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ARTICLE

Analysis of Hydroperoxides, Aldehydes and Epoxides by ^{1}H Nuclear Magnetic Resonance in Sunflower Oil Oxidized at 70 and 100 °C

Encarnación Goicoechea and María D. Guillen*

Department of Food Technology, Faculty of Pharmacy, University of the Basque Country (UPV-EHU), Paseo de la Universidad 7, 01006 Vitoria, Spain

A global study of sunflower oil oxidation at two different temperatures (100 and 70 °C) with aeration was carried out by ^{1}H nuclear magnetic resonance (^{1}H NMR), paying attention not only to the degradation of the main components but also to the formation of others. The functional groups of compounds monitored simultaneously during the oxidation were, in addition to acyl groups, the following: hydroperoxides, conjugated dienic systems of hydroperoxy acyl groups, aldehydes including the genotoxic and cytotoxic oxygenated alpha,beta-unsaturated aldehydes, and mono- and diepoxides. Chemical shifts of protons of all mentioned groups were given. Differences between both oxidation processes were found from the initial stages to the end. The formation of diepoxides in the oxidation process of edible oils was shown for the first time, as was the influence of temperature on the formation of epoxy acyl groups, some of these latter related to leukotoxin and isoleukotoxin.

KEYWORDS: Sunflower oil thermoxidation; ^{1}H nuclear magnetic resonance; oxygenated alpha,beta-unsaturated aldehydes; mono- and diepoxides; leukotoxin

INTRODUCTION

Edible oil degradation by heating in the presence of air is a subject of great interest, as oils are subjected to thermal treatments in both industrial and culinary processes, and because toxic compounds are known to be generated in these processes. Although the knowledge of edible oil thermoxidation mechanisms has made great advances, these are not totally well-known, nor are all the intermediate and final compounds formed. Most of the general knowledge on this subject has been deduced from the determination, in the oil submitted to degradative conditions, of the concentration of the so-called primary oxidation products, such as hydroperoxides (peroxide value) and conjugated dienic systems (absorbance at 232 nm in UV spectroscopy), or of secondary oxidation products, such as certain carbonylic compounds (anisidine value, or thiobarbituric acid reactive substances (TBARS) test). These classical methods only give concentration values for some compounds in a generic way, but do not provide information on the specific nature of the compounds involved in each determination. There are even doubts raised about the specific compounds really determined by these methods (*1*). In addition, it has recently been proved that hydroperoxides and conjugated dienic systems can also be present among secondary oxidation compounds (*2*−*4*). This could be why the results provided by these classical determinations do not shed further light on the thermo-oxidative mechanisms of oils.

*Corresponding author. Tel: 34-945-013081. Fax: 34-945-013014. E-mail: mariadolores.guillen@ehu.es.

It is generally agreed that oil thermal oxidation provokes the formation of intermediate structures (hydroperoxides and conjugated dienic groups) in the acyl group chains of triglycerides, which evolve to give other compounds, among which there are small volatile molecules and dimers, oligomers and polymers of triglycerides, as well as degraded triglycerides, some of them supporting epoxy, hydroxy or keto groups, among others (*2*−*7*). With reference to oxidation products, over recent years there has been growing interest in two groups of compounds, due to their high toxicity and possible absorption by means of diet (*8*, *9*). The first are oxidized triacylglycerols with keto, epoxy and hydroxyl groups supported on modified acyl groups. Among these compounds it is worth noting the presence of 9,10-epoxy-12-octadecenoate groups, derived from linoleic acyl groups, whose hydrolysis gives 9,10-epoxy-12-octadecenoic acid or leukotoxin; this last compound is so named as it is able to be formed endogeneously, causes degeneration and necrosis of leukocytes and has been associated with multiple organ failure, breast cancer, cell proliferation *in vitro* and disruption of reproductive functions in rats (*10*).

The second group of oxidation compounds to which special attention is being paid is oxygenated alpha,beta-unsaturated aldehydes (O$\alpha\beta$UAs), which are well-known genotoxic and cytotoxic compounds (*11*); they can be generated in the oxidation of omega-3 and omega-6 polyunsaturated fatty acids and esters, both in biological systems and foodstuffs (*11*), and can be absorbed through the diet (*8*, *12*).

In this context, this paper deals with the development of an approach, based on ^{1}H nuclear magnetic resonance (^{1}H NMR)

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data, in order to study sunflower oil thermoxidation processes at 100 and 70 °C in the presence of air until total polymerization. The aim of the study is to help in extending the current knowledge about the mechanisms occurring in the thermo-oxidative processes of edible oils at different temperatures, as well as to elucidate the nature of some of the compounds formed and determine their concentrations throughout these processes. Special attention will be paid to the tendency to form toxic oxidized triacylglycerol structures with epoxy groups and oxygenated alpha, beta-unsaturated aldehydes (O$\alpha\beta$UAs).

MATERIALS AND METHODS

Materials and Heating Procedure. The oil subject of study was sunflower oil acquired in a local supermarket (six bottles of the same batch), which contained no added antioxidants. The oil was submitted to two thermoxidation experiments: one at 100 °C and one at 70 °C, both with aeration. To this aim, several 10 g samples of sunflower oil held in crystal Petri dishes of 80 mm diameter and 15 mm height were placed in a Selecta convection oven, with circulating air and without being stirred. Its temperature was maintained either at 100 °C or at 70 °C with a stability of ±0.5%. The Petri dishes were introduced into the oven without their lids to facilitate exposure to the circulating air. The thermoxidation experiments were carried out in duplicate. These processes were monitored by ^{1}H NMR on an hourly (at 100 °C) or a daily (at 70 °C) basis, until total polymerization of the samples was reached (55 h at 100 °C and 264 h at 70 °C). These heating times were considered to be the end point of these processes, because the samples were practically solid.

Methods. The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra of the oil samples were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. Each oil sample (200 μL) was mixed with 400 μL of deuterated chloroