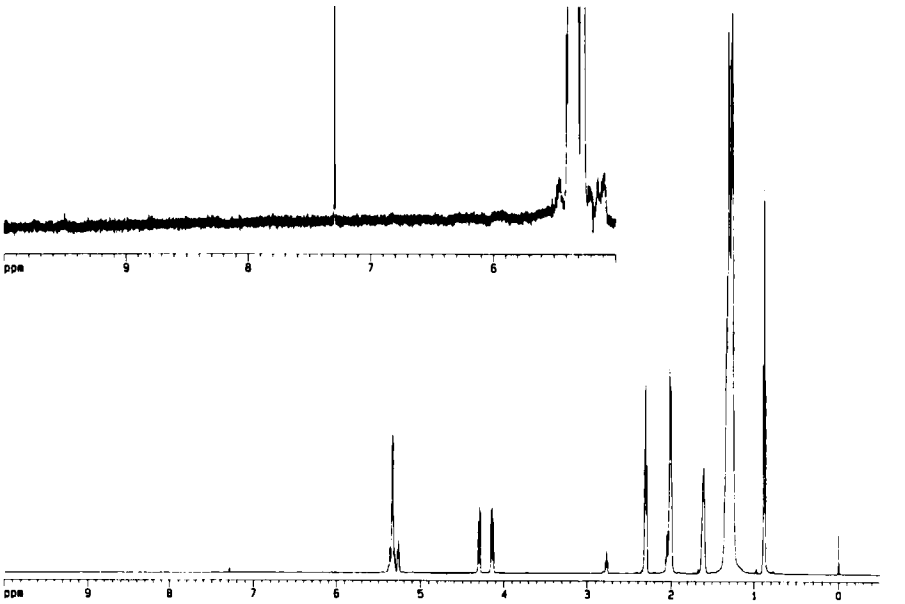
(a)



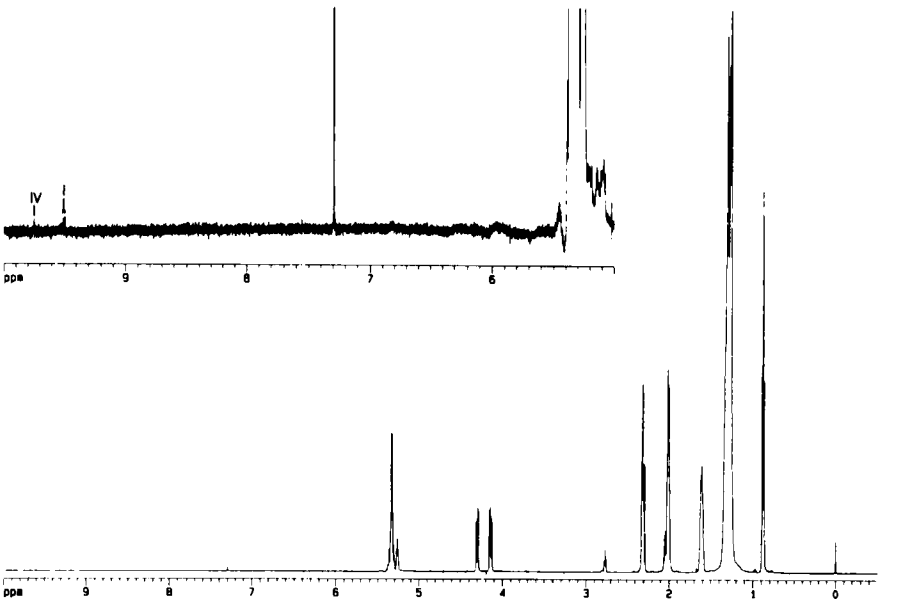
(b)

