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

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Abstract

Thermal stressing of polyunsaturated fatty acid (PUFA)-rich culinary oils according to routine frying or cooking practices generates high levels of cytotoxic aldehydic products (predominantly *trans*-2-alkenals, *trans*,*trans*-alka-2,4-dienals, *cis*,*trans*-alka-2,4-dienals, and *n*-alkanals), species arising from the fragmentation of conjugated hydroperoxydiene precursors. In this investigation we demonstrate that typical *trans*-2-alkenal compounds known to be produced from the thermally induced autoxidation of PUFAs are readily absorbed from the gut into the systemic circulation in vivo, 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. Since such aldehydic products are damaging to human health, the results obtained from our investigations indicate that the dietary ingestion of thermally, autoxidatively stressed PUFA-rich culinary oils promotes the induction, development, and progression of cardiovascular diseases. (*J. Clin. Invest.* 1998. 101:1210–1218.) Key words: culinary oils • lipid peroxidation products • α,β-unsaturated aldehydes • LDL modification • atherosclerosis

Introduction

Despite recent advances in therapeutics, coronary heart disease remains one of the major causes of mortality in the developed world. The frequent dietary consumption of saturated fats has, in recent years, been regularly cited as a major factor in the pathogenesis of atherosclerosis and its associated pathological sequelae, i.e., ischemic heart disease (IHD),[1] and peripheral vascular disease. However, it is now generally recognized that the replacement of saturated fats by vegetable oils containing high levels of polyunsaturated fatty acids (PUFAs) may also render individuals susceptible to cardiovascular lesions (1–3). For example, PUFA-rich groundnut oil has been shown to be proatherogenic, whereas cocoa butter, which contains a high level of saturated fatty acids, is much less atherogenic than expected (a phenomenon that researchers have attributed previously to the fatty acid substitutional status of the glycerol moiety of triacylglycerols present in these materials [4]).

In view of putative associations between the dietary ingestion of culinary oils and fats and the induction and progression of cardiovascular diseases, the Ministry of Agriculture, Fisheries, and Food (MAFF) (UK) recently invited proposals for the funding of research programs aimed at clarifying such research problems (5), e.g., is the dietary intake of glycerol-bound polyunsaturates positively correlated with the development of selected disease processes in humans?

In previous investigations using high resolution proton (^{1}H) NMR analysis we have demonstrated that thermal stressing of culinary oils and fats according to standard frying practices (domestic or otherwise) gives rise to and/or perpetuates the oxygen radical–mediated autoxidation of PUFAs therein, primarily generating conjugated hydroperoxydiene (CHPD) species which, in turn, produce aldehydes via a process which involves the β-scission of preformed alkoxyl radicals (6, 7). The concentrations of such PUFA-derived autoxidation products generated (≤ 20 and 30×10^{-3} mol·kg^{-1} for saturated and α,β-unsaturated aldehydes, respectively) are critically dependent on the PUFA content of the oil, the nature and capacity of the heating vessel used, and the duration and conditions of heating and storage (7). Such aldehydic species (*n*-alkanals, *trans*-2-alkenals, *trans*,*trans*- and *cis*,*trans*-alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde [MDA]) have the capacity to exert a variety of toxicological effects in view of

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1. *Abbreviations used in this paper:* α-TOH, α-tocopherol; CHPD, conjugated hydroperoxydiene; COSY, correlation spectroscopy; GSH, glutathione; HNE, 4-hydroxy-*trans*-2-nonenal; IHD, ischemic heart disease; MDA, malondialdehyde; MLHP, methyl linoleate hydroperoxide; pH*, pH + 0.40; PUFA, polyunsaturated fatty acid; *t*-2N, *trans*-2-nonenal; *t*-2P, *trans*-2-pentenal; TOCSY, total correlation spectroscopy; TSP, 3-(trimethylsilyl)-[1,1,2,2-d_4] propionate.

their extremely high reactivity with critical biomolecules (DNA base adducts, proteins such as LDLs, peptides, free amino acids, and endogenous thiols such as glutathione). Indeed, a wealth of information concerning the generation and toxicity of these agents as terminal products of in vivo lipid peroxidation processes is currently available (for reviews see references 8 and 9).

Despite the availability of much epidemiological and experimental evidence relating the dietary consumption of saturated or PUFAs to the development and progression of atherosclerosis, the precise autoxidation status of culinary oils and fats ingested (i.e., the molecular nature and levels of CHPDs, aldehydes, etc. therein) has not yet been sufficiently considered by researchers conducting animal feeding studies designed to mimic the induction of vascular lesions in humans. Indeed, the toxicological hazards associated with the regular ingestion of unheated and/or thermally stressed culinary oils and fats may, at least in part, be ascribable to products generated from the autoxidation of PUFAs.

However, 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, Cortesi and Privett (10) showed that a single intravenous dose of 20 mg/kg methyl linoleate hydroperoxide (MLHP) to rats gave rise to a high mortality within 24 h (animals dying from severe lung damage), whereas an oral dose of $\sim$ 200 mg/kg was without effect. Similarly, Holman and Greenberg (11) showed that whereas the intravenous LD_{50} for ethyl linoleate hydroperoxide in mice was 12 mg/kg, similar doses given orally were nonlethal, a finding supported by the later studies of Olcott and Dolev (12). 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 (13). These authors found that a substantial proportion of an oral dose of [1-^{14}C]-labeled MLHP remained in the gastrointestinal tract for periods of up to 31 h after dosing, and from analysis of lipid extracts derived from mesenteric adipose tissue, they suggested that MLHP was transformed to both hydroxydiene and oxodiene derivatives during uptake. However, no direct evidence for the nature of the species absorbed was obtained. Subsequently, Nakatsugawa and Kameda (14) 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 conjugated hydroperoxydienes, some limited evidence for the absorption of complex secondary products of lipid autoxidation has been found by Kanazawa et al. (15). Thus, although $\sim$ 50% of an oral dose of autoxidized [^{14}C]-labeled linoleic acid was excreted in the feces, the majority (75%) of the remaining activity was absorbed and excreted in the urine, or as 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.

