Application of High-Resolution, Two-Dimensional ^{1}H and ^{13}C Nuclear Magnetic Resonance Techniques to the Characterization of Lipid Oxidation Products in Autoxidized Linoleoyl/Linolenoylglycerols

Christopher J.L. Silwood and Martin Grootveld*

Bone and Joint Research Unit, St. Bartholomews and the Royal London Hospitals School of Medicine and Dentistry, London E1 2AD, United Kingdom

ABSTRACT: Subjection of polyunsaturated fatty acid (PUFA)-rich culinary oils to standard frying episodes generates a range of lipid oxidation products (LOP), including saturated and α,β-unsaturated aldehydes which arise from the thermally induced fragmentation of conjugated hydroperoxydiene precursors. Since such LOP are damaging to human health, we have employed high-resolution, two-dimensional ^{1}H-^{1}H relayed coherence transfer, ^{1}H-^{1}H total correlation, ^{1}H-^{13}C heteronuclear multiple quantum correlation, and ^{1}H-^{1}H J-resolved nuclear magnetic resonance (NMR) spectroscopic techniques to further elucidate the molecular structures of these components present in (i) a model linoleoylglycerol compound (1,3-dilinolein) allowed to autoxidize at ambient temperature and (ii) PUFA-rich culinary oils subjected to repeated frying episodes. The above techniques readily facilitate the resolution of selected vinylic and aldehydic resonances of LOP which appear as complex overlapping patterns in conventional one-dimensional spectra, particularly when employed in combination with solvent-induced spectral shift modifications. Hence, much useful multicomponent information regarding the identity and/or classification of glycerol-bound conjugated hydroperoxydiene and hydroxydiene adducts, and saturated and α,β-unsaturated aldehydes, present in autoxidized PUFA matrices is provided by these NMR methods. Such molecular information is of much value to researchers investigating the deleterious health effects of LOP available in the diet.

Paper no. L7951 in *Lipids 34,* 741–756 (July 1999).

The autoxidation of polyunsaturated fatty acids (PUFA) is an autocatalytic, self-perpetuating chain reaction system that has been implicated in the pathogenesis of many human diseases, e.g., atherosclerosis and inflammatory joint diseases (1–3). PUFA 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 of the methylene group C–H bonds. Subsequently, one major reaction pathway for the resulting resonance-stabilized carbon-centered 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 hydroperoxydiene (CHPD) 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), the process is repeated many times. CHPD are subsequently degraded to a wide variety of secondary autoxidation products which include saturated and unsaturated aldehydes, di- and epoxyaldehydes, lactones, furans, ketones, oxo and hydroxy acids, and saturated and unsaturated hydrocarbons.

Thermal stressing of culinary oils according to standard frying/cooking practices (domestic or otherwise) gives rise to and/or perpetuates the radical-dependent autoxidation of PUFA therein. Indeed, a wide range of aldehydes arises from the thermally induced decomposition of CHPD *via* several processes, including the β-scission of pre-formed alkoxyl radicals. Such aldehydic fragments (*n*-alkanals, *trans*-2-alkenals, *trans,trans*- and *cis,trans*-alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde) have the capacity to exert a variety of toxicological effects in view of their extremely high reactivity with critical biomolecules [DNA base adducts, proteins such as the apolipoprotein B moiety of low density lipoprotein, peptides, free amino acids, endogenous thiols such as glutathione, etc. (4)]. Interaction of these aldehydes with DNA can give rise to genotoxic events and possibly cancer.

Our laboratory recently reported the detection and quantification of PUFA-derived autoxidation products (notably aldehydes and their conjugated hydroperoxy/hydroxydiene precursors) in culinary oils and fats by high-field proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy (5,6). Results obtained demonstrated that the thermally induced oxida-

*To whom correspondence should be addressed at Bone and Joint Research Unit, London Hospital Medical College, ARC Building 25-29 Ashfield St., London E1 2AD, United Kingdom. E-mail: c.j.silwood@mds.qmw.ac.uk

Abbreviations: CHPD, conjugated hydroperoxydiene; HMQC, heteronuclear multiple quantum coherence transfer; LOP, lipid oxidation products; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acids; RCT, relayed coherence transfer; RD, relaxation delay; TMS, tetramethylsilane; TOCSY, total correlation.

tive stressing of PUFA-rich culinary oils produces much higher levels of cytoxic aldehydes than in corresponding samples of predominantly saturated fats (lard, drippings, etc.). The concentrations of aldehydes generated in culinary oils are critically dependent on PUFA content, the nature and capacity of the heating vessel employed, and the duration/conditions of heating and subsequent storage. More recently, we demonstrated that typical *trans*-2-alkenal compounds generated from the thermally induced autoxidation of PUFA are readily absorbed from the gut into the systemic circulation, metabolized (primarily *via* the addition of glutathione across their electrophilic carbon-carbon double bonds), and excreted in the urine as C-3 mercapturate conjugates in rats (7). Hence, such aldehydes have the ability to covalently modify lysine residues of the apolipoprotein B moiety of low density lipoprotein, rendering it susceptible to uptake by macrophages, a critical step in the production of foam cells *in vivo* (2). The hypothesis that dietary-derived lipid oxidation products (LOP) contribute significantly to the pathogenesis of atherosclerosis is further supported by reports that such agents can accelerate all three stages of the disease process, i.e., endothelial injury, accumulation of plaque, and thrombosis (8). Moreover, animal feeding studies showed that diets containing thermally-stressed [and therefore LOP-rich (5,6)] PUFA-laden culinary oils have a greater atherogenicity than those containing corresponding unheated oils (9). Indeed, Straprans *et al.* (10) recently examined the ability of oxidized dietary lipids to accelerate the development of atherosclerosis in New Zealand White rabbits and found that feeding with an oxidized lipid-rich diet gave rise to a 100% increase in fatty streak lesions in the aorta.

In view of the above observations, the detection and quantification of specific products arising from the autoxidation of culinary oil PUFA during standard frying practices are of paramount importance, and the multicomponent analytical ability of high-resolution ^{1}H NMR spectroscopy offers major advantages over alternative laboratory methods since it permits the rapid, simultaneous study of many primary and secondary LOP present in such samples (5,6). Indeed, the development of high-field NMR spectrometers with increased resolution, dynamic range, and sensitivity has given rise to rapid advances in the analysis of complex, multicomponent samples such as foodstuffs, human biofluids, and pharmaceutical formulations (11–14). The principles of this spectroscopic technique involve the absorption of energy from the radio frequency region of the electromagnetic spectrum to detect changes in the alignment of nuclear magnets during their exposure to a powerful external magnetic field. The absorption frequencies of such nuclei [for example, those of ubiquitous hydrogen nuclei (^{1}H)] present in the ^{1}H NMR spectrum of a particular chemical compound are critically dependent on their magnetic (and therefore, chemical) environment, and the appearance (multiplicity) of a resonance (signal) is influenced by neighboring ^{1}H nuclei in a well-characterized manner. Moreover, the intensity of each signal is directly proportional to the product of the number of magnetically equivalent nuclei in the structural/functional group responsible for it and the concentration of the molecule containing that group. Hence, much valuable molecular information regarding the nature and levels of a wide variety of compounds present in biological or biologically derived matrices can be obtained from high-field, high-resolution NMR investigations.

Although numerous prominent ^{1}H NMR signals ascribable to aldehydes and their CHPD precursors are observed in the less-crowded, higher-frequency regions of one-dimensional spectra acquired on such materials, there is a high degree of overlap between selected vinylic (isomeric CHPD and conjugated hydroxydienes, and α,β-unsaturated aldehydes) and aldehydic group proton resonances, even at operating frequencies as high as 600 MHz. However, the recent availability of spectrometers of operating frequencies ≥600 MHz has led to a synchronous increase in the development and application of both homonuclear and heteronuclear two-dimensional NMR methods in order to resolve the many overlapping multiplet resonances (many with higher-order coupling patterns) now detectable in very high field ^{1}H NMR spectra acquired on complex multicomponent samples. Indeed, the battery of such two-dimensional techniques employable facilitates the establishment of the molecular structures of specified organic compounds (15–19). Proton-proton and proton-carbon scalar couplings readily determine groups of resonances arising from individual components, and the unambiguous structural information derived therefrom is, in general, not obtainable from alternative analytical techniques.

Therefore, in this investigation we employed ^{1}H-^{1}H relayed coherence transfer (RCT), ^{1}H-^{1}H total correlation (TOCSY), ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC), and ^{1}H-^{1}H *J*-resolved NMR spectroscopic techniques for the purpose of clarifying our spectral assignments and hence provide further useful information regarding the molecular structures of LOP present in thermally/oxidatively stressed culinary oils and model PUFA.

MATERIALS AND METHODS

Culinary oil and model linoleoylglycerol samples. Control (unheated) and repeatedly utilized culinary frying oil samples were kindly supplied by a fast-food retail establishment. Electronic integration of the bis-allylic-CH_2 , ω-CH_3, and highly unsaturated fatty acid acyl chain terminal-CH_3 group ^{1}H resonances (δ = 2.76, 0.90, and 0.95 ppm, respectively) present in single-pulse 400 MHz proton NMR spectra of the control (unheated) sample acquired immediately after collection revealed that the material had a polyunsaturate content of 42 molar %, of which *ca.* one-quarter consisted of highly unsaturated fatty acids, i.e., those with ≥3 unconjugated double bonds (predominantly linolenoylglycerol adducts).

The above repeatedly used culinary oil samples were placed in glass vessels and stored in the dark at ambient temperature (22°C) for a period of 30 d prior to NMR analysis.

Sealed vials containing authentic samples of the model linoleoylglycerol species 1,3-dilinolein (Sigma-Aldrich

Chemical Co., Gillingham, Dorset, United Kingdom) were opened, and the compound therein allowed to autoxidize in the presence of atmospheric O_2 at an ambient temperature for a period of 2.0 h prior to subjecting it to NMR experiments.

Portions (300 g) of chipped potatoes and bacon were fried in 60- and 20-mL vol, respectively, of a commercially available sample of sunflower seed oil [PUFA content 62% (w/w)] in a 15-cm diameter shallow frying pan at 180°C for a period of 30 min in the presence of atmospheric O_2. A 60-mL volume of this culinary oil subjected to an identical thermal stressing episode in the absence of added food material, together with an unheated sample of this frying medium, served as controls. Subsequently, samples (1.00 mL) of oil were removed for ^{1}H NMR analysis.

Reagents. Tetramethylsilane (TMS) and the model autoxidized PUFA-derived aldehydes pentanal, hexanal, heptanal, octanal, *trans*-2-pentenal, *trans*-2-heptenal, *trans,trans*-nona-2-4-dienal, and *trans,trans*-deca-2,4-dienal were purchased from Sigma-Aldrich Chemical Co. Deuterated NMR solvents (C^2HCl_3 and ^2H_2O) were purchased from Goss Scientific Ltd. (Great Baddow, Essex, United Kingdom).