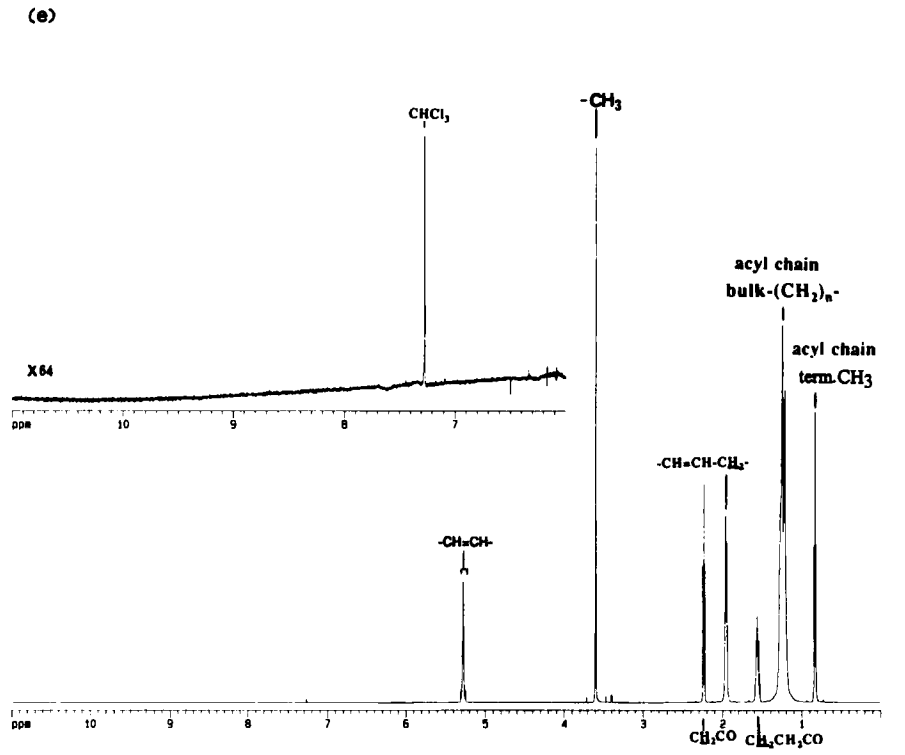


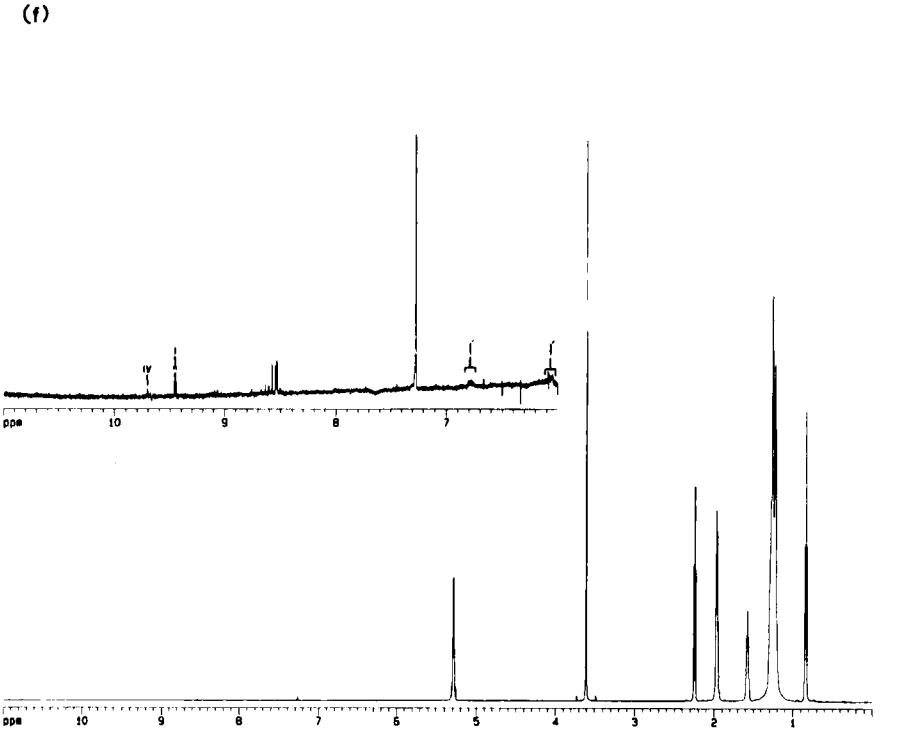
(c)



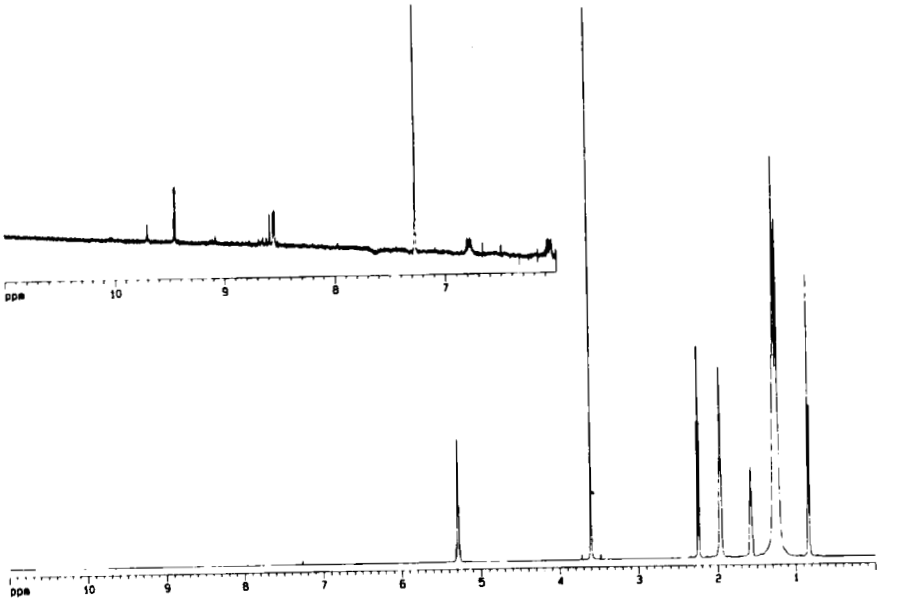
(d)



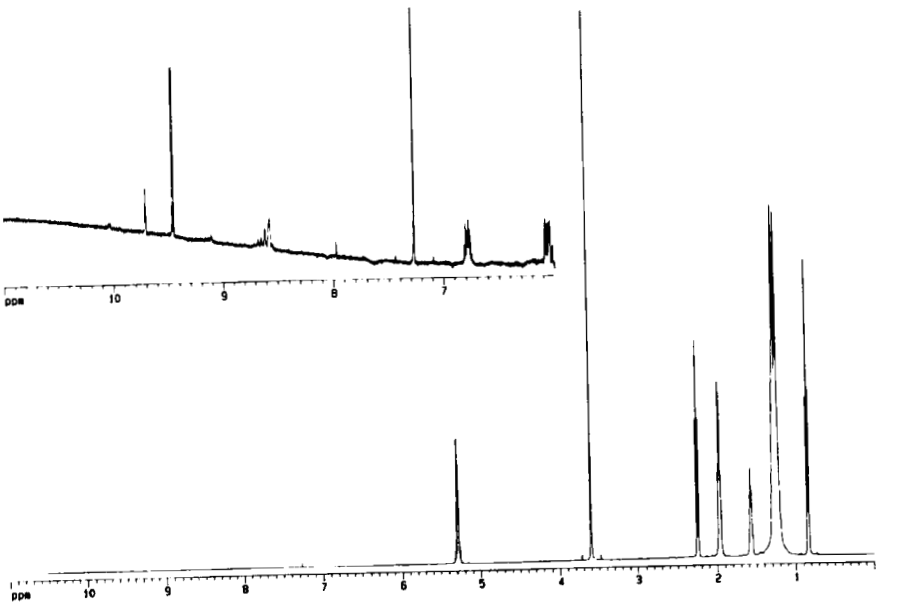




(g)