Further information available in this research area concerns the in vivo absorption, metabolism, and urinary excretion of MDA (16) and acrolein (CH_2=CH·CHO) (17) (the latter being an industrially derived air pollutant which is also present in cigarette smoke). However, to date, there are no reports available regarding the in vivo absorption and biotransformation of the longer chain *trans*-2-alkenals, species which represent the predominant α,β-unsaturated aldehydes generated from the peroxidation of linoleyoylglycerols (18).

In this communication we report the in vivo absorption, metabolism, and urinary excretion of typical autoxidized PUFA-derived α,β-unsaturated aldehydes (*trans*-2-nonenal [*t*-2N] and *trans*-2-pentenal [*t*-2P]). The dietary, physiological, biochemical, and toxicological significance of the results obtained are discussed in detail.

Methods

Materials. *t*-2P, *t*-2N, 3-*S*-(methylthio)-hexan-1-ol, and HPLC-grade ethyl acetate were purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK) and sodium 3-(trimethylsilyl)-[1,1,2,2-d_4] propionate (TSP) was obtained from Sigma Chemical Co. (Poole, Dorset, UK). Deuterated NMR solvents (^2H_2O, C^2HCl_3, and $C^2H_3SOC^2H_3$) were purchased from Goss Scientific Ltd. (Great Baddow, Essex, UK).

Animal experiments involving oral administration of trans-2-alkenals. Groups of healthy male Wistar albino rats with initial weights of $\sim$ 180 g (n = 10) were housed individually in metabolism chambers equipped for the collection of urine and feces, and were fasted for a 24-h period before the direct oral administration of *t*-2N or *t*-2P (animals were allowed free access to tap water before and after dosing). Cages were placed in well-ventilated animal rooms with regular light cycles (12 h: 0700 to 1900). The α,β-unsaturated aldehydes *t*-2N or *t*-2P in an unheated olive oil vehicle were introduced directly into the stomach of the experimental animals via a gavage tube at a dose of 100 mg/kg. A control group of the above animals (n = 10) received the unheated olive oil vehicle in the same manner.

Urine samples (24 h) were collected for ^{1}H NMR analysis before and after oral administration of *t*-2N, *t*-2P, or their unheated olive oil vehicle. Urinary volumes and pH values were recorded and samples were stored at −20°C for a maximum duration of 48 h before analysis.

^{1}H NMR analysis of urine collected from control and trans-2-alkenal-treated experimental animals. ^{1}H NMR measurements on rat urine samples were conducted on Bruker spectrometers (model AMX-600 or AMX-400; University of London Intercollegiate Research Services [ULIRS], London, UK) operating at frequencies of 600.13 and 400.13 MHz, respectively. A 0.60-ml aliquot of each urine sample was placed in a 5-mm diameter NMR tube and 0.07 ml of ^2H_2O was added to provide a field frequency lock. Typical pulsing conditions for the single-pulse spectra acquired were: 45° pulse angle, 2.730 s acquisition time, 2.270 s pulse delay, 32,768 computer points, and 128 transients. Chemical shift values were referenced to preadded sodium TSP (δ = 0.00 ppm), typically present at a concentration of 5.22×10^{-4} mol·dm^{-3}. The relative intensities of selected resonances were determined by electronic integration. 400 MHz two-dimensional shift-correlated ^{1}H NMR (COSY) spectra were acquired using 1,024 data points in the t_2 dimension, 256 increments of t_1, a relaxation delay of 2.00 s, and 64 transients. Pulsing conditions for the 600 MHz COSY spectra obtained were 1,024 data points in the t_2 dimension, 512 increments of t_1, a relaxation delay of 2.00 s, and 40 transients. Total correlation spectroscopy (TOCSY) spectra were acquired on intact rat urine and the sodium glucuronate solution using 2,048 data points in the t_2 dimension, 512 increments of t_1, spin locks of 50 and 70 ms, respectively, recycle delays of 1.22 and 1.71 s, respectively, and 64 and 8 transients, respectively.

^2H_2O-reconstituted ethyl acetate extracts of rat urine were obtained by adjusting the pH of intact 24-h urine samples (0.50–4.00 ml) to a value of 2.0 with aqueous HCl and adding HPLC grade ethyl acetate (1.0–1.5 ml). The mixtures were thoroughly rotamixed, left to