NMR measurements. NMR measurements on the above samples were conducted on Bruker AMX-600 (University of London Intercollegiate Research Services, Queen Mary and Westfield College Facility, University of London, United Kingdom) or Bruker AMX-400 (ULIRS; King's College Facility, University of London, United Kingdom) spectrometers, the former operating at frequencies of 600.13 (^{1}H) and 150.93 (^{13}C) MHz, the latter at 400.13 (^{1}H) MHz. ^{1}H-^{1}H *J*-resolved, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C transfer (HMQC) experiments were performed on the 600 MHz facility, while ^{1}H-^{1}H RCT experiments were performed on the 400 MHz spectrometer. The probe temperature was 298 K. Typically, 0.30-mL aliquots of culinary oil or 1,3-dilinolein samples were diluted to a volume of 0.90 mL with deuterated chloroform (C^2HCl_3) [or deuterated methanol (C^2H_3OH), where appropriate], which provided a field frequency lock, the solutions were thoroughly rotamixed and then were transferred to either 5- (one-dimensional ^{1}H, ^{13}C, ^{1}H-^{1}H RCT, ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H *J*-resolved experiments) or 8-mm (^{1}H-^{13}C HMQC experiments) diameter NMR tubes.

Chemical shifts were referenced to internal TMS ($\delta = 0.00$ ppm) and/or the residual chloroform proton signal ($\delta = 7.262$ ppm), [and for samples in C^2H_3OH solution, the residual methanol methyl group signal ($\delta = 3.210$ ppm)]. For ^{13}C spectra recorded in C^2HCl_3 solution, chemical shifts were referenced to internal TMS ($\delta = 0.00$ ppm) and/or the chloroform carbon signal ($\delta = 77.00$ ppm). Resonances present in single-pulse ^{1}H NMR spectra of culinary oil and model PUFA compound samples were routinely assigned by a consideration of chemical shift values, coupling patterns, and coupling constants, where appropriate. The molecular nature of particular classes of aldehydes detectable was confirmed by performing standard additions of authentic, commercially available compounds [*n*-alkanals, *trans*-2-alkenals, and *trans,trans*-alka-2,4-dienals, the latter also containing trace (although ^{1}H NMR-detectable) levels of corresponding *cis,trans*-isomer impurities].

^{1}H-^{1}H RCT experiments employed the standard sequence 90°-t_1-90°-τ-180°-τ-90°-ACQ (20,21), 90° on the AMX-400 spectrometer being equivalent to 9.5 μs. Acquisition parameters were: 256 t_1 increments, each of magnitude 1,024 data points; spectral width 4,902 Hz in each dimension; 64 transients in each case; 4 dummy scans; relaxation delay (RD) 2.0 s; acquisition time 0.21 s. Unshifted sine-bell window functions were applied in each dimension, with zero-filling twice in f_1, prior to transformation of the matrix of size 1,024 × 1,024 data points. The delay period τ (0.05 s) was optimized for the appropriate vicinal coupling constants and rotameric equilibria existing in the expected constituent glyceride moieties.

^{1}H-^{1}H TOCSY spectra were recorded using the pulse sequence RD-(90°-t_1-spin lock)-ACQ (22), 90° on the AMX-600 spectrometer for that experiment being equivalent to 8.4 μs. The spin lock employed the MLEV-17 sequence (23), with a typical mixing time of 70 ms. For experiments where a restriction of carbon chain correlations was required, this mixing time was only 20 ms. Acquisition parameters were: 256 t_1 increments, each of magnitude 2,048 data points; spectral width 12,019 Hz in each dimension; 64 transients in each case; 4 dummy scans; RD 3 s; acquisition time 0.17 s. Sine-bell squared window functions shifted by π/2 (i.e., a cosine squared function) were applied in each dimension, with zero-filling twice in f_1, prior to transformation of the matrix of size 2,048 × 1,024 data points. The standard phase-sensitive (time-proportional phase incrementation) method (24) for optimal detection along the second dimension was utilized in such experiments.

^{1}H-^{1}H *J*-resolved experiments employed the standard sequence 90°-t_1/2-180°-t_1/2-ACQ (16), 90° on the AMX-600 spectrometer for that experiment being equivalent to 7.9 μs. Acquisition parameters were: 128 t_1 increments, each of magnitude 2,048 data points; spectral width 8,621 Hz in f_2 and 100 Hz in f_1; 64 transients in each case; 4 dummy scans; RD 2.0 s; acquisition time 0.24 s. Unshifted sine-bell window functions were applied in each dimension before transformation of the matrix of eventual size 2,048 × 256 data points.

The time-proportional phase incrementation phase-sensitive method was also utilized in the ^{1}H-^{13}C HMQC (25) experiments for optimal detection of ^{13}C frequencies in the second dimension, ^{1}H detection being achieved through the employment of a dedicated 8-mm inverse geometry probe in order to maximize sample information. Acquisition parameters were: 200 t_1 increments each of magnitude 2,048 data points; spectral width 13,228 Hz in f_2 and 63,381 Hz in f_1; 960 transients in each case; 4 dummy scans; RD 1.0 s. A delay equivalent to 3.7 ms (1/2 $^1J_{CH}$) was utilized for optimal refocusing of heteronuclear information, and composite pulse broadband ^{13}C decoupling (GARP) (26) was employed during the ^{1}H acquisition period. A sine-bell-squared function, shifted by π/2 (i.e., a cosine squared function) was applied in each dimension, with forward complex linear prediction from

200 to 400 data points and subsequent zero-filling to 1,024 data points in f_1, prior to transformation of the matrix of size 2,048 × 1,024 data points.

Confirmation of NMR assignments using computational methods. ^{1}H NMR assignments and appropriate chemical shift values and coupling constants of resonances of LOP possessing complex spin systems were confirmed with the HNMR prediction software (version 3.0) from the Advanced Chemistry Development (ACD Inc., Toronto, Canada) software suite. This software was employed to generate a theoretical spectrum which did not incorporate an allowance for solvent-mediated shielding effects, and the ^{1}H chemical shift and ^{1}H-^{1}H spin-spin coupling constant values generated therefrom were then adjusted to reflect those obtained from the experimental spectrum *via* a first-order analysis. The theoretical spectrum was then recalculated, a correct first-order analysis giving rise to an exact match between the experimentally and theoretically derived spectra. Calculations also made an allowance for the concentration of each LOP within sample mixtures. The ^{13}C facility of the ACD suite of programs (ACD Inc.), CNMR, was employed for predicting spectra of LOP expected to be contributing to ^{13}C spectra of autoxidized culinary oils and model PUFA compounds exhibiting a high degree of resonance overlap. For example, the ^{1}H simulations were based around an internal database containing data for >81,000 experimental ^{1}H spectra, the associated algorithms employing intramolecular interaction parameters for >300 structural fragments, the associated subalgorithms estimating initial values for unique structural fragments. Fragment lists are handled with a modified HOSE (Hierarchical Organization of Shells Expert)-code which allowed for explicit substituent charge and stereo bond conventions, optimizing to the maximal number of spheres. The subsequent quantum mechanical shielding calculations allowed for the number of these codes found in the internal database search, as well as the number of those sought. Calculational errors were determined as the standard deviations of the experimental values found within the database. Typically, predicted ^{1}H chemical shifts were accurate to within 0.05 ppm, and predicted coupling constants were accurate to within 0.2 Hz. Predicted ^{13}C chemical shifts were accurate to within 3 ppm.

RESULTS

Figure 1A shows the 0.5–6.8 ppm region of a 400 MHz ^{1}H-^{1}H RCT spectrum of a sample of the model linoleoylglycerol compound 1,3-dilinolein which was allowed to autoxidize in the presence of atmospheric oxygen as described in the Materials and Methods section, and subsequently dissolved in C^2HCl_3 solution. CHPD vinylic proton multiplets are present in the 5.4–6.6 ppm chemical shift range, whereas the 9- and/or 13-position proton bonded to the hydroperoxy group-bearing carbon has a chemical shift value of 4.38 and 4.30 ppm for the *cis,trans*- and *trans,trans*-isomers, respectively. Cross-peaks refer to protons **a–j** in Figure 2, the chemical shift values of which are listed in Table 1. Figure 1B shows the 0.40–6.60 ppm region of a 600 MHz ^{1}H-^{1}H TOCSY spectrum of the same sample, this time dissolved in C^2H_3OH solution. The selection of a comparatively short mixing time in this experiment yields a two-dimensional spectrum that is equivalent to that generated with the RCT method. Solvent-induced spectral shifts of the appropriate protons encountered in the spectrum served as a convenient means of improving the resolution of overlapping signals observed in spectra acquired in C^2HCl_3 solution. These shift changes are indicated in parentheses within the table. Such two-dimensional spectra afford the complete, unambiguous (and to our knowledge, the first) indexing of all vinylic, allylic, and neighboring aliphatic protons of both *cis,trans*- and *trans,trans*-isomers of 9- and 13-hydroperoxy-octadecadienoylglycerol species. Utilization of the RCT and short mixing time TOCSY methods in this case is particularly suited to the ^{1}H NMR study of these primary LOP derived from the peroxidation of PUFA, furnishing spin system information that is largely restricted to protons separated by five bonds, and thus generating less-crowded spectra. A predicted spectrum (ACD Inc.), based on spectral parameters derived from the *cis,trans*- and *trans,trans*-hydroperoxydiene assignments in the chloroform solution (corroborated with the further resolution of overlapped signal information in the methanol solution), was in excellent agreement with typical experimentally acquired spectra (Fig. 3).

A number of broad hydroperoxide-OO<u>H</u> group resonances were detectable in the 8.1–8.9 ppm chemical shift range of spectra acquired on autoxidized 1,3-dilinolein (data not shown). As expected, these signals were completely removed from spectra following treatment of samples with ^2H_2O (0.030 mL was added to C^2HCl_3 solutions, the mixture rotamixed, equilibrated at ambient temperature for 30 min, centrifuged, and the predominant, lower C^2HCl_3 phase removed for further ^{1}H NMR analysis).