(h)



which only become NMR-detectable after rigorous thermal stressing (90 min. at 180°C). As expected, no conjugated diene system olefinic proton signals were detectable in the spectra acquired. The thermally-induced production of the 9.48 ppm aldehydic proton resonance was accompanied by those characteristic of *trans*-2-alkenal olefinic protons [6.85 (*dt*) and 6.10 ppm (*dd*)]. Although the results obtained may partially reflect the low levels of PUFAs present in this culinary oil, ^{1}H NMR analysis demonstrated that heating of the model *mono*-unsaturated fatty acid methyl oleate at 180°C for 30, 60 and 90 min. induced the generation of the above two classes of aldehyde. Figures 7(e)–(h) exhibit the complete and expanded 6.00–11.00 ppm regions of 600 MHz ^{1}H NMR spectra of control (unheated) and thermally-stressed samples of this compound. Elevations in the intensity of the 9.48 ppm aldehydic resonance with increasing time of heating at 180°C were accompanied by corresponding rises in those of the α,β-*mono*-unsaturated aldehyde olefinic proton signals centred at 6.85 and 6.10 ppm. Moreover, a variety of singlet ^{1}H resonances in the 8.50–8.80 and 9.00–9.20 ppm spectral regions are also noteworthy. In view of the data shown in Figures 3(e) and (f), these singlet resonances are assignable to hydroperoxy (–OO<u>H</u>) and/or hydroxy (–O<u>H</u>) group protons present in hydroperoxy/hydroxy-*mono*-ene adducts which presumably act as precursors of selected aldehydes (specifically *trans*-2-alkenals and alkanals). A small reduction in the intensity of the *mono*-allylic-CH_2 group signal located at 1.95 ppm (ca. 3%) was also observed, consistent with the limited peroxidative susceptibility of this *mono*-unsaturated fatty acid methyl ester. The relative rates of autoxidation of *mono*, *di*- and *tri*-unsaturated fatty acids (i.e., oleic, linoleic and linolenic acid derivatives) are in the ratio 1:12:25, respectively, under a specified set of experimental conditions.[26]

DISCUSSION

The non-destructive analysis of culinary oils and fats by high resolution ^{1}H NMR spectroscopy both prior and subsequent to heating at standard frying temperatures provides much useful information regarding thermally-induced chemical modifications to a variety of lipidic components detectable, with special reference to the autoxidation of PUFAs. Indeed, the advent of the 600 MHz spectrometer with its high level of sensitivity of detection and increased spectral dispersion has facilitated the rapid identification of such modifications.

In view of their high volatilities, the concentration of each aldehyde determined in heated culinary oils and fats represents that remaining in the sample after thermal stressing for a specified period of time (i.e., that available for human consumption) and therefore is considered to be a lower limit for the total amount of each of these components generated from the thermally-induced autoxidation of glycerol-bound PUFAs (and, to a lesser extent, *mono*-unsaturated fatty acids) present.

FIGURE 7. 600 MHz ^{1}H NMR spectra of a commercially-available sample of virgin olive oil heated at 180°C in the presence of atmospheric O_2 for periods of (a) 0 (control sample), (b) 30, (c) 60 and (d) 90 min. The insets show the expanded 8.00–10.40 ppm regions of these spectra. A 25g quantity of this culinary oil was heated in a 50 ml volume conical flask (oil surface area 15.91 cm^2) and samples removed at the above time-points were stored in the dark at ambient temperature for a period of 168 hr. prior to ^{1}H NMR analysis.

The complete and expanded 6.00–11.00 ppm regions of the 600 MHz ^{1}H NMR spectra of methyl oleate obtained after heating at 180°C in the presence of atmospheric O_2 for periods of 0 (control sample), 30, 60 and 90 min. are shown in (e), (f), (g) and (h) respectively (these samples were stored in the dark at ambient temperature for a 96 hr. period prior to analysis). Typical spectra are shown. Abbreviations: as Figures 1 and 2, with -CH_3, methyl ester functional group protons (–C00C<u>H</u>$_3$) of methyl oleate.

A further important consideration is the physical state of culinary oil and fat PUFAs when subjected to common frying practices. For example, the peroxidation rate of PUFAs is lower in globules than when they are dispersed in emulsions,[28] a phenomenon of much relevance to the frying of water-laden foodstuffs. Hence, the influence of increasing amounts of pre-added water on the generation of ^{1}H NMR-detectable, PUFA-derived autoxidation products in culinary oils and fats during episodes of thermal stressing requires investigation, and experiments to evaluate this are currently in hand.