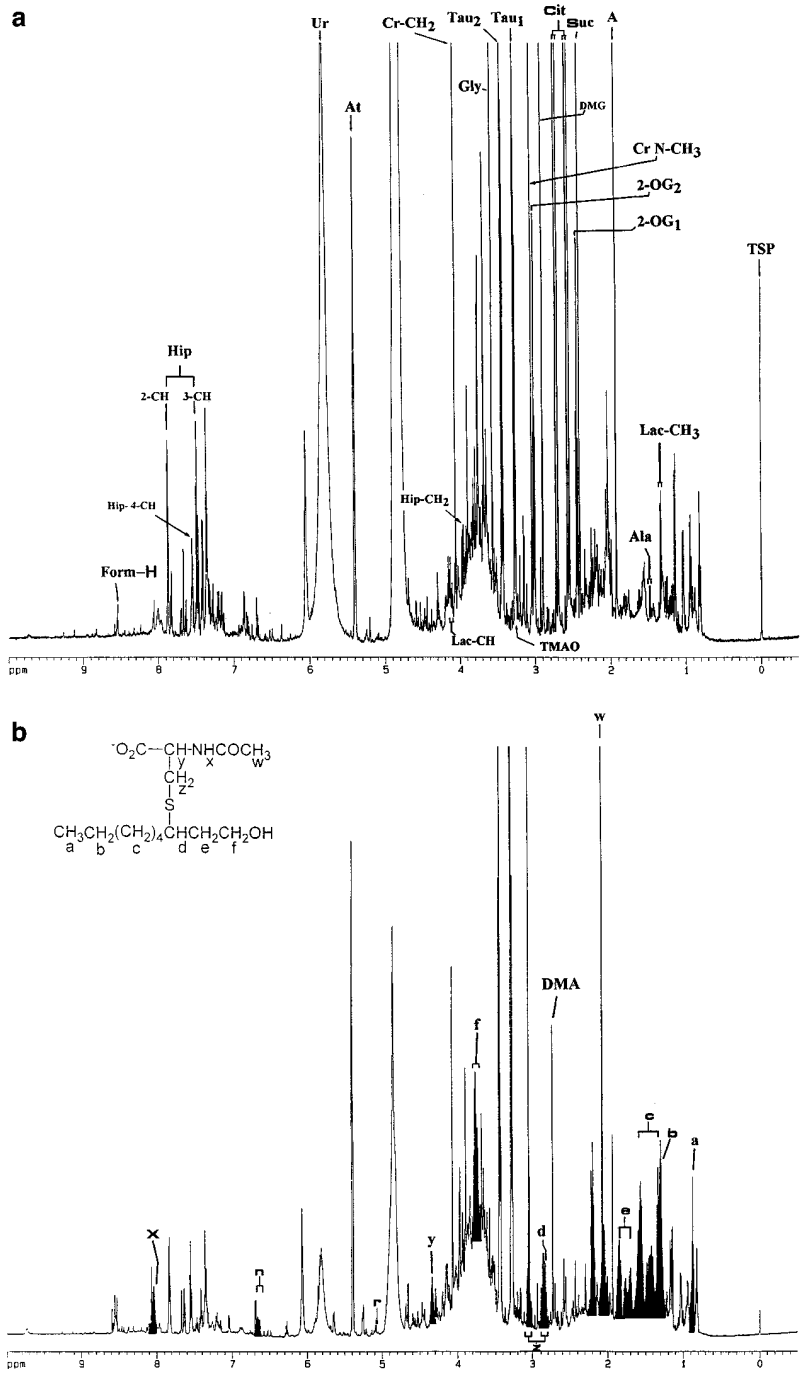

Figure 1. Single-pulse (one-dimensional) urinary ^{1}H NMR profiles of an experimental animal (male Wistar albino rat) (*a*) before, and (*b*) after, oral dosing with *t*-2N (100 mg/kg). Typical spectra are shown. *A*, acetate-CH_3; *Ala*, alanine-CH_3; *At*, allantoin-CH; *Cit*, citrate-CH_2; *Cr N-CH3* and *-CH2*, creatinine N-CH_3 and -CH_2- groups; *DMA*, dimethylamine-$N(CH_3)_2$; *DMG*, N,N-dimethylglycine-CH_3; *Form*, formate ^-O_2C-H; *Gly*, glycine-CH_2; *Hip-CH2*, -2-CH, -3-CH, and *-4-CH*, hippurate-CH_2, and aromatic ring 2-CH, 3-CH, and 4-CH protons, respectively; *Lac-CH3* and *-CH*, lactate-CH_3 and -CH respectively; *2-OG1* and *2-OG2*, 2-oxoglutarate $^-O_2C{\cdot}CH_2$ and $CH_2COCO_2^-$ group protons, respectively; *Suc*, succinate-CH_2; *Tau1* and *Tau2*, taurine $H_3NCH_2^-$ and $-CH_2SO_3^-$ group protons, respectively; *TMAO*, trimethylamine oxide $-N(CH_3)_3$; *Ur*, urea $-CONH_2$; *TSP* (internal chemical shift reference [δ = 0.00 ppm] and quantitative standard $-Si(CH_3)_3$); *a-f* and *w-z*, resonances arising from the 3-*S*-(*N*-acetylcysteinyl) nonan-1-ol metabolite of *t*-2N, as denoted in *b*; *r*, anomeric sugar ring proton(s) of *O*-glucuronide adduct(s) arising from the in vivo metabolism of *t*-2N; *n*, C-3 position vinylic proton resonance of a further NMR-detectable *t*-2N metabolite. The ^{1}H resonances depicted in black in the 24 h after dose urinary ^{1}H NMR profile shown in *b* represent those arising from all *t*-2N metabolites detectable.

stand at ambient temperature for 30 min, and centrifuged at 7,000 *g* (30 min at 4°C). Subsequently, the upper ethyl acetate phase was removed and the solvent was evaporated under N_2 in a fume hood. The residue was then reconstituted in 0.60 ml of ^2H_2O (final pH + 0.40 [pH*] value 7.0) or hexadeutero (d_6) DMSO and samples were stored at −20°C for a maximum duration of 18 h before ^{1}H NMR analysis.

Results

High resolution ^{1}H NMR spectroscopy was used as an analytical tool for probing the in vivo absorption and subsequent metabolic fate of the model α,β-unsaturated aldehyde *t*-2N. For this purpose, we orally administered *t*-2N at a dose level of 100 mg/kg to male Wistar albino rats (n = 10) and then housed the animals in metabolism chambers equipped for the collection of urine and feces.

^{1}H NMR analysis of deuterated chloroform (C^2HCl_3) extracts of the feces of rats predosed with *t*-2N showed that little or none of this aldehyde was detectable therein, although an examination of stomach contents obtained 16 h after dosing (under Sagatal anesthesia) revealed that ~15% of the remaining *t*-2N had been oxidized to its corresponding carboxylic acid

derivative, *trans*-2-nonenoic acid (characteristic olefinic proton resonances located at 5.81 [*d*, *j* = 16.9 Hz] and 7.09 ppm [*dt*, *j* 6.6, 16.9 Hz]).

Fig. 1 shows the urinary ^{1}H NMR profiles acquired on samples collected from an experimental rat before and after oral administration of *t*-2N, confirming the in vivo absorption of this aldehyde from the gut into the systemic circulation, metabolism, and excretion as water-soluble metabolites (typical spectra are shown). These data provided evidence for the urinary excretion of *t*-2N as one or more C-3 mercapturate conjugates (Fig. 2), consistent with a previous report by Winter et. al. (19) who utilized a tandem mass spectroscopic technique to examine the metabolic fate of 4-hydroxy-*trans*-2-hexenal after injection into the hepatic portal vein of male Sprague-Dawley rats.