A consideration of spectral parameters for the CHPD components derived from autoxidation of the above model dilinoleoylglycerol compound facilitated assignment of such LOP resonances present in two-dimensional ^{1}H-^{1}H NMR spectra acquired on thermally stressed culinary oil samples. Figure 4A exhibits the 5.0–10.0 ppm region of the 600 MHz ^{1}H-^{1}H TOCSY spectrum of a typical sample of repeatedly utilized frying oil obtained from a fast-food restaurant. The cross-peak signals indicated confirm the presence of the CHPD LOP components in this sample together with signals concordant with the spin systems expected for *trans*-2-alkenals and 13-hydroxy-*cis*-9,*trans*-11 and 9-hydroxy-*trans*-10,*cis*-12 hydroxydiene derivatives, their ^{1}H chemical shift values being consistent with previously published data (27). The confirmation of the presence of such aldehydes is of particular clinical importance since they, rather than the conjugated hydroperoxydienes, are absorbed from the gut into the systemic circulation (*in vivo*) (7), where they have the capacity to exert a range of deleterious health effects. Other correlations involving the aldehydic proton region highlighted the presence of two further alkenal species represented by spin systems with chemical shift values of 9.57, 6.83 and 6.35 ppm, and 9.55,

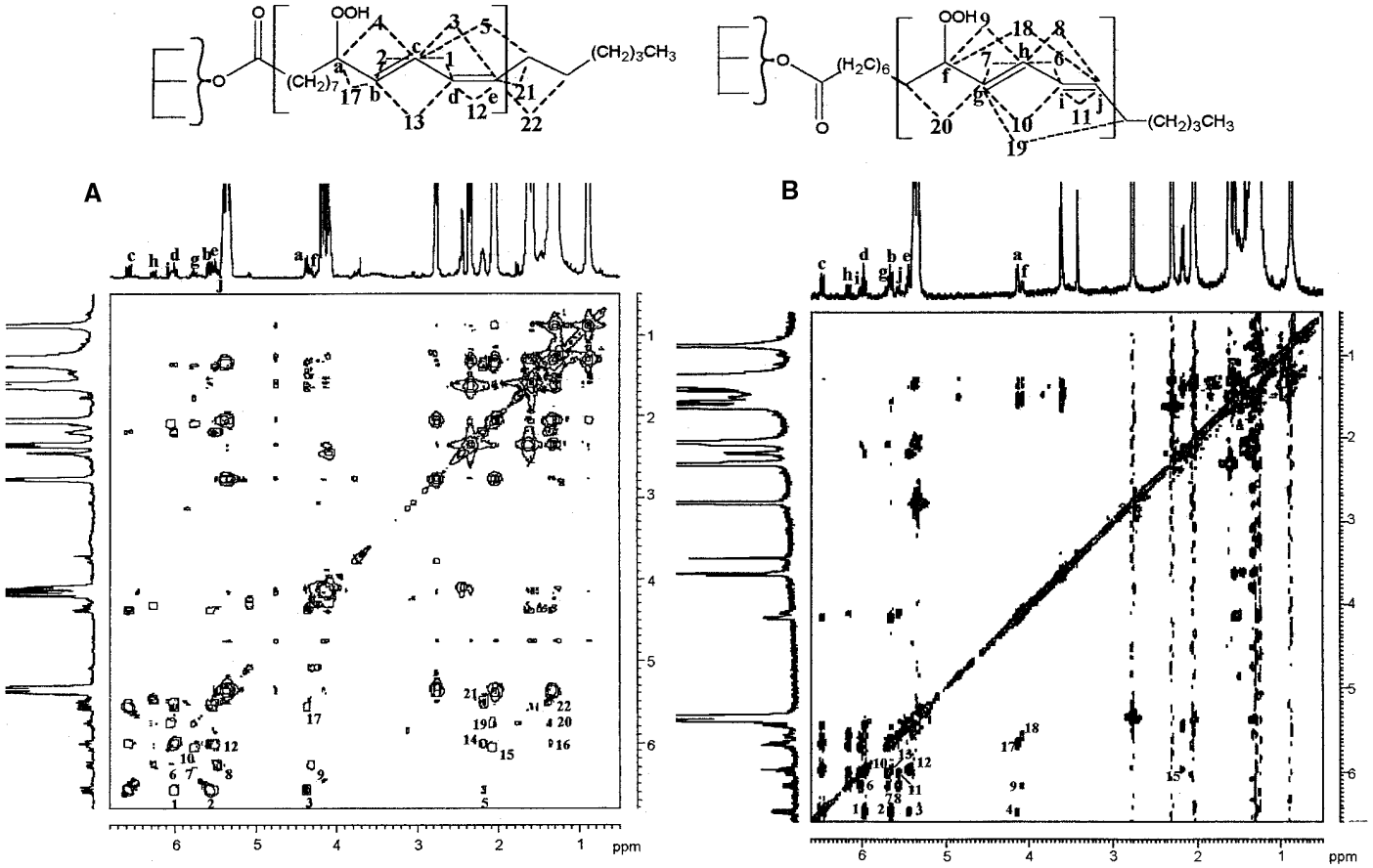

FIG. 1. (A) 0.50–6.80 ppm region of a 400 MHz ^{1}H-^{1}H relayed coherence transfer (RCT) spectrum of an autoxidized sample of 1,3-dilinolein in C^2HCl_3 solution, and (B) 0.40–6.60 ppm region of a 600 MHz ^{1}H-^{1}H total correlation (TOCSY) spectrum of the same sample in C^2H_3OH solution, the latter acquired with a short mixing time (20 ms). Typical spectra are shown. *Cis,trans*- and *trans,trans*-conjugated hydroperoxydiene [or more specifically 9-hydroperoxy-*trans*-10,*cis*-12- (and, in an analogous manner 13-hydroperoxy-*cis*-9,*trans*-11-) and 9-hydroperoxy-*trans*-10,*trans*-12- (and, in an analogous manner 13-hydroperoxy-*trans*-9,*trans*-11-) octadecadienoylglycerol isomers] cross-peak assignments are indicated as connectivities involving protons **a–j** in the accompanying molecular structures.

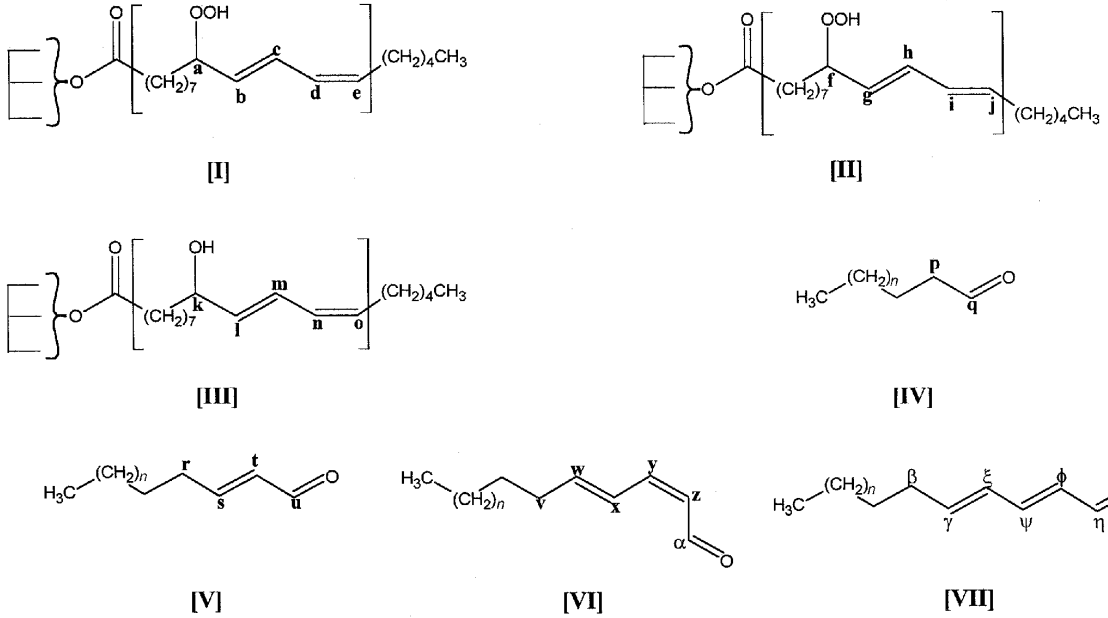

FIG. 2. Structural units of lipid oxidation products (LOP) detectable in peroxidized polyunsaturated fatty acids (PUFA) by high-resolution nuclear magnetic resonance (NMR) spectroscopy. The lower case letter labels correspond to ^{1}H (and/or) ^{13}C nuclei distinguishable by the two-dimensional techniques employed in this investigation. Key: **[I]**, 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoylglycerol adduct; **[II]**, 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoylglycerol adduct; **[III]**, 9-hydroxy-*trans*-10,*cis*-12-octadecadienoylglycerol adduct; **[IV]**, *n*-alkanals; **[V]**, *trans*-2-alkenals; **[VI]**, *cis,trans*-alka-2,4-dienals; **[VII]**, *trans,trans*-alka-2,4-dienals.