The facile, simultaneous detection of a range of aldehydic species, and their conjugated hydroperoxydiene precursors in thermally-stressed culinary oils and fats by the technique outlined here is of much significance with regard to the possible health risks posed by their dietary consumption. The toxicological properties of aldehydes derived from the peroxidation of PUFAs are understood to be attributable to their ability to inhibit protein synthesis, inactive enzymes, block macrophage action and stimulate thrombin production *in vivo*.[29,34] These adducts react with free amino functional groups of proteins and DNA (i.e., Maillard reactions), and α,β-unsaturated aldehydes are particularly reactive as electrophiles in this context. Moreover, Maillard reactions involving the bi-functional aldehyde MDA cause intermolecular cross-linking of proteins and DNA.[4,35]

Toxic effects which putatively arise from the short-term feeding of heated and/or oxidised oils and fats to experimental animals include loss of appetite, diarrhoea, growth retardation, cardiomyopathy, hepatomegaly, haemolytic anaemia, and accumulation of peroxides in adipose tissue (highly oxidised cod liver oil);[36] elevated kidney and liver weights, cellular damage in various organs and a modified fatty acid composition of tissue lipids (oils and fats subjected to the heat and oxidation associated with normal usage).[37,38] Moreover, in one long-term investigation, Kaunitz[39] reported that the consumption of mildly oxidised oils by rats throughout their lifespan gave rise to an increase in the frequency of hepatic bile duct and cardiac fibrotic lesions.

Recent studies indicate that dietary-derived lipid peroxidation products may contribute significantly to the pathogenesis of atherosclerosis,[40] a hypothesis supported by reports that such species can accelerate all three stages of the disease process, i.e., endothelial injury, accumulation of plaque and thrombosis.[41] The observation that concentrations of lipid peroxides in blood serum obtained from humans and animals with atherosclerosis are significantly higher than those in corresponding samples collected from relevant controls has provided further evidence consistent with this proposal, and consumption of peroxidised oils has been suggested to enhance the accumulation of oxidised lipids in macrophages and monocytes,[42] a critical event in the disease process. Furthermore, animal studies have shown that diets containing thermally-stressed, PUFA-laden culinary oils exhibit a greater atherogencity than those containing unheated oils.[43]

Further toxicological investigations concerning heated oils and fats have focused on their mutagenic properties. Mutagen formation of repeatedly-used deep-frying fats has been previously evaluated using the Ames test.[44] Fat samples were fractionated into polar and non-polar fractions by column chromatography, and the former were found to increase the number of revertants without *S*-9 mix in various strains, strain *TA*97 being the most sensitive (the non-polar fractions showed no mutagenicity). Moreover, the mutagenic activity of polar fractions was positively correlated with thiobarbituric acid-reactive substances, suggesting the involvement of lipid oxidation products in mutagenicity.[44] Polar fractions of lipids extracted from pre-fried and fried potatoes were also investigated and the frying process was found to marginally increase the

number of revertants in strain *TA*97 without *S*-9 mix.[45] However, the mutagenicity of the lipid fractions of fried potatoes was not related to the heating time of the fat. Methanol extracts of fat-free residues of fried potatoes increased numbers of revertants in strain *TA*97 after metabolic activation, indicating that different classes of mutagens had been isolated. Urine samples from a small number (6) of healthy, non-smoking volunteers collected during the 24 hr. period following consumption of portions of potatoes fried in repeatedly-used fat showed no increase in mutagenicity compared with control samples. However, the precise molecular nature of mutagens formed during deep frying as well as their metabolic fate in humans remains unclear.[45]

The toxicological properties putatively associated with each of the above PUFA-derived peroxidation products are, of course, critically dependent on the rate and extent of their *in vivo* absorption from the gut into the systemic circulation. Although systemically-administered conjugated lipid hydroperoxydienes are acutely toxic to rodents, their effects tend to be less severe after oral administration. Thus, for example, Cortessi and Privett[46] showed that a single intravenous (*i.v.*) dose of 20 mg/kg methyl linoleate hydroperoxide (MLHP) to rats gave rise to a high mortality within 24 hr. (animals dying from severe lung damage), whereas an oral dose of approximately 200 mg/kg was without effect. Similarly, Holman and Greenberg[47] showed that whereas the *i.v.* LD_{50} value for ethyl linoleate hydroperoxide in mice was 12 mg/kg, similar doses given orally were non-lethal, a finding supported by the later studies of Olcott and Dolev.[48] Indirect evidence to suggest that the lack of effect of orally-administered conjugated hydroperoxydienes is attributable to the failure of these compounds to be absorbed across the gastric or intestinal epithelium was obtained by Bergen and Draper.[49] These researchers found that a substantial proportion of an oral dose of (1-^{14}C)-labelled MLHP remained in the gastrointestinal tract for periods of up to 31 hr. post-dosing, and from analysis of lipid extracts derived from mesenteric adipose tissue, they suggested that MLHP was transformed to both hydroxy- and oxodiene derivatives during uptake. However, no direct evidence for the nature of the species absorbed was obtained. Subsequently, Nakatsugawa and Kameda[50] found that a small proportion (0.23%) of an oral dose of MLHP was present in the lymphatic secretions of treated rabbits.

In contrast to the lack of absorption of the conjugated hydroperoxydienes, some limited evidence for the absorption of complex secondary lipid autoxidation products has been found by Kanazawa *et al.*[51] Thus, although approximately 50% of an oral dose of autoxidised [^{14}C]-labelled linoleic acid was excreted in the faeces, the majority (75%) of the remaining activity was absorbed and excreted in urine, or as $^{14}CO_2$. No indication of the nature of the absorbed materials was obtained, although fractionation of the products into low- (aldehyde-containing) and high- (endoperoxide-containing) molecular-mass fractions confirmed that both classes of compound were absorbed. Other compounds produced during deep oil/fat frying include cyclic fatty acid monomers and dimers. Feeding studies in rats with deep-fried fat showed that a high proportion of the fat was absorbed and that it contained a fraction which exerted toxicological properties.[52]

The biological actions and associated biochemical affects of 4-hydroxy-*trans*-2-nonenal have previously been investigated in some detail. Indeed, this aldehyde induces the lysis of erythrocytes,[53] diminishes the generation of superoxide anion ($O_2^{\cdot}$) by human neutrophils,[54] potentiates human platelet aggregation,[55] influences the chemotaxis of rat neutrophils[56] and exerts cytotoxic actions towards human umbilical cord vein endothelial cells,[57] Ehrlich tumour ascites cells[58] and the human malarial parasite,[59] and is both genotoxic and cytotoxic to rat hepatocytes.[60] 4-Hydroxy-*trans*-2-nonenal also inactivates cytochrome P-450,[53] adenylate cyclase,[61] 5′-nucleotidase[61] and glucose-

6-phosphatase,[53] inhibits the proliferative response to phytohaemagglutinin and alloantigens,[62] and the expression of c-myconcogene,[63] and modifies the structure of low-density-lipoprotein[64] (a phenomenon giving rise to an enchanced uptake of it by macrophages). Interestingly, Benedetti *et al.*[65] found that injection of a mixture of peroxidised PUFA-derived carbonyl compounds (predominantly 4-hydroxy-*trans*-2-nonenal at a level of 1.5×10^{-7} mol.dm^{-3}) gave rise to an inflammatory response (i.e., oedema) in the hind paw of rats.

Selley *et al.*[66] have recently employed gas chromatography combined with mass spectrometric detection to determine levels of 4-hydroxy-*trans*-2-nonenal in the blood plasma of healthy human subjects and patients with rheumatoid and osteoarthritis. Intriguingly, this lipid peroxidation end-product is present at a concentration of ca. 1×10^{-7} mol.dm^{-3} in healthy and osteoarthritic human plasma samples (but significantly elevated in those collected from rheumatoid arthritis patients), and although at least some of this could arise from the oxidative deterioration of PUFAs *in vivo*, there may be a relationship existing between these levels and the frequency of thermally/oxidatively-stressed culinary oil or fat consumption in the diet.

Oral administration of 4-hydroxy-*trans*-2-nonenal to rats at a dose level of only 2.6×10^{-7} mol.dm,$^{-3}$ a concentration not dissimilar to those found in healthy human plasma, was found to induce peptic ulcers in rats.[67] Hence, an elevated frequency and/or level of thermally-stressed culinary oil and, to a lesser extent, fat consumption in the diet may render individuals more susceptible to gastropathic conditions.

The chemical nature and concentrations of Maillard reaction products generated from the reactions of heated culinary oil- or fat-derived aldehydes with proteins and low-molecular-mass amino acids present in foodstuffs during standard frying practices is also of much toxicological importance since such species have mutagenic properties.[68] Moreover, chemical modification of proteins by aldehydes and alternative carbonyl compounds (i.e., ketones) may yield antigenic species, and the intermolecular cross-linking of proteins by MDA[40] is a process of much significance in this research area. Such Maillard reactions may be of relevance to the recent incidence of very severe and potentially fatal allergic reactions (anaphylactic shocks) experienced by a small but significant number of individuals who consume peanuts in their diet. Such a reaction arises from a response to one or more peanut proteins, and it is conceivable that chemical modification and/or cross-linking of these macromolecules by aldehydes present in peanut (groundnut) oil gives rise to antigens which are responsible for this phenomenon. Indeed, we have recently detected significant levels of proteins and aldehydes (alkanals, *trans*-2-alkenals and MDA) in commercially-available samples of this culinary oil that were not subjected to episodes of heating in the laboratory, the former by gel electrophoresis techniques (data not shown). The aldehydes may arise from an isolation or processing operation employed by manufacturers, possibly one involving a mild level of thermal-stressing. Experiments to further investigate this are currently in progress and results derived therefrom will be published in detail elsewhere.

Maillard reactions involving dietary-derived carbonyl compounds are also of a special significance in relation to dental aesthetics, cariology and periodontology since many independent observations have indicated that non-enzymic browning is of much aetiological importance in the development of extrinsic tooth discolouration.[69] Indeed, brown-pigmented melanoidins arise from the interactions of aldehydes and ketones with proteins containing a surplus of free amino groups,[70] and such browning products could result from the reactions of carbonyl compounds consumed in the diet with proteins located at the primary site for extrinsic tooth discoloration (the acquired pellicle). Hence, dietary consumption of thermally-stressed culinary oils and fats

containing high levels of reactive aldehydes may contribute to the discolouration of human teeth.

The results presented here also indicate that the inclusion of appropriate concentrations of α-TOH, or alternative lipid-soluble dietary antioxidants (e.g., BHT), in commercially-available frying oils and fats may render PUFAs therein less sensitive to thermally-induced peroxidation. However, it should be noted that α-TOH naturally present in the materials utilised for these investigations (ca. 2×10^{-3} mol.kg^{-1} in corn oil[71]) failed to prevent the generation of potentially cytotoxic aldehydes in samples after heating at 180°C for periods of 30 min., and therefore it is of much importance to establish a minimum effective level of such added antioxidants. Experiments to evaluate this are currently underway.

In conclusion, multicomponent analysis of thermally-stressed culinary oils and fats by high field ^{1}H NMR spectroscopy provides much useful information regarding the chemical nature and levels of PUFA-derived autoxidation products present. Such information is a critical primary requirement for future evaluations of the toxicological hazards putatively associated with the regular consumption of these materials.