Further possible metabolic products of *t*-2N include its corresponding carboxylic acid anion [$CH_3(CH_2)_5{\cdot}CH{=}CH{\cdot}CO_2^-$] and alcohol [$CH_3(CH_2)_5{\cdot}CH{=}CH{\cdot}CH_2OH$] derivatives. Indeed, previous investigations conducted by various laboratories have established that the carboxylate adduct is enzymatically generated via the actions of liver aldehyde dehydrogenases (i.e., NAD(P)-dependent oxidation of *t*-2N's aldehydic functional group), whereas the alcohol arises from electron-transfer mediated by NADH-dependent alcohol dehydrogenase present in liver cytosol (9). Such transformations may occur after the formation of C-3 position-substituted thiol conjugates (e.g., those of glutathione [GSH], cysteine, and *N*-acetylcysteine involved in the sequential metabolic process depicted in Fig. 2). However, previous metabolism of the intact aldehyde to its carboxylate or alcohol derivatives in the manners described above precludes the secondary generation of thiol conjugate Michael addition products (primarily those of GSH) in view of the critical role of the aldehydic functional group in rendering the C-3 position olefinic carbon susceptible to nucleophilic attack (9).

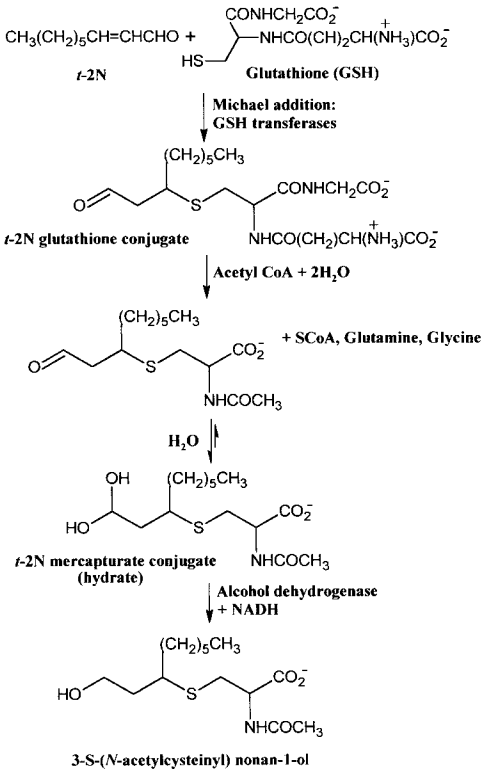

Figure 2. Mechanism for the generation of *t*-2N mercapturate conjugates in vivo.

Of course, the *t*-2N carboxylate and alcohol adducts (and/or their conjugates with endogenous thiols) may also be further metabolically transformed to *O*-glucuronides, and/or *O*-sulfates in the case of *t*-2N–derived alcohol derivatives, processes which further complicate investigations of the molecular nature of its urinary excretion products.

Nucleophilic attack of the amino groups of selected biomolecules at *t*-2N's C-1 position carbonyl carbon center can, in principle, give rise to Schiff base products in vivo, and previous investigations have established that *N*-(2-propenal)lysine (20), *N*-acetyl-(2-propenal)lysine (16), *N*-(2-propenal)serine (21), *N*-(2-propenal)ethanolamine (22), and a guanine-MDA adduct (23) are urinary metabolites of the bifunctional aldehyde MDA in the rat. Furthermore, it is conceivable that Michael addition products arising from the attack of reactive endogenous amino groups at the C-3 olefinic carbon atom are also generated in vivo. However, for α,β-unsaturated aldehydes, the rates of such Maillard and Michael addition reactions are slow when expressed relative to those involving thiolate sulfur centers (9), and hence the latter interactions are expected to predominate in physiological environments.

The molecular nature of putative *t*-2N–derived metabolic products was further investigated using two additional procedures. Firstly, two-dimensional COSY and TOCSY ^{1}H NMR spectra of intact urine samples collected from animals predosed with *t*-2N were acquired (Fig. 3), and the data obtained concurred with the presence of 3-*S*-(*N*-acetylcysteinyl) nonan-1-ol as a major urinary metabolite (i.e., clear connectivities between (*a*) the acetamido amide (-N<u>H</u>-CO-CH_3), -NH-C<u>H</u>-CO_2^-, and -CH-C<u>H</u>$_2$-S- group proton resonances of its *N*-acetylcysteine moiety (δ = 8.03, 4.33, and 2.92 ppm, respectively); (*b*) the -CH_2-C<u>H</u>$_2$OH, -CH(*S*-NAcCys)-C<u>H</u>$_2$-CH_2OH, and -CH_2-C<u>H</u>(S-NAcCys)-CH_2- group multiplets centered at 3.75, 1.82, and 2.85 ppm, respectively; (*c*) the 2.85 ppm methine proton signal of the *N*-acetylcysteine sulfur-substituted carbon and the five acyl chain bulk-CH_2- group resonances located in the 1.2–1.7 ppm chemical shift range; and (*d*) the acyl chain terminal-CH_3 triplet (δ = 0.89 ppm) and its adjacent -CH_2- group multiplet (δ = 1.28 ppm). Indeed, the chemical shift values and coupling patterns of the C-1–C-4 position proton resonances are very similar to those of the -CH_2-CH(SCH_3)-CH_2-CH_2OH system of the model compound 3-*S*-(methylthio)-hexan-1-ol (in C^2HCl_3 solution). Secondly, aliquots of the above urine samples were preadjusted to a pH value of 2.0 and then extracted twice with ethyl acetate (mercapturate conjugates of xenobiotics are effectively partitioned into the higher, ethyl acetate phase under these conditions and hence this technique affords a high level of selectivity regarding the analysis of such metabolites). After the removal of the organic solvent under N_2, samples were reconstituted in ^2H_2O (pH* value adjusted to 7.0), and one- and two-dimensional ^{1}H NMR analysis of the resulting solutions at an operating frequency of 600 MHz further ratified the above resonance assignments (Fig. 4). The absence of a -CH_2-C<u>H</u>O aldehydic group proton resonance (*t*, δ ∼ 9.8 ppm) from a high resolution ^{1}H NMR spectra of the d_6-DMSO-reconstituted ethyl acetate extracts confirmed that little or none of the *t*-2N–derived mercapturate conjugate(s)