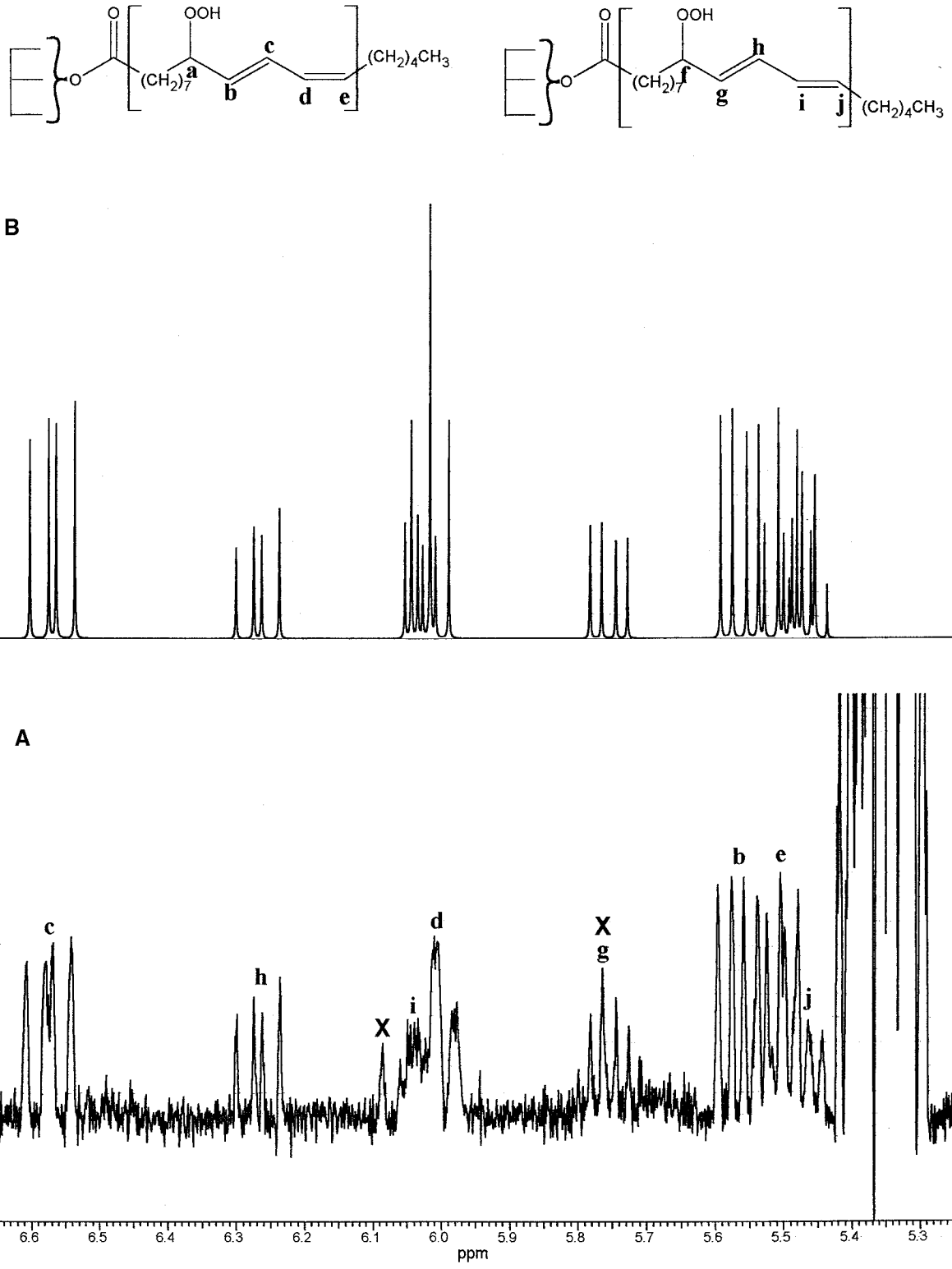

FIG. 3. Expanded 5.20–6.70 ppm regions of (A) an experimental 400 MHz one-dimensional ^{1}H NMR spectrum of an autoxidized sample of 1,3-dilinolein (in $C^{2}HCl_{3}$ solution), and (B) a corresponding computer-simulated spectrum generated using the software suite described in the Materials and Methods section, a system which employed spectral parameters obtained from all LOP assignments. A typical spectrum/simulation is shown. Abbreviations: ^{1}H nucleus labels of isomeric conjugated hydroperoxydienes (CHPD) correspond to those given in the molecular structures depicted above the spectra. Signals labeled as "X" indicate spectral artifacts. See Figure 2 for other abbreviations.

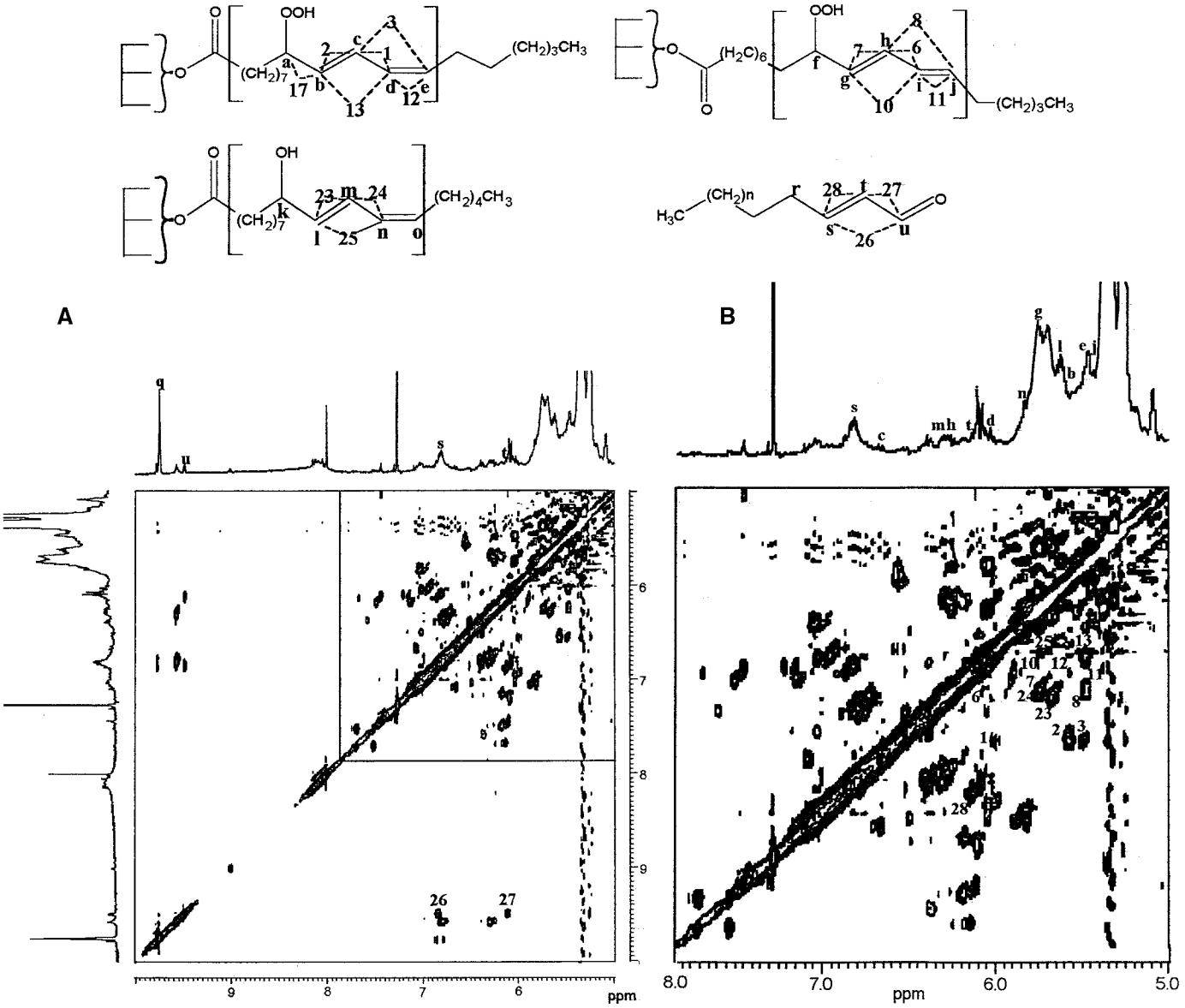

FIG. 4. (A) 5.0–10.0 ppm region of a 600 MHz ^{1}H-^{1}H TOCSY spectrum of a sample of repeatedly used culinary frying oil obtained from a fast-food retail outlet in C^2HCl_3 solution, acquired with a short mixing time (20 ms). The expanded 5.0–7.9 ppm region of this spectrum is shown in (B). A typical spectrum is shown. Abbreviations: ^{1}H nucleus labels of isomeric CHPD, 9-hydroxy-*trans*-10, *cis*-12-octadecadienoylglycerol adducts, and saturated and α,β-unsaturated aldehydes correspond to labeled protons in the accompanying molecular structures. See Figures 1 and 3 for abbreviations.

6.80 and 6.25 ppm. Moreover, the expanded 5.0–8.0 ppm region of this spectrum (Fig. 4B) revealed a complex range of additional overlapping signals, none of which corresponded to those expected for other known LOP such as *trans*-4,5-epoxyhept-*trans*-2-enal (28), various epoxides, hydroxyepoxides, 5- and 6-membered hydroperoxyepidioxides of linolenoylglycerol species [e.g., 5.40, 5.62, 6.00, and 6.64 ppm for the conjugated diene vinylic protons and characteristic 4.45 and 4.75 ppm multiplets for the methine group protons of the epidioxide ring of *cis,trans*-9- and 16-hydroperoxy epidioxides (29)], and conjugated oxodienes (30–32). This observation indicates that such LOP (important precursors of volatile components which reflect the oxidative degradation of linolenoylglycerols) are readily fragmented to peroxidation end products when exposed to a source of oxidative stress (i.e., prolonged thermal stressing episodes). Further experiments to establish the identities of these resonances are currently in progress.

Figure 5 exhibits complete and expanded regions of the ^{1}H-^{13}C HMQC spectrum of a further repeatedly utilized culinary oil sample (also obtained from a fast-food retail outlet), acquired in C^2HCl_3 solution, the full spectrum in (Fig. 5A) highlighting signals expected for glycerol backbones and their esterified fatty acid units. Consideration of the nature of the vinylic carbon ^{13}C signals and the average chain length as deduced from the single-pulse ^{1}H spectrum confirmed that