Acknowledgements

We are very grateful to the Ministry of Agriculture, Fisheries and Food (UK) and the Commission of the European Communities (Agriculture and Agro-industry, including Fisheries) for financial support, the University of London Intercolleigate Research Services for the provision of NMR facilities, Peter Haycock [Department of Chemistry, Queen Mary and Westfield College, London, UK] for excellent technical assistance, and Jean Gatehouse and Janet Shaw for typing the manuscript.

References

1. Kubow, S. (1990) Toxicity of dietary lipid peroxidation products. *Trends in Food Science and Technology*, **1(3)**, 67–71.
2. Esterbauer, H. (1982) Aldehydic products of lipid peroxidation. In McBrien, D.C.H. and Slater, T.F. (eds.) *Free Radicals, Lipid Peroxidation and Cancer* pp. 101–128, Academic Press, London and New York.
3. Esterbauer, H., Jürgens, G., Quenberger, O. and Doller, E. (1987) Autoxidation of human low-density-lipoprotein: loss of polyunsaturated fatty acids and vitamin E, and generation of aldehydes. *Journal of Lipid Research*, **28**, 495–509.
4. Addis, P.B. (1986) Occurrence of lipid oxidation products in food. *Food Chemistry and Toxicology*, **24**, 1021–1030.
5. Iles, R.A., Buckingham, M.J. and Hawkes, G.E. (1983) Spin-echo proton NMR detection of normal and abnormal metabolites in plasma and urine. *Biochemical Society Transactions*, **11**, 374–375.
6. Nicholson, J.K., O'Flynn, M.P., Sadler, P.J., MacLeod, A.F., Juul, S.M. and Sönksen, P.H. (1984) Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects. *Biochemical Journal*, **217**, 365–375.
7. Grootveld, M., Henderson, E.B., Farrell, A., Blake, D.R., Parkes, H.G. and Haycock, P. (1991) Oxidative damage to hyaluronate and glucose in synovial fluid during exercise of the inflamed rheumatoid joint. Detection of abnormal low-molecular-mass metabolities by proton-n.m.r. spectroscopy. *Biochemical Journal*, **273**, 459–467.
8. Renou, J.P., Brigeut, A., Gattellier, P. and Kopp, J. (1985) *International Journal of Food Science and Technology*, **22**, 169–172.
9. Grootveld, M., Jain, R., Claxson, A.W.D., Naughton, D.P. and Blake, D.R. (1990) The detection of irradiated foodstuffs. *Trends in Food Science and Technology*, **1(1)**, 7–14.
10. Guillou, C., Remaud, G. and Martin, G.J. (1992) Applications of NMR to the characterisation and authentification of foods and beverages. *Trends in Food Science and Technology*, **3(8)**, 197–201.
11. Aue, W.P., Bartholdi, E., and Ernst, R.R., (1976) Two dimensional spectroscopy: application to nuclear magnetic resonance. *Journal of Chemical Physics*, **64**, 2229–2246.