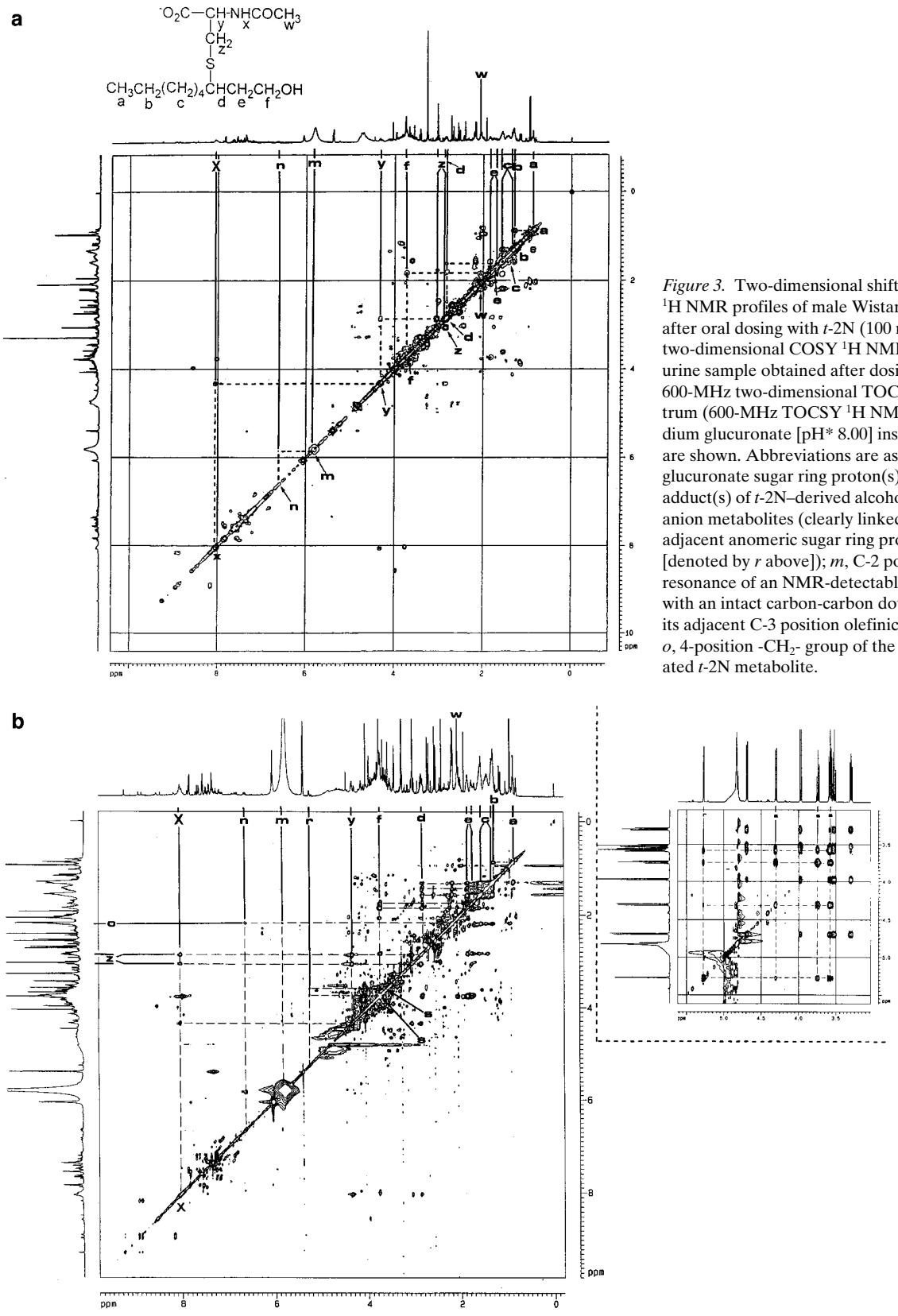

Figure 3. Two-dimensional shift-correlated urinary ^{1}H NMR profiles of male Wistar albino rats acquired after oral dosing with *t*-2N (100 mg/kg). (*a*) 400-MHz two-dimensional COSY ^{1}H NMR spectrum of a 24-h urine sample obtained after dosing; *b* as *a*, but a 600-MHz two-dimensional TOCSY ^{1}H NMR spectrum (600-MHz TOCSY ^{1}H NMR spectrum of sodium glucuronate [pH* 8.00] inset). Typical spectra are shown. Abbreviations are as in Fig. 1, with *s*, glucuronate sugar ring proton(s) of *O*-glucuronide adduct(s) of *t*-2N–derived alcohol and carboxylic acid anion metabolites (clearly linked to corresponding adjacent anomeric sugar ring proton resonance(s) [denoted by *r* above]); *m*, C-2 position vinylic proton resonance of an NMR-detectable *t*-2N metabolite with an intact carbon-carbon double bond (linked to its adjacent C-3 position olefinic proton signal, *n*); *o*, 4-position -CH_2- group of the above α,β-unsaturated *t*-2N metabolite.

excreted contained an intact aldehydic/aldehydic hydrate functional group.