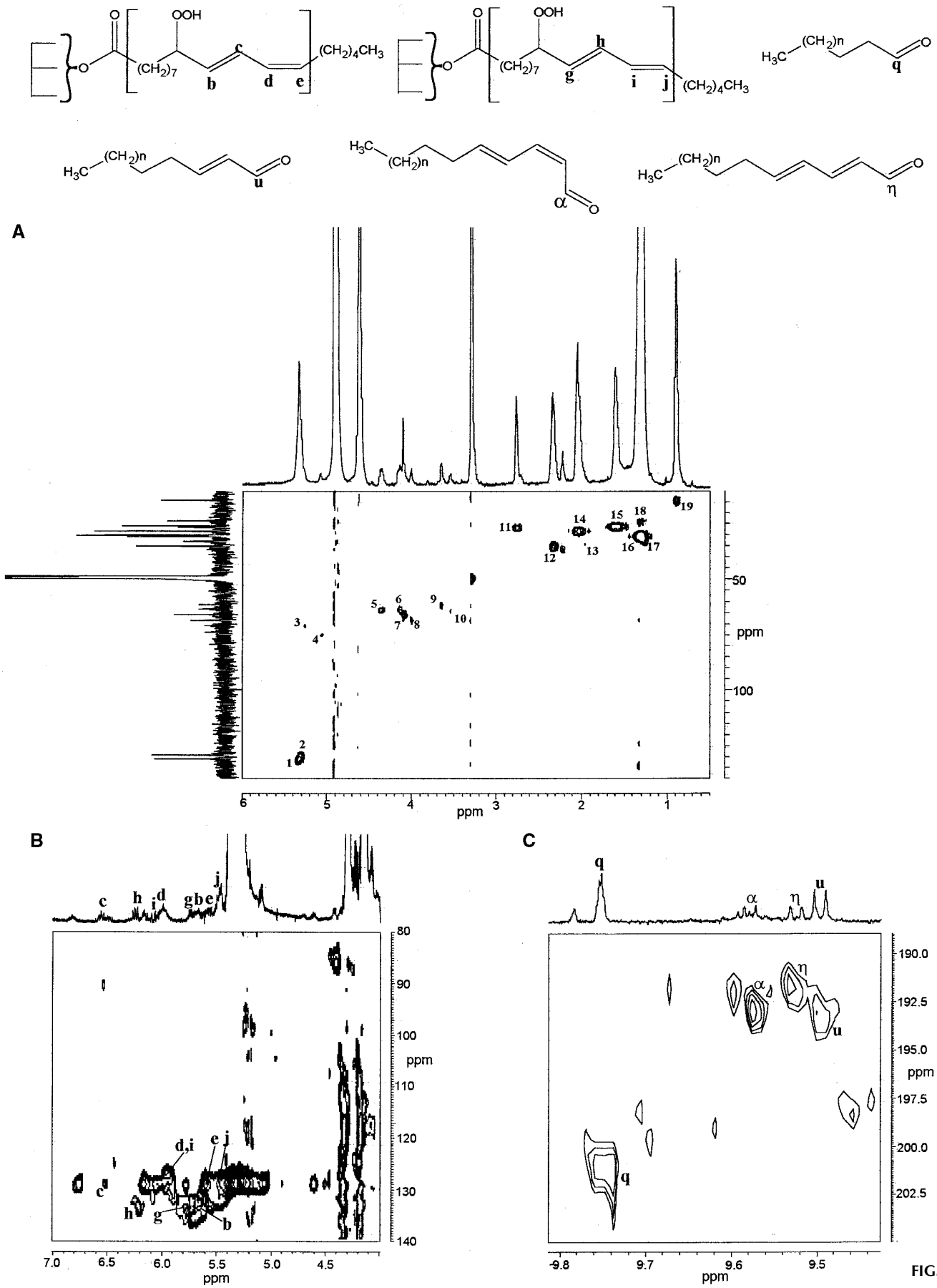

FIG. 5

glycerol-bound linoleate was the major PUFA constituent present in this material. Resolution of all glyceride acyl signal correlations provides information concerning the nature and distribution of glyceride esterification. The ^{1}H-^{13}C chemical shift correlations observed for 1-, 1,2-, 1,3- and 1,2,3-substituted glycerol backbone units were verified utilizing either previously reported data (33,34), or from predicitive software (ACD Inc.). Knowledge of the distribution of these species is of much importance since the nature of glyceride substitution was shown to significantly influence the rate of PUFA autoxidation. Indeed, dilenilenoyl-linoleoylglycerols are slightly less resistant to oxidation when linolenate is in the 1,2-rather than the 1,3-triacylglycerol positions, and dilinoleoyl-linolenoylglycerols are less resistant to oxidation when linoleate is in the 1,3- rather than the 1,2-triacylglycerol positions (35).

Glycerol, an end-product arising from the hydrolysis of triacylglycerols (a process occurring during standard frying practices), was undetectable in the HMQC spectra acquired. This observation is presumably a consequence of its volatilization during thermal stressing episodes since its boiling point (182°C) (36) is very similar to the temperature recommended for this process (180°C).

Comparisons of the one-dimensional ^{1}H NMR spectra of repeatedly-utilized frying oils with that of a corresponding control (unheated) material provided evidence consistent with the thermally-induced autoxidation of glycerol-bound PUFA therein. Indeed, the ratios of the intensities of the *bis*-allylic-C$\underline{H}_2$ group and the total unsaturated fatty acid vinylic-–C$\underline{H}$=C$\underline{H}$– proton resonances (δ = 2.76 and 5.38 ppm, respectively) to that of the acyl chain terminal-C$\underline{H}_3$ group protons (*t*, δ = 0.90 ppm) were markedly lower in repeatedly-employed samples. Moreover, the low-field-shifted highly unsaturated fatty acid acyl chain terminal-C$\underline{H}_3$ group triplet resonance located at 0.95 ppm was found to almost completely disappear from spectra following subjection of the culinary oil to repeated frying episodes (together with perpetuation of the autoxidation process *via* storage in the manner described in the Materials and Methods section), an observation concordant with the fact that linolelenoylglycerols autoxidize at a faster rate than linoleoylglycerols (35).

Figure 5B in particular demonstrates the ability of the HMQC method to resolve overlapping signals, the use of the 8-mm ^{13}C probe in this case undoubtedly facilitating the detection and identification of many low-level LOP components. Corroboration of previous ^{1}H assignments (5) with the appropriate ^{13}C NMR data is also important for the purpose

TABLE 1
^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Assignments for the Lipid Oxidation Products (LOP) in $C^{2}HCl_3$ Solution[a]

LOP nucleus positional code	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)
a	4.38 (4.15)	86.0
b	5.57 (5.61)	132.5
c	6.57 (6.49)	131.0
d	6.02 (5.97)	130.0
e	5.48 (5.43)	132.0
f	4.30 (4.02)	87.5
g	5.76 (5.68)	132.5
h	6.27 (6.14)	133.0
I	6.05 (6.03)	130.0
j	5.46 (5.52)	131.0
k	4.09	
l	5.64	
m	6.28	
n	5.83	
o	5.30	
p	2.44	
q	9.74	201.0
r	2.33	
s	6.85	
t	6.10	
u	9.48	193.2
v	2.25	
w	6.20	
x	6.30	
y	7.39	
z	6.15	
α	9.63	192.8
β	2.25	
γ	6.20	
ξ	6.30	
ψ	7.07	
ϕ	6.04	
η	9.52	191.5

[a]Where appropriate, corresponding ^{1}H assignments in $C^{2}H_3OH$ solution are indicated in parentheses. The LOP nucleus (nuclei) positional labels correspond to those indicated in Figure 2. ^{13}C assignments for 13- and/or 9-hydroxy-substituted octadecadienoylglycerol adducts (the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers, respectively) are absent since the very low levels of this LOP present in autoxidized model polyunsaturated fatty acids (PUFA)/thermally stressed PUFA-containing culinary oil samples preclude their detection (i.e., their ^{13}C features are unreceptive to NMR analysis under such conditions). Vinylic and bulk chain –CH_2– group ^{13}C resonance assignments for each class of aldehyde were unobtainable in view of a high degree of overlap with those of their primary LOP precursors and PUFA substrates, together with further glycerol-bound fatty acids.

FIG. 5. (See previous page) (A) Complete 600 MHz ^{1}H-^{13}C heteronuclear multiple quantum coherence transfer (HMQC) spectrum of a sample of repeatedly-used culinary frying oil obtained from a fast-food restaurant outlet. In (B), the spectral display was pre-set to emphasize ^{1}H-^{13}C correlations of lower intensity (i.e., those of LOP). (C) Expanded aldehydic group region of the above ^{1}H-^{13}C HMQC spectrum showing clear correlations between the ^{1}H and ^{13}C nuclei of the –CHO groups of saturated and α,β-unsaturated aldehydes. A typical spectrum is shown. Abbreviations for (A) (C_A, C_B, and C_C refer to substituted/unsubstituted glycerol carbon centers as indicated, and the indices 1,2 or 1,2,3 indicate substitution mode, i.e., 1,2 represents a diacylglycerol with fatty acids esterified at the 1- and 2-glycerol backbone positions, and 1,2,3 represents a triacylglycerol): 1, olefinic –CH = CH–; 2, as 1; 3, C_B 1,2,3; 4, C_B 1,2; 5, C_A/C_C 1,2,3; 6, as 5; 7, C_A 1,2; 8, C_A/C_C 1,3; 9, C_C 1,2; 10, C_A/C_C 1/3 (i.e., 1 or 3 positions unsubstituted); 11, bis-allylic-C$\underline{H}_2$ groups of PUFA; 12, fatty acid C-2; 13, fatty acid C-16; 14, allylic-C$\underline{H}_2$ (i.e., –CH = CH–C$\underline{H}_2$) group of unsaturated fatty acids ; 15, fatty acid C-3; 16, fatty acid bulk chain C-4 to C-15 –(C$\underline{H}_2)_n$– groups ; 17, as 16; 18, fatty acid C-17; 19, fatty acid terminal –CH_3. ^{1}H-^{13}C correlated signal labels in (B) and (C) correspond to those indicated on the molecular structures displayed above the spectra. See Figure 2 for abbreviations.