12. Pouchert, C.J., and Behnke, J. (1992) *The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra*, Aldrich Chemical Co. Inc., U.S.A.
13. Kubow, S. (1992) Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radicals in Biology and Medicine*, **12**, 63–81.
14. Gardner, H.W. and Weisleder, D. (1972) Hydroperoxides from oxidation of linoleic and linolenic acids by soybean lipoxygenase: proof of the *trans*-11 double bond. *Lipids*, **7(3)**, 191–193.
15. Chan, H.W. and Levett, G. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. (1977) *Lipids*, **12(1)**, 99–104.
16. Neff, W.E., Frankel, E.N. and Miyashita, K. (1990) Autoxidation of polyunsaturated triacylglycerols. Trilinoleoylglycerol. *Lipids*, **25(1)**, 33–39.
17. Frank, H., Thiel, D. and MacLeod, J. (1989) Mass spectrometric detection of cross-linked fatty acids formed during radical-induced lesion of lipid membranes. *Biochemical Journal*, **260**, 873–878.
18. Gardner, H.W., Kleiman, R. and Weisleder, D. (1974) Homolytic decomposition of linoleic acid hydroperoxide: identification of fatty acid products. *Lipids*, **9(9)**, 696–706.
19. Barenghi, L., Bradamante, S., Giudici, G.A., and Vergani, C. (1990) NMR analysis of low-density-lipoprotein oxidatively modified *in vitro*. *Free Radical Research Communications*, **8(3)**, 175–183.
20. Neff, W.E., Frankel, E.N., Selke, E. and Weisleder, D. (1983) Photosensitised oxidation of methyl linoleate monohydroperoxides: hydroperoxy cyclic peroxides, dihydroperoxides, keto esters and volatile thermal decomposition products. *Lipids*, **18(12)**, 868–876.
21. Frankel, E., Neff, W.E. and Miyashita, K. (1990) Autoxidation of polyunsaturated triacylglycerols. II. Trilinolenoylglycerol. *Lipids*, **25(1)**, 40–47.
22. Wills, E.D. (1969) Lipid peroxide formation in microsomes. General Considerations. *Biochemical Journal*, **113**, 315–324.
23. Graveland, A. (1970) Enzymic oxidations of linoleic acid and glycerol-l-monolinoleate in doughs and water flour suspensions. *Journal of the American Oil Chemists Society*, **47**, 352–361.
24. Corongiu, F.P., Poli, G., Diansani, M.U., Cheeseman, K.H. and Slater, T.F. (1986) Lipid peroxidation and molecular damage to polyunsaturated fatty acids in rat liver. Recognition of two classes of hydroperoxide formed under conditions *in vivo*. *Chemico-Biological Interactions*, **59**, 147–155.
25. Binder, R.G., Applewhite, T.H., Diamond, M.J. and Goldblatt, L.A. (1964) Chromatographic analysis of seed oils II: fatty acid composition of dimorphoteca oil. *Journal of the American Oil Chemists Society*, **41**, 108–111.
26. de Man, J.M. (1976) *Principles of Food Chemistry*, p. 60, AVI, Westport, C.T.
27. Tallent, W.H., Harris, J., Wolf, I.A. and Lundin, R.E. (1966) (R)-13-hydroxy-*cis*-9, *trans*-11-octadecadienoic acid, the principle fatty acid from *Coriaria nepalensis* seed oil. *Tetrahedron Letters*, 4329.
28. Wills, E.D. (1965) Mechanisms of lipid peroxide formation in tissues. Role of metals and hematin proteins in the catalysis of the oxidation of unsaturated fatty acids. *Biochim. Biophys. Acta.*, **98**, 238–251.
29. Gutteridge, J.M.C., Lamport, P. and Dormandy, T.L. (1976) The antibacterial effect of water-soluble compounds from autoxidising linolenic acid. *Journal of Medical Microbiology*, **9**, 105–110.
30. Tappel, A.L. (1975) Lipid peroxidation and fluorescent molecular damage to membrances. In Trump, B.J. and Arstila, A.V. (eds.). *Pathobiology of Cell Membranes Vol. 1*, pp. 145–170, Academic Press, London and New York.
31. Turner, S.R., Campbell, J.A. and Lynn, W.S. (1975) Polymorphonuclear leukocyte chemotaxis toward oxidised lipid components of cell membranes. *Journal of Experimental Medicine*, **141**, 1437–1441.
32. Schauenstein, E., Esterbauer, H. and Zollner, H. (1977) *Aldehydes in Biological systems*, Pion Press.
33. Barrowcliffe, T.W., Gray, E., Kerry, P.J. and Gutteridge, J.M.C. (1984) Trigylceride-rich lipoproteins are responsible for thrombin generation induced by lipid peroxides. *Thrombosis and Haemostasis*, **52**, 7–10.
34. Van Hinsbergh, V.W.H. (1984) LDL cytotoxicity. The state of the art. *Atherosclerosis*, **53**, 113–118.
35. Kristal, B.S. and Yu, B.P. (1992) An emerging hypothesis: synergistic induction of ageing by free radicals and Maillard reactions. *Journal of Gerontology*, **47(4)**, B107–B114.
36. Sanders, T.A.B. (1983) Nutritional significance of rancidity. In Allen, J.C. and Hamilton, R.J. (eds.) *Rancidity in Foods* pp. 59–66, Elsevier, Amsterdam.
37. Yoshida, H. and Kujimoto, G. (1989) Effect of dietary vitamin E on the toxicity of autoxidised oil to rats. *Annals of Nutrition and Metabolism*, **33**, 153–161.
38. Alexander, J.C., Valli, V.E., and Chanin, B.E. (1987) Biological observations from feeding corn oil and heated peanut oil to rats. *Journal of Toxicology and Environmental Health*, **21**, 295–309.
39. Kaunitz, H. (1978) In Kabara, J.J. (ed.) *The Pharmacological Effect of Lipid*, pp. 203–210, The American Oil Chemists Society.
40. Addis, P.B. (1990) Occurrence of lipid peroxidation products in food. *Food and Nutrition News*, **62**, 1–4.
41. Yagi, K. (1988) Lipid peroxides in atherosclerosis. In Ando, W. and Morooka, Y. (eds.) *The Role of Oxygen in Chemistry and Biology* pp. 383–390, Elsevier, Amsterdam.