Additional, clearly linked multiplet resonances present in the above spectra at δ = 2.22, 2.86, and 1.61 ppm are conceivably attributable to the -CH(S-NAcCys)-C<u>H</u>$_2$-CO_2^-, -CH_2C<u>H</u>-(S-NAcCys)-CH_2-, and -CH_2-C<u>H</u>$_2$-CH(S-NAcCys)- protons of 3-*S*-(*N*-acetylcysteinyl) nonan-1-oate, or its corresponding methyl ester [$CH_3(CH_2)_5$·CH(S-NAcCys)·$CH_2CO_2CH_3$].

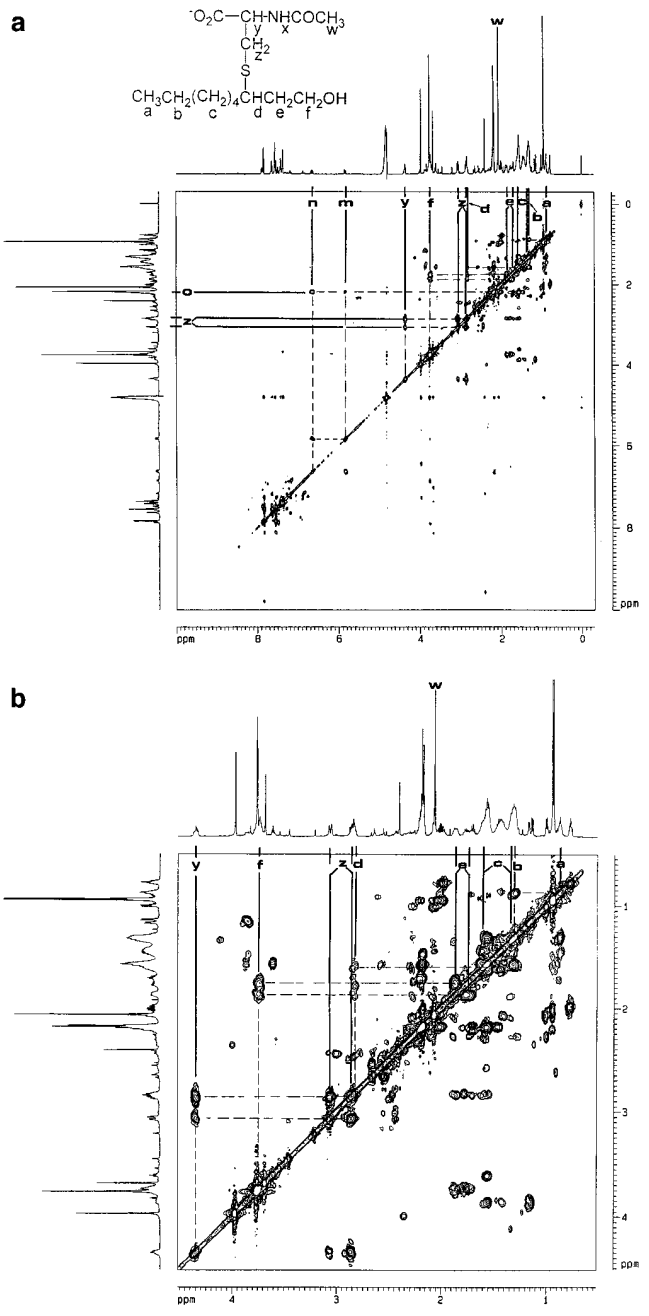

Figure 4. (*a*) Complete and (*b*) expanded 0.50 – 4.50 ppm regions of the 600-MHz two-dimensional COSY ^{1}H NMR spectrum of a ^2H_2O-reconstituted ethyl acetate extract of a 24-h urine sample collected after oral administration of 100 mg/kg *t*-2N (pH* value of sample on analysis = 7.00). A typical spectrum is shown. Abbreviations are as in Fig. 1.

Evidence for the urinary excretion of subcutaneously administered acrolein (CH_2=CH·CHO) as *N*-acetyl-*S*-(3-hydroxypropyl)-cysteine [CH_2(S-NAcCys)-CH_2-CH_2OH] in rats has been previously obtained by Kaye (17), and Draminski et al. (24) found that oral administration of this simple alkenal to such experimental animals gave rise to the urinary excretion of *S*-carboxyethyl-*N*-acetylcysteine and/or *S*-(propionic acid methylester) mercapturate. These observations are consistent with our ^{1}H NMR data obtained on the metabolic transformation of *t*-2N. Moreover, Kaye (17) did not detect any allylmercapturate, nor its corresponding sulfoxide, in urine samples collected from animals subcutaneously dosed with acrolein.

A comparison of the TOCSY ^{1}H NMR spectrum of sodium glucuronate (pH* 8.00) with those of urine obtained from animals predosed with *t*-2N revealed the presence of characteristic, clearly connected glucuronide moiety anomeric proton resonances in the latter (Fig. 3 *b*, inset) demonstrating the presence of relatively low levels of *O*-glucuronide adducts derived from the metabolic transformation of *t*-2N. Such glucuronide species presumably arise via a sequential metabolic pathway involving (*a*) previous thiol conjugation in the C-3 position and (*b*) enzymic reduction or oxidation of the remaining aldehydic functional group as described above.

Intriguingly, minor but clearly connected olefinic multiplet resonances centered at 5.86 (*dd*, *J* 13.95, 9.3 Hz) and 6.65 ppm are detectable in the one- and two-dimensional spectra shown in Figs. 1 and 3, respectively, providing evidence for a partial retention of *t*-2N's carbon-carbon double bond throughout the in vivo metabolic processes. These signals are further linked to a C-4 position methylene (-CH_2-) group resonance located at 2.125 ppm.

Similar results were obtained from experiments involving the ^{1}H NMR analysis of urine samples collected from male Wistar albino rats predosed with *t*-2P via the oral route, e.g., excretion of 3-*S*-(*N*-acetylcysteinyl)-pentan-1-ol as a major metabolic product.