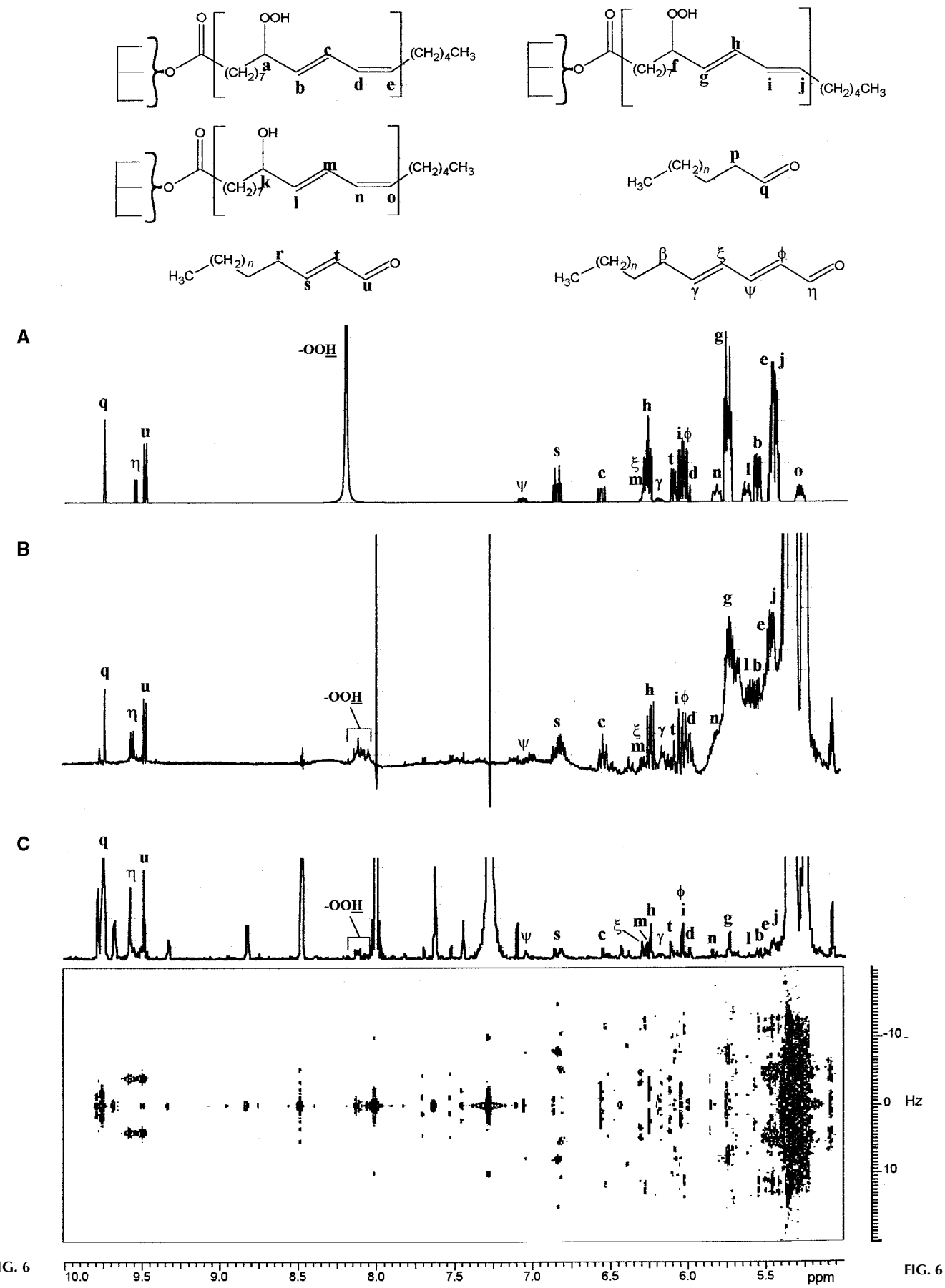

FIG. 6

FIG. 6

of confirming the identity of particular LOP known to exert adverse toxicological (e.g., proatherogenic and proinflammatory) properties (1–3,37). The ^{13}C assignments and related HMQC correlated signals were confirmed with the aid of known chemical shifts for the appropriate model compounds (33,34) and predicted spectra (ACD Inc.). The conjugated hydroperoxydiene unsaturated carbon ^{13}C signals appear in the virtually exclusive 130–140 ppm region, their chemical shift values being in good agreement with those obtained in previous investigations (38). Correlated signals found in the region around 200 ppm serve to offer a useful confirmation of our earlier ^1H NMR assignments of different classes of aldehyde, i.e., *n*-alkanals, *trans*-2-alkenals, *cis,trans*- and *trans,trans*-alka-2,4-dienals (5) [the identity of each of these linked signals was verified by a combination of simulated (ACD Inc.) and reference (36) data]. None of the ^1H-^{13}C HMQC spectra acquired demonstrated the presence of the epoxide, hydroxyepoxide, hydroperoxyepidioxide, and oxodiene species described above, although there remains some unavoidable coincidence of ^{13}C resonances of similar frequencies.

^1H-^1H *J*-resolved NMR spectroscopy is a further important technique which can be employed for the purpose of precisely determining the chemical shift values of overlapping ^1H resonances (ideally in the absence of second-order coupling effects) through utilization of the "skyline" f_2 projection, and Figure 6 shows the expanded 5.0–10.0 ppm region of the 600 MHz ^1H-^1H *J*-resolved spectrum (f_2 "skyline" projection and corresponding contour plot of the two-dimensional matrix) of an additional repeatedly used culinary frying oil sample (the contour plot depicts the separation of signal splittings into the second dimension). The corresponding one-dimensional spectrum, also exhibited in Figure 6, illustrates the generation of "homonuclear proton-decoupled" signals in the f_2 "skyline" projection. With the exception of resonances ascribable to *cis,trans*-alka-2,4-dienals, all LOP signals were readily visible. After making appropriate allowances for the differing concentrations of each LOP, the computer-predicted spectrum of this particular mixture (Fig. 6A) has a high level of agreement with the experimentally obtained one-dimensional spectrum, demonstrating the applicability of the spectral simulation program employed as an aid to the analysis of complex, multicomponent LOP matrices.

The NMR techniques outlined above are, of course, also readily applicable to the analysis of LOP present in culinary oils employed in further food preparation methods, e.g., domestic frying episodes. Figure 7A and B display the 9.00–10.00 ppm aldehydic group proton regions of one-dimensional ^1H NMR spectra acquired on a sample of sunflower seed oil [polyunsaturate and monounsaturate contents 63 and 12% (w/w), respectively] both prior and subsequent, respectively, to heating at a temperature of 180°C for a period of 30 min. Corresponding spectra acquired on samples of this culinary oil utilized for the purpose of frying potato chips and bacon (30 min at 180°C) are illustrated in Figure 7C and D, respectively. Clearly, resonances ascribable to the aldehydic group protons of *trans*-2-alkenals, *trans,trans*- and *cis,trans*-alka-2,4-dienals, and *n*-alkanals are clearly visible in these spectra, demonstrating the ready generation of these cytotoxic LOP from the autoxidative deterioration of PUFA during standard frying practices. The ^1H-^1H TOCSY spectrum of the oil sample employed for frying potato chips is exhibited in Figure 7E. This two-dimensional spectrum displays clear connectivities between (i) ^1H resonances of the conjugated diene systems of CHPDs, (ii) the aldehydic, olefinic, and local bulk alkyl chain ($-CH_2-$) group resonances of α,β-unsaturated aldehydes, and (iii) the aldehydic and adjacent bulk *n*-alkyl chain ($-CH_2-$) group signals.

DISCUSSION

Employment of a combination of two-dimensional NMR spectroscopic methods for the analysis of products arising from the oxidative degradation of glycerol-bound PUFA in culinary oils (thermally stressed or otherwise) provides much valuable molecular information which is an essential requirement for future evaluations of the toxicological hazards putatively associated with the dietary consumption of LOP. Moreover, the identification of specific LOP also highlights particular reaction pathways which can be related to the degree of PUFA unsaturation, together with the nature of glyceride substitution.

The RCT and TOCSY spectroscopic techniques permit the transfer of magnetization from an ^1H nucleus [or magnetically equivalent group (2 or 3) of such nuclei] bonded to one specific carbon atom (C_1), (the former through a relayed spin), to one or more magnetically distinct nuclei located two or more carbon positions further along a molecular chain (C_3, C_4, C_5 position, etc.), i.e., the latter nucleus/nuclei is/are not directly coupled to the C_1-bearing ^1H nucleus. Hence, the technique is extremely useful for the identification of LOP, particularly isomeric CHPD which have complex overlapping vinylic resonance patterns in their one-dimensional ^1H NMR spectra.

Direct observation of ^{13}C nuclei is poorly sensitive in view of its low natural abundance (1.11%), especially for relatively low concentrations of components such as LOP present in

FIG. 6. (See previous page) (A) Computer-simulated one-dimensional spectrum generated by the software suite described in the Materials and Methods section, utilizing spectral parameters derived from the assignment of all the detected lipid oxidation products and making allowance for their relative concentrations within the mixture. (B) One-dimensional ^1H NMR spectrum of a repeatedly utilized culinary frying oil sample collected from a fast-food restaurant, and (C) corresponding "skyline" f_2 projection and two-dimensional matrix contour plot of a 600 MHz ^1H-^1H *J*-resolved spectrum. Typical spectra are shown. Abbreviations: ^1H nucleus labels of isomeric CHPD, conjugated hydroxydienes, and saturated and α,β-unsaturated aldehydes correspond to labeled protons in the accompanying molecular structures. The label –OO<u>H</u> represents hydroperoxide-OO<u>H</u> group proton resonances. See Figures 2 and 3 for abbreviatons.

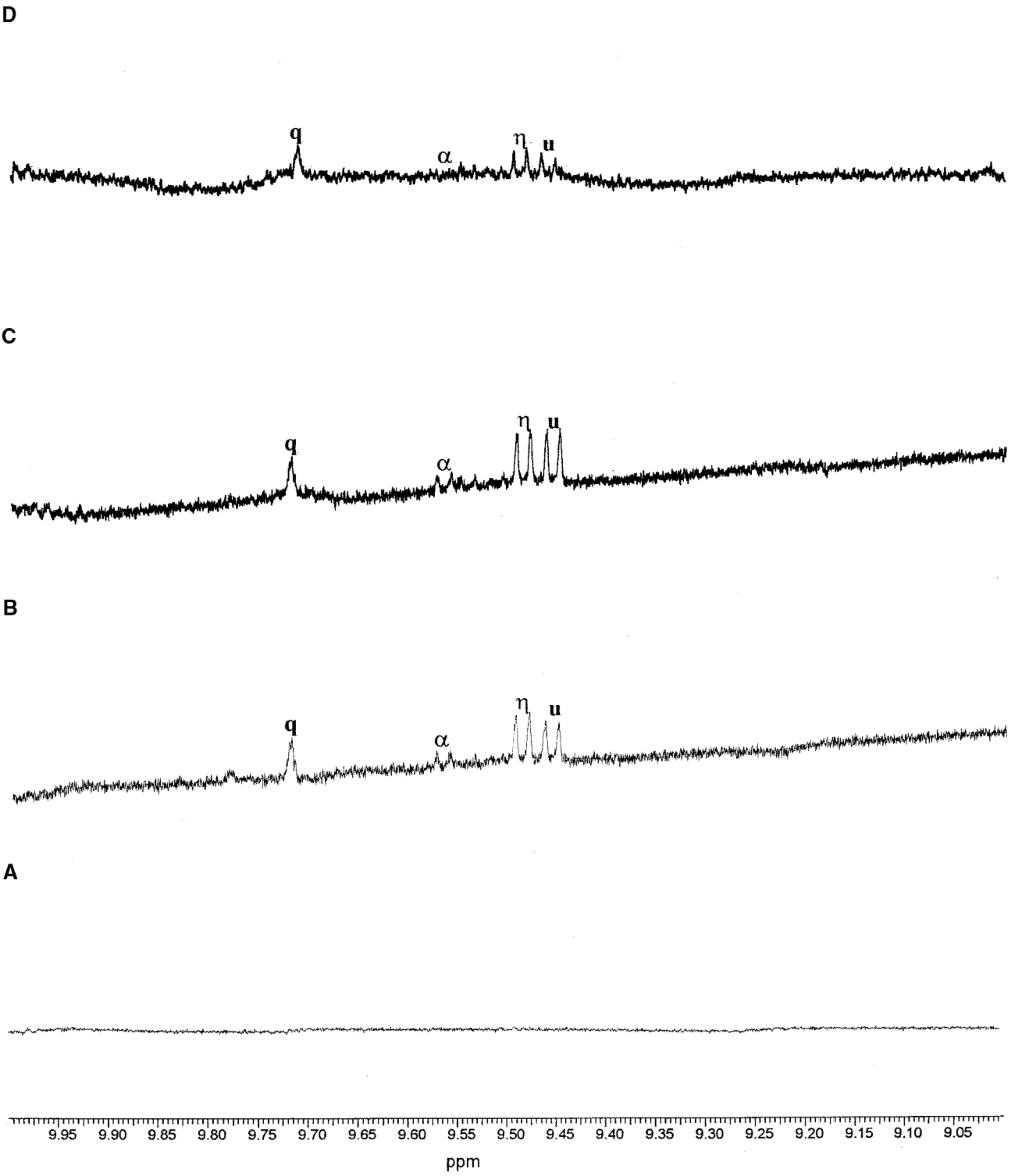

FIG. 7. Expanded 9.0–10.0 ppm regions of experimental 600 MHz one-dimensional ^{1}H NMR spectra of $C^{2}HCl_{3}$ solutions of (A) control (unheated) sunflower seed oil, (B) the above sample heated at a temperature of 180°C for a period of 30 min, (C) a sample of this culinary oil that had been utilized for the purpose of frying potato chips at 180°C for a period of 30 min (the Materials and Methods section) and (D) as (C), but employed for the purpose of frying bacon under the conditions outlined in the Materials and Methods section (30 min at 180°C), (E) 5.0–10.0 ppm region of a corresponding 600 MHz ^{1}H-^{1}H TOCSY spectrum of the sunflower seed oil sample giving rise to the one-dimensional spectrum shown in (C) above. Typical spectra are shown. Abbreviations: ^{1}H nucleus labels of saturated and α,β-unsaturated aldehydes correspond to labeled protons in the molecular structures depicted above the spectra. See Figure 2 for abbreviation.