42. Smith, T., and Kummerow, F.A. (1987) The role of lipids in heart disease and ageing. In Watson, R.R. (ed.) *Nutrition and Heart Disease Vol. 1*, pp. 45–64, CRC Press.
43. Kritchevsky, D. and Tepper, S.A. (1967) Cholesterol vehicle in experimental atherosclerosis. 9. Comparison of heated corn oil and heated olive oil. *Journal of Atherosclerosis Research*, **7**, 647–651.
44. Hageman, G., Hermans, R., Ten Hoor, F. and Kleinjans, J. (1988) Mutagenicity of deep frying fat, and evaluation of urine mutagenicity after consumption of fried potatoes. *Journal of Mutation Research*, **204**, 593–604.
45. Hageman, G., Hermans, R., Ten Hoor, F. and Kleinjans, J. (1990) Mutagenicity of deep frying fat, and evaluation of urine mutagenicity after consumption of fried potatoes. *Journal of Food Chemistry and Toxicology*, **28**, 75–80.
46. Cortesi, R. and Privett, O.S. (1972) Toxicity of fatty ozonides and peroxides. *Lipids*, **7(11)**, 715–721.
47. Holman, R.T. and Greenberg., S.I. (1958) The toxicities of methyl oleate peroxide and ethyl linoleate peroxide. *Journal of the American Oil Chemists Society*, **35**, 707.
48. Olcott, H.S. and Dolev, A. (1963) Toxicity of fatty acid ester hydroperoxides. *Proceedings of the Society of Experimental Biology and Medicine*, **114**, 820.
49. Bergan, J.G. and Draper, H.H. (1970) Absorption and metabolism of 1-^{14}C-methyl linoleate hydroperoxide. *Lipids*, **5**, 975–982.
50. Nakatsugawa, K. and Kaneda, T. (1980) Absorption of methyl linoleate hydroperoxides in rabbits. *Yukayaku*, **30**, 74–77.
51. Kanazawa, K., Kanazawa, E. and Natake, M. (1985) Uptake of secondary autoxidation products of linoleic acid by the rat. *Lipids*, **20**, 412–419.
52. Nolen, G.A., Alexander, J.C. and Artman, N.R. (1967) Long-term rat feeding study with used frying fats. *Journal of Nutrition*, **93**, 337–348.
53. Benedetti, A., Comporti, M. and Esterbauer, H. (1980) Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim. Biophys. Acta*, **620**, 281–296.
54. Witz, G., Lawrie, N.J., Ambruso, M.A. and Goldstein, B.D. (1985) Inhibition by reactive aldehydes of superoxide anion radical production in stimulated human neutrophils. *Chemico-Biological Interactions*, **53**, 13–23.
55. Selley, M.L., McGuiness, J.A., Jenkin, L.A., Bartlett, M.R. and Ardlie, N.G. (1988) Effect of 4-hydroxy-2,3-*trans*-nonenal on platelet function. *Thrombosis and Haemostasis*, **59**, 143–146.
56. Curzio, M., Esterbauer, H., Di Mauro, C., Cecchini, G. and Dianzani, M.U. (1986) Chemotactic activity of the lipid peroxidation product 4-hydroxynonenal and homologous hydroxynonenals. *Biological Chemistry Hoppe-Seyler*, **367**, 321–329.
57. Kaneko, T., Kaji, K. and Matsum, M. (1988) Cytotoxicities of a linoleic acid hydroperoxide and its related aliphatic aldehydes toward cultured human umbilical vein endothelial cells. *Chemico-Biological Interactions*, **67**, 295–304.
58. Hauptlorenz, S., Esterbauer, H., Moll, W., Pumpel, R. Schauenstein, E. and Puschendorf, B. (1985) Effects of the lipid peroxidation product 4-hydroxynonenal and related aldehydes on proliferation and viability of cultured Ehrlich ascites tumour cells. *Biochemical Pharmacology*, **34**, 3802–3809.
59. Clark, I.A., Butcher, G.A., Buffington, G.D. and Cowden, W.B. (1987) Toxicity of certain products of lipid peroxidation to the human malaria parasite plasmodium falciparum. *Biochemical Pharmacology*, **36**, 543–546.
60. Griffin, D.S. and Segall, H.J. (1986) Genotoxicity and cytotoxicity of selected pyrrolizidine alkaloids, a possible alkenal metabolite of the alkaloids, and related alkenals. *Toxicology and Applied Pharmacology*, **86**, 227–234.
61. Paradisi, L., Panagini, G., Parola, M., Barrera, G. and Dianzani, M.U. Effects of 4-hydroxynonenal on adenylate cyclase and 5′-nucleotidase activities in rat liver plasma membranes (1985) *Chemico-Biological Interactions*, **53**, 209–217.
62. Tessitore, L., Matera, L., Bonelli, G., Baccino, F.M. and Dianzani, M.U. (1987) Aliphatic aldehydes inhibit the proliferative response of human peripheral blood lymphocytes to phytohaemagglutinin and alloallergens. *Chemico-Biological Interactions*, **62**, 217–226.
63. Barrera, G., Martinotti, S., Fazio, V., Manzari, V., Paradisi, L., Parola, M., Frati, L. and Dianzani, M.U. (1987) Effect of 4-hydroxynonenal on c-myc expression. *Toxicological Pathology*, **15**, 238–240.
64. Hoff, H.F., O'Neil, J., Chisolm, G.M., Cole, T.B., Quehenberger, D., Esterbauer, H. and Jurgens, G. (1987) Modification of low density lipoprotein with 4-hydroxynonenal induces uptake by macrophages. *Arteriosclerosis*, **9**, 539–549.
65. Benedetti, A., Ferrali, M., Casini, A.F., Peiri, S. and Comporti, M. (1980) Foot edema induced by carbonyl compounds originating from the peroxidation of liver microsomal lipids. *Biochemical Pharmacology*, **29**, 121–124.
66. Selley, M.L., Bourne, D.J., Bartlett, M.R., Tymms, K.E., Brook, A.S., Duffield, A.M. and Ardlie, N.G.

(1992) Occurrence of (E)-4-hydroxy-2-nonenal in plasma and synovial fluid of patients with rheumatoid and osteoarthritis. *Annals of the Rheumatic Diseases*, **51**, 481–484.
67. Jayaraj, A.P., Rees, K.R., Tovey, F.EI. and White, J.S. (1986) A molecular basis of peptic ulceration due to diet. *British Journal of Experimental Pathology*, **67**, 149–155.
68. Skog, K., Knize, M.G., Felton, J.S. and Jagerstad, M. (1992) Formation of new heterocyclic amine mutagens by heating creatinine, alanine, threonine and glucose. *Mutation Research*, **268(2)**, 191–197.
69. Eriksen, H.M., Nordbo, H., Kantanen, H. and Ellingsen, J.E. (1985) Chemical plaque control and extrinsic tooth discolouration. A review of possible mechanisms. *Journal of Clinical Periodontology*, **12**, 345–350.
70. Berk, Z (1976). In Berk, Z (ed.) Non-enzymic browning. *Braveman's Introduction to the Biochemistry of Foods* pp. 149–167, Elsevier, Amsterdam.
71. Hammer, C.T. and Wills, E.D. (1978) The role of lipid components of the diet in the regulation of the fatty acid composition of the rat liver endoplasmic reticulum and lipid peroxidation. *Biochemical Journal*, **174**, 585–593.

Accepted by Professor B. Halliwell