Discussion

The results obtained in this investigation demonstrate that typical *trans*-2-alkenal compounds known to be produced from the thermally induced autoxidation of PUFAs are readily absorbed from the gut into the systemic circulation in vivo, metabolized (primarily via the addition of glutathione across their electrophilic carbon-carbon double bonds), and excreted in the urine as C-3 mercapturate conjugates. Such aldehydic products have the ability to exert a range of toxicological effects and previous investigations have shown that they interfere with the growth of cultured animal and bacterial cells, block macrophage action, express chemotactic actions upon neutrophils in biofluids, stimulate thrombin production in vivo, and are mutagenic in bacterial test systems (25–30). Moreover, these adducts inhibit protein synthesis and inactivate enzymes (31, 32).

Peroxidation of the PUFA components of LDL in vivo and the subsequent production of foam cells from macrophages represents a critical step in the pathogenesis of atherosclerosis (3, 30). Modification of LDL in vivo is thought to occur via a mechanism which comprises (*a*) previous generation of aldehydes from the decomposition of preformed, PUFA-derived CHPDs, and (*b*) alteration of the apo B moiety's structure via Maillard reactions involving the above carbonyl compounds. Indeed, 4-hydroxy-*t*-2N (HNE), a major aldehydic product arising from the autoxidation of PUFAs, reacts with selected amino acids of apo B, rendering it susceptible to uptake by the macrophage scavenger receptor (33). Indeed, apo B lysine residues are readily derivatized by aldehydic CHPD fragmentation products (34), and since a critical number of these modified residues are required for the recognition of the oxidized

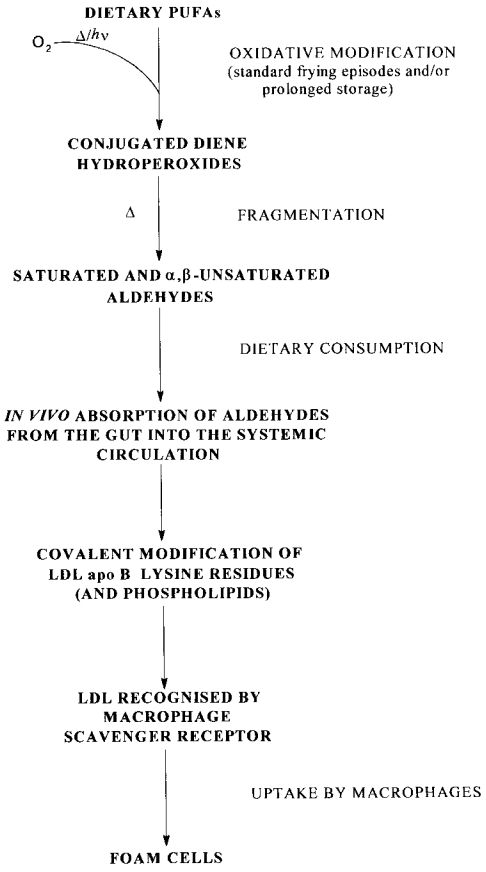

Figure 5. Simplified representation of events giving rise to the diet-induced, covalent (nonoxidative) modification of LDL in vivo and the subsequent generation of foam cells. *apo B*, LDL apolipoprotein B moiety.

LDL by the acetyl LDL receptor, its uptake by macrophages increases with an increasing level of such derivatization (35). Therefore, after their in vivo absorption, the aldehydes present in thermally, oxidatively stressed culinary oils ingested in the diet will have the capacity to directly affect structural modifications of the apo B component of LDLs, a process which induces the generation of foam cells from macrophages (Fig. 5).

Selley et al. (36) measured the concentrations of HNE in human biofluids by a method involving gas chromatography coupled with mass spectrometric detection, and found that it is present at a level of $\sim 10^{-7}$–10^{-6} mol·dm^{-3} in the blood plasma of healthy human subjects. While a significant proportion of human plasma HNE may arise from the oxidative degradation of PUFAs in vivo, these levels may also be dependent on the dietary consumption of oxidatively, thermally stressed, PUFA-rich culinary oils. The above observations also indicate that in vivo absorption of autoxidized PUFA-derived aldehydes would exert a major influence on results obtained from bioanalytical investigations which use such molecules as markers of in vivo lipid peroxidation (e.g., detection and measurement of *n*-alkanals, MDA, etc., in human biofluids and tissues), and the authors recommend adequate fasting of experimental subjects/animals before the collection of samples for analysis.

While the toxic effects of PUFA-derived aldehydes are well described in animal models and in vitro, there is currently much controversy regarding the role of PUFA-rich diets in promoting, regressing, or preventing cardiovascular diseases in humans. Some epidemiological investigations have demonstrated apparent paradoxes in the relationship between dietary lipid consumption and cardiovascular mortality. In 1920, the typical American diet was rich in saturated fat (and cholesterol), yet contained only one-third the level of PUFAs consumed today. In that year, death was seldom attributed to myocardial infarction. Natural and refined vegetable oils containing high concentrations of PUFAs were subsequently introduced, and by 1960 deaths arising from myocardial infarction in the USA had substantially escalated to a figure of 600,000 per year (37). In 1978, the demographic yearbook of the United Nations reported that the Sri-Lankan population had the lowest documented mortality rate from IHD of all the countries surveyed. Their predominant dietary fat is coconut oil (38), which contains $\sim$ 95% (wt/wt) saturates and a very low concentration of PUFAs (subjection of commercially available samples of this oil to episodes of thermal stressing generates little or no aldehydic products [6]). Other populations demonstrating this paradox are the saturated fat–eating Masai tribe (39) and that of southern France (40). These observations support a pathogenic role for PUFA-derived aldehydes in the pathogenesis of IHD.