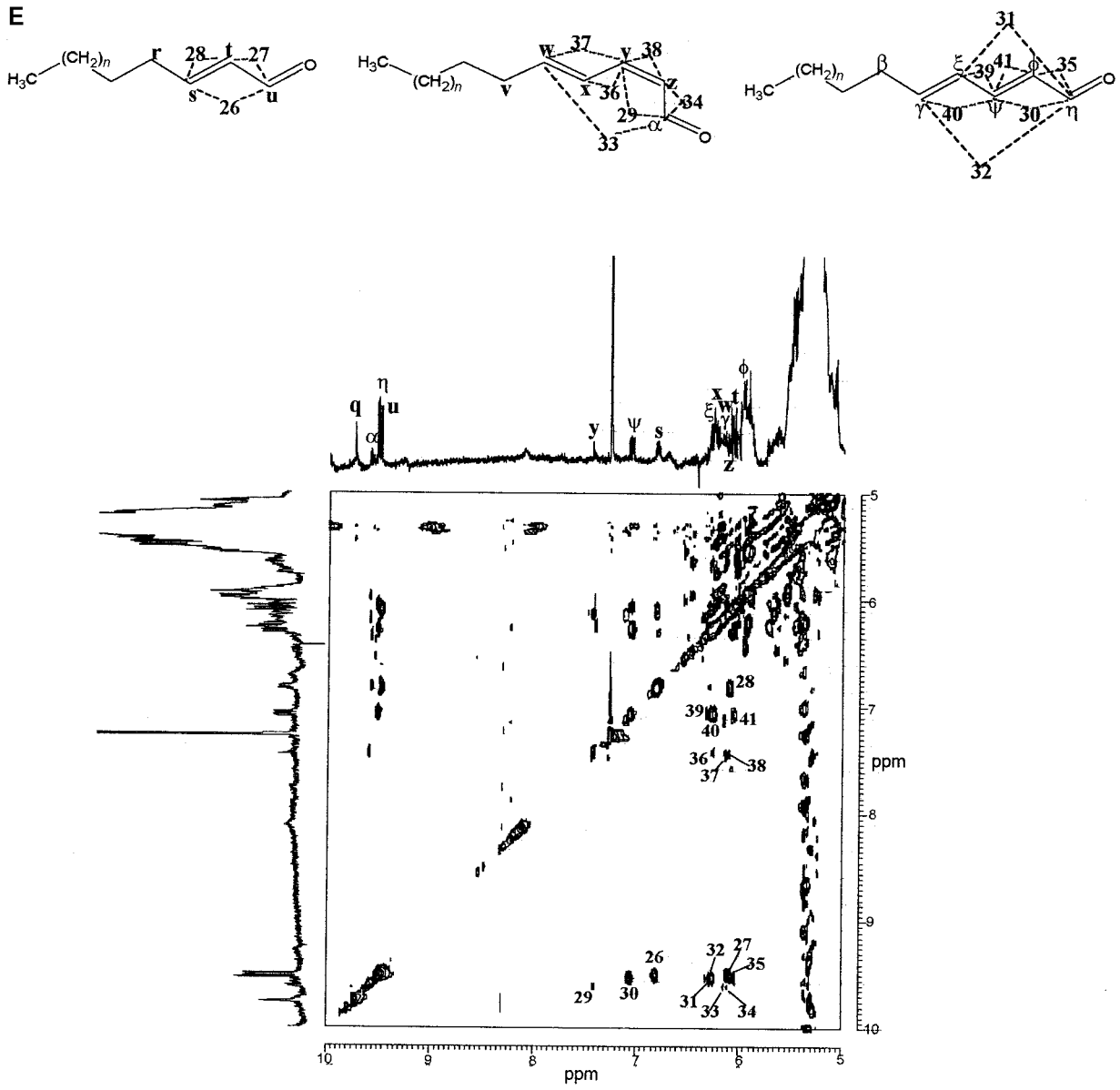

FIG. 7. (continued)

peroxidized triacylglycerol mixtures (the one-dimensional ^{13}C spectrum depicted as the f_1 projection of the HMQC profile displayed in Figure 5A required *ca.* 5,000 scans). However, the recent development of inverse-geometry probes and relevant accompanying pulse sequences has largely overcome this problem since HMQC spectroscopy and related techniques offer a substantial gain in sensitivity over conventional, one-dimensional spectroscopy. Hence, the HMQC technique is a particularly attractive one which readily facilitates investigations of the structure and dynamics of LOP.

Furthermore, application of the ^1H-^1H *J*-resolved spectroscopic method also serves to clarify the molecular nature of LOP present in autoxidized PUFA mixtures. Indeed, the technique is especially advantageous for the purpose of making resonance assignments in crowded spectral regions and enables the accurate determination of chemical shift and coupling constant values.

A high percentage of humans are frequently and continually exposed to LOP in the diet (arising, for example, from the shallow- or deep-frying of PUFA-rich culinary oils), and the possibility that regular consumption of such agents may be deleterious to human health has recently attracted much

interest. The short-term feeding of heated and/or oxidized oils and fats to experimental animals can give rise to loss of appetite, diarrhea, cardiomyopathy, hepatomegaly, hemolytic anemia, growth retardation, and an apparent accumulation of peroxides in adipose tissue (highly oxidized cod liver oil) (39). Moreover, cellular damage in various organs, elevated liver and kidney weights, and a modified fatty acid composition of tissue lipids in rats were shown to result from the short-term feeding of oils and fats subjected to the heat and oxidation associated with normal usage (40,41), and one long-term study revealed that the consumption of mildly oxidized culinary oils by rats throughout their lifespans produced an increased frequency of cardiac fibrotic and hepatic bile-duct lesions (42). Further toxicological investigations concerning thermally stressed oils and fats focused on their mutagenic properties (43,44). However, the precise molecular nature of mutagens formed during frying processes and their metabolic fate in humans were not considered by these researchers.

Although there is much epidemiological and experimental evidence available regarding the influence of the dietary consumption of saturated or PUFA to the development and/or progression of human diseases, the precise autoxidation status of the culinary oils and fats ingested (i.e., the structure and concentrations of CHPD, conjugated hydroxydienes, aldehydes, etc., therein) has not hitherto been sufficiently considered, and the two-dimensional NMR techniques employed here serve to provide a multicomponent profile of PUFA-derived autoxidation products, information not readily attainable from alternative analytical methods which generally require much prior information regarding the molecular nature of such LOP.

CHPD are acutely toxic to rodents when administered systemically, but such effects are much less severe when given orally. Indeed, a single intravenous (i.v.) dose of methyl linoleate hydroperoxide administered to rats gave rise to a high mortality within 24 h (animals dying from severe lung damage), whereas an oral dose of *ca.* 200 mg/kg was without effect (45). Holman and Greenberg (46) demonstrated that although the intravenous LD_{50} value for ethyl linoleate hydroperoxide in mice was 12 mg/kg, similar dose levels administered orally were nonlethal, an observation supported by the subsequent investigations of Olcott and Dolev (47). Furthermore, Bergen and Draper (48) obtained indirect evidence indicating that the lack of effect of orally administered CHPD is explicable by their failure to be absorbed across the gastric or intestinal epithelium.

Notwithstanding, CHPD have the capacity to exert damage to the gastrointestinal epithelium, and Jayaraj *et al.* (49) showed that oral administration of the aldehydic lipid oxidation end product 4-hydroxy-*trans*-2-nonenal to rats at a dose level of only 2.6×10^{-7} mol·dm^{-3}, a concentration similar to that found in healthy human blood plasma, induced peptic ulcers.

As noted in the introduction, aldehydic LOP have been implicated in the induction, development, and progression of atherosclerosis (2) (and therefore its associated pathological sequelae such as ischemic heart disease and peripheral vascular disease), and additional investigations established that these cytotoxic agents exert genotoxicological (50) and proinflammatory (51) actions.

The above two-dimensional NMR techniques are also applicable to the analysis of appropriate lipidic extracts of biofluids and tissues obtained from patients with clinical conditions in which the *in vivo* oxidation of PUFA (and/or the dietary ingestion of chemically reactive aldehydes) has been implicated (1–3,7–10), and experiments to investigate this are currently in progress.

ACKNOWLEDGMENTS

We thank the University of London Intercollegiate Research Services for the provision of NMR facilities; Vladimir Shilay, Dmitry Mitushev (ACD, Moscow, Russian Federation) for helpful discussions regarding the calculation of ^{1}H NMR spectra, Andrew Claxson for excellent technical assistance, and Colette Troy for typing the manuscript. We are very grateful to the Commission of the European Communities (Agriculture and Agro-industry, including Fisheries: DG-XII) for financial support.

REFERENCES

1. Smith, T., and Kummerow, F.A. (1987) The Role of Oxidized Lipids in Heart Disease and Ageing, in *Nutrition and Heart Disease Vol. 1* (Watson, R.R., ed.), pp. 45–64, CRC Press, FL.
2. Witzum, J.L., and Steinberg, D. (1991) Role of Oxidised Low Density Lipoprotein in Atherogenesis, *J. Clin. Invest. 88*, 1785–1792.
3. 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, *Ann. Rheum. Dis. 51*, 481–484.
4. Esterbauer, H. (1985) Lipid Peroxidation Products: Formation, Chemical Properties and Biological Activities, in *Free Radicals in Liver Injury* (Poli, G., Cheeseman, K.H., Diazani, M.U., and Slater, T.F., eds.), pp. 29–47, IRL Press, London.
5. Haywood, R.M., Claxson, A.W.D., Hawkes, G.E., Richardson, D.P., Coumbarides, G., Hawkes, J., Lynch, E.J., and Grootveld, M.C. (1995) Detection of Aldehydes and Their Conjugated Hydroperoxydiene Precursors in Thermally-Stressed Culinary Oils and Fats: Investigations Using High Resolution Proton NMR Spectroscopy, *Free Radical Res. 22*, 441–482.
6. Claxson, A.W.D., Hawkes, G.E., Richardson, D.P., Naughton, D.P., Haywood, R.M., Chander, C.L., Atherton, M., Lynch, E.J., and Grootveld, M.C. (1994) Generation of Lipid Peroxidation Products in Culinary Oils and Fats During Episodes of Thermal Stressing: A High Field ^{1}H NMR Study, *FEBS Lett. 355*, 81–90.
7. Grootveld, M., Atherton, M.D., Sheerin, A.N., Hawkes, J., Blake, D.R., Richens, T.E., Silwood, C.J.L., Lynch, E., and Claxson, A.W.D. (1998) *In vivo* Absorption, Metabolism, and Urinary Excretion of α,β-Unsaturated Aldehydes in Experimental Animals. Relevance to the Development of Cardiovascular Diseases by the Dietary Ingestion of Thermally Stressed Polyunsaturate-Rich Culinary Oils, *J. Clin. Invest. 101*, 1210–1218.
8. Yagi, K. (1988) Lipid Peroxides in Atherosclerosis, in *The Role of Oxygen in Chemistry and Biology* (Ando, W., and Morooka, Y., eds.), pp. 383–390, Elsevier, Amsterdam.
9. Kritchevsky, D., and Tepper, S.A. (1967) Cholesterol Vehicle in Experimental Atherosclerosis. IX. Comparison of Heated Corn Oil and Heated Olive Oil, *J. Atheroscler. Res. 7*, 647–651.
10. Straprans, I., Rapp, J.H., Pan, X.M., Hardman, D.A., and Feingold, K.R. (1996) Oxidised Lipids in the Diet Accelerate the De-

velopment of Fatty Streaks in Cholesterol-Fed Rabbits, *Arterioscler. Thromb. Vasc. Biol. 16*, 533–538.
11. Bell, J.D., Brown, J.C., and Sadler, P.J. (1989) NMR Studies of Biofluids, *NMR Biomed. 2*, 246–256.
12. Grootveld, M., Sheerin, A., Atherton, M., Millar, A.D., Lynch, E.J., Blake, D.R., and Naughton, D.P. (1996). Applications of High Resolution NMR Analysis to the Study of Inflammatory Diseases at the Molecular Level, in *Biomedical Applications of Spectroscopy*, Advances in Spectroscopy Vol. 25 (Clark, R.J.H., and Hester, R.E., eds.) pp. 295–327, John Wiley & Sons Ltd., Chichester.
13. Nicholson, J.K., and Wilson, I.D. (1989). High-Resolution Proton Magnetic Resonance Spectroscopy of Biological Fluids, *Prog. NMR Spectrosc. 21*, 449–501.
14. Grootveld, M., Jain, R., Claxson, A.W.D., Naughton, D., and Blake, D.R. (1990) The Detection of Irradiated Foodstuffs, *Trends Food Sci. Technol. 1*, 7–14.
15. Willker, W., and Liebfritz, D. (1998) Assignment of Mono- and Polyunsaturated Fatty Acids in Lipids of Tissues and Body Fluids, *Magn. Reson. Chem. 36*, S79–S84.
16. Silwood, C.J.L., and Davies, D.B. (1991) ^{1}H NMR Structural Analysis of Isomeric Tetrazole-Substituted Spirostan Steroids—Shielding Effects and Conformational Study, *Magn. Reson. Chem. 29*, 63–72.
17. Maschke, S., Azaroual, N., Wieruszeski, J.-M., Lippens, G., Imbenotte, M., Mathieu, D., Vermeersch, G., and Lhermitte, M. (1997) Diagnosis of a Case of Acute Chloroquine Poisoning Using ^{1}H NMR Spectroscopy: Characterisation of Drug Metabolites in Urine, *NMR Biomed. 10*, 277–284.
18. Nicholson, J.K., Foxall, P.J.D., Spraul, M., Farrant, R.D., and Lindon, J.C. (1995) 750 MHz ^{1}H and ^{1}H-^{13}C NMR Spectroscopy of Human Blood Plasma, *Anal. Chem. 67*, 793–811.
19. Sweatman, B.C., Farrant, R.D., Holmes, E., Ghauri, F.Y., Nicholson, J.K., and Lindon, J.C. (1993) 600 MHz ^{1}H-NMR Spectroscopy of Human Cerebrospinal Fluid: Effects of Sample Manipulation and Assignment of Resonances, *J. Pharm. Biomed. Anal. 11*, 651–664.
20. Eich, G., Bodenhausen, G., and Ernst, R.R. (1982) Exploring Nuclear-Spin Systems by Relayed Magnetization Transfer, *J. Amer. Chem. Soc. 104*, 3731–3732.
21. Bax, A., and Drobny, G. (1985) Optimisation of 2-Dimensional Homonuclear Relayed Coherence Transfer NMR-Spectroscopy, *J. Mag. Reson. 61*, 306–320.
22. Braunschweiler, L., and Ernst, R.R. (1983) Coherence Transfer by Isotropic Mixing—Application to Proton Correlation Spectroscopy, *J. Magn. Reson. 53*, 521–528.
23. Bax, A., and Davis, D.G. (1985) MLEV-17-Based Two-Dimensional Homonuclear Magnetization Transfer Spectroscopy, *J. Magn. Reson. 65*, 355–360.
24. Marion, M., and Wüthrich, K. (1983). Application of Phase Sensitive Two-Dimensional Correlated Spectroscopy (COSY) for Measurements of ^{1}H-^{1}H Spin-Spin Coupling-Constants in Proteins, *Biochem. Biophys. Res. Commun. 113*, 967–974.
25. Bax, A., Griffey, R.H., and Hawkins, B.L. (1983) Correlation of ^{1}H and ^{15}N Chemical Shifts by Multiple Quantum NMR, *J. Mag. Reson. 64*, 547–552.
26. Shaka, A.J., Barker, P.B., and Freeman, R. (1985) Computer Optimised Decoupling Scheme for Wideband Applications and Low-Level Operation, *J. Mag. Reson. 64*, 547–552.
27. 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,* 191–193.
28. Swoboda, P.A.T., and Peers, K.E. (1978) *Trans*-4,5-epoxyhept-*trans*-2-enal. The Major Volatile Compound Formed by the Copper and α-Tocopherol Induced Oxidation of Butterfat, *J. Sci. Food Agric. 29*, 803–807.
29. Frankel, E., Neff, W.E., and Miyashita, K. (1990) Autoxidation of Polyunsaturated Triacylglycerols. II. Trilinolenoylglycerol, *Lipids 25*, 40–47.
30. Gardner, H.W., Kleinman, R., and Weisleder, D. (1974) Homolytic Decomposition of Linoleic Acid Hydroperoxide: Identification of Fatty Acid Products, *Lipids 9*, 696–706.
31. Neff, W.E., Frankel E.N., Selke E., and Weisleder, D. (1983) Photosensitized Oxidation of Methyl Linoleate Monohydroperoxides: Hydroperoxy Cyclic Peroxides, Dihydroperoxides, Keto Esters and Volatile Thermal Decomposition Products, *Lipids 18*, 868–876.
32. Graveland, A. (1970) Enzymic Oxidations of Linoleic Acid and Glycerol-1-monolinoleate in Doughs and Flour Water Suspensions, *J. Am. Oil Chem. Soc. 47*, 352–361.
33. Lie Ken Jie, M.S.F., Lam, C.C., and Yan, B.F.Y. (1992) Carbon-13 Nuclear Magnetic Resonance Studies on Some Synthetic Saturated Glycerol Triesters, *J. Chem. Res. (S),* 12–13.
34. Henderson, J.M., Petersheim, M., Templeman, G.J., and Softly, B.J. (1994) Quantitation and Structural Elucidation of the Positional Isomers in a Triacylglycerol Mixture Using Proton and Carbon One- and Two-Dimensional NMR, *J. Agric. Food Chem. 42*, 435-441.
35. Miyashita, K., Frankel, E.N., Neff, W.E., and Awl, R.A. (1990) Autoxidation of Polyunsaturated Triacylglycerols. III. Synthetic Triacylglycerols Containing Linoleate and Linolenate, *Lipids 25*, 48–53.
36. Pouchert, C.J., and Behnke, J. (1993) *The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra*, Aldrich Chemical Co. Inc., Milwaukee.
37. Jurgens, G., Hoff, H.F., Chisolm, G.M., III, and Esterbauer, H. (1987) Modification of Human Serum Low-Density-Lipoprotein with 4-Hydroxynonenal Induces Uptake by Macrophages, *Chem. Phys. Lipids 45*, 315–316.
38. Neff, W.E., Frankel, E.N., and Miyashita, K. (1990) Autoxidation of Polyunsaturated Triacylglycerols. I. Trilinoleoylglycerol, *Lipids*, *25*, 33–39.
39. Sanders, T.A.B. (1983) Nutritional Significance of Rancidity, in *Rancidity in Foods* (Allen, J.C., and Hamilton, R.J., eds.) pp. 59–66, Elsevier, Amsterdam.
40. Yoshida, H., and Kujimoto, G. (1989) Effect of Dietary Vitamin E on the Toxicity of Autoxidised Oil to Rats, *Ann. Nutr. Metab. 33*, 153–161.
41. Alexander, J.C., Valli, V.E., and Chanin, B.E. (1987) Biological Observations from Feeding Corn Oil and Heated Peanut Oil to Rats, *J. Toxicol. Environ. Health 21*, 295–309.
42. Kaunitz, H. (1978) Toxic Effects of Polyunsaturated Vegetable Oils, in *The Pharmacological Effect of Lipid* (Kabara, J.J., ed.) pp. 203–210, American Oil Chemists' Society, Champaign.
43. Hageman, G., Hermans, R., Ten Hoor, F., and Kleinjans, J. (1988) Assessment of the Mutagenicity of Repeatedly-Used Deep Frying Fats, *Mutation Res. 204*, 593–604.
44. 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, *Food Chem. Toxicol. 28*, 75–80.
45. Cortesi, R., and Privett, O.S. (1972) Toxicity of Fatty Acid Ozonides and Peroxides, *Lipids 7*, 715–721.
46. Holman, R.T., and Greenberg, S.I. (1958) The Toxicities of Methyl Oleate Peroxide and Ethyl Linoleate Peroxide, *J. Am. Oil Chem. Soc. 35*, 707.
47. Olcott, H.S., and Dolev, A. (1963) Toxicity of Fatty Acid Ester Hydroperoxides, *Proc. Soc. Exp. Biol. Med. 114*, 820.
48. Bergen, J.G., and Draper, H.H. (1970) Absorption and Metabolism of 1-^{14}C-Methyl Linoleate Hydroperoxide, *Lipids 5*, 975–982.
49. Jayaraj, A.P., Rees, K.R., Tovey, F.E.I., and White, J.S. (1986) A Molecular Basis of Peptic Ulceration Due to Diet, *Br. J. Exp. Pathol. 67*, 149–155.

50. 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, *Toxicol. Appl. Pharmacol. 86*, 227–234.
51. 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, *Biochem. Pharmacol. 29*, 121–124.

[Received June 18, 1998, and in final revised form March 10, 1999; revision accepted May 13, 1999]