With the exception of direct damage to the gastrointestinal epithelium, the toxicological hazards putatively posed by aldehydes generated from the autoxidation of PUFA-rich culinary oils are, of course, critically dependent on the rate and extent of their in vivo absorption from the gut into the systemic circulation. Our data concerning the in vivo absorption and metabolism of typical autoxidized PUFA-derived *trans*-2-alkenals, and their urinary excretion as water-soluble mercapturate conjugates (Fig. 2), lend further support to the hypothesis that diet-derived aldehydes play an important role in atherogenesis. Interestingly, depletion of intracellular GSH via this metabolic pathway has been suggested to be responsible for the cytotoxicity of α,β-unsaturated aldehydes (9), and in vivo exposure to acrolein (CH_2=CH·CHO) has been previously shown to cause a dose-dependent depletion of GSH in the liver (41). Since the reactions of *trans*-2-alkenals with endogenous thiols are readily reversible (42), β-thiyl-substituted saturated aldehyde metabolites serve as a latent source of bioactive unsaturated carbonyl compounds, potentially prolonging their deleterious actions in vivo.

Previous investigations have indicated that such secondary PUFA peroxidation products are indeed absorbed (15), unlike their CHPD precursors which failed to penetrate the gastrointestinal epithelium (10–12). Further evidence for the in vivo absorption and potential pathogenicity of PUFA-derived aldehydes comes from Smith and Kummerow (43), who recently demonstrated that dietary consumption of peroxidized culinary oils enhances the accumulation of oxidized lipids in macrophages and monocytes. In addition, animal feeding studies have shown that diets containing thermally stressed, PUFA-laden oils are more atherogenic than those containing unheated oils (44), and recently, Staprans et al. (45) 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. These observations clearly indicate that consumption of PUFA peroxidation products in the diet may represent an important risk

factor for the induction and development of atherosclerosis in humans.

The autoxidative consumption of PUFAs in culinary oils subjected to episodes of thermal stressing is therefore of much significance in view of orthodox medical recommendations, i.e., the perceived notion of these molecules as protective agents against atherosclerosis. Therefore, the results obtained in our investigations pose a critical question: is there a culinary oil that is safe to use when subjected to common frying or cooking practices?

Experiments conducted previously in our laboratory demonstrated that heating of oleic acid–rich olive oil according to standard frying practices generates little or no aldehydic products (6, 7), results consistent with the low-risk status of this monounsaturated fatty acid regarding the development of atherosclerosis in humans or experimental animal model systems. Monounsaturated fatty acids are far less susceptible to autoxidation than PUFAs (the relative rates of peroxidation of oleic, linoleic, and linolenic acid derivatives are in the ratio of 1:12:25, respectively, under a specified set of experimental conditions [46]). Intriguingly, Kritchevsky and Tepper (44) showed that thermal stressing of olive oil (20 min at 215± 15°C) failed to elevate its atherogenicity in rabbits, whereas a diet containing corn oil heated in the same manner was substantially more atherogenic than that containing unheated corn oil.

It has also been reported recently that dietary sources of oleic acid may offer a high level of protection against atherosclerosis. Parthasarathy et al. (47) conducted experiments involving the feeding of a newly developed monounsaturated fatty acid–rich variant sunflower oil (containing 80% [wt/wt] oleic acid and 8% [wt/wt] linoleic acid) to rabbits, and found that LDLs isolated from their plasma (substantially enriched in oleic acid and markedly depleted in its linoleic acid content) was highly resistant to copper(II)- or cultured endothelial cell–mediated oxidative modification, results which markedly contrasted with those obtained from animals fed a conventional sunflower oil (containing only 20% [wt/wt] oleic acid and 67% [wt/wt] linoleic acid). As expected, macrophage uptake of the oleate-enriched LDL particles was only marginally elevated after prolonged exposure to the above oxidizing systems. Consistently, Whitman et al. (48) showed that *n*-3 fatty acid incorporation into LDL particles renders them more vulnerable to oxidation in vitro and possibly more atherogenic in vivo.

Another approach to solving the dietary problems outlined above may be the commercial fortification of vegetable-derived culinary oils with lipid-soluble, chain-breaking, dietary antioxidants. Results obtained from preliminary investigations conducted in this laboratory indicate that the inclusion of adequate levels of α-tocopherol (α-TOH, vitamin E) or butylated hydroxytoluene in commercial preparations of culinary frying oils may render PUFAs more resistant to oxidation (thermally induced or otherwise) (Grootveld et al., unpublished data).[2] Unfortunately, concentrations of α-TOH naturally present in the culinary oils examined in our investigations (e.g., $\sim 2 \times 10^{-3}$ mol·kg^{-1} in corn oil) are clearly insufficient to protect PUFAs against oxidative deterioration induced by heating at a temperature of 180°C for only a 30-min period. Moreover, recent pilot studies conducted by the authors also suggest that the α-TOH content of culinary oils subjected to thermal stressing episodes is substantially depleted when subjected to thermal stressing episodes (Grootveld et al., unpublished observations),[3] a phenomenon negating any claims concerning the supplementation of requirements for this nutrient with these frequently utilized products.

The wealth of specific structural (i.e., qualitative) and quantitative analytical data provided by high resolution ^{1}H NMR analysis in this study is, of course, a critical primary requirement for future investigations of the toxicological/proatherogenic effects associated with the regular dietary consumption of culinary oils and fats. Such data should provide valuable information regarding the average daily intake of PUFA-derived oxidation products, information which will eventually be of much use to those undertaking epidemiological research.

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3. The α-TOH phenolic-OH group proton resonance (*s*, δ = 4.635 ppm) detectable in ^{1}H NMR spectra of samples of sunflower seed oil pretreated with this antioxidant was markedly diminished in intensity after thermal stressing according to standard frying practices. Moreover, the intensity of its heterocyclic ring 4-position methylene group proton resonance (*t,* δ = 2.59 ppm) was also substantially reduced after heating. Although decreases in the intensities of the α-TOH resonances may be partially ascribable to the volatilization of this antioxidant (boiling point 210°C), these ^{1}H NMR-detectable observations were accompanied by the development of an intense brown coloration (λ_{max} 270 nm in hexane) consistent with the generation of α-TOH oxidation products such as α-tocopherylquinone (49) (the intensity of this coloration was much greater than that observed on heating untreated sunflower seed oil in the same manner). Hence, data acquired are in accordance with the consumption of α-TOH via its involvement in peroxidative chain reactions and/or its direct thermally induced oxidation.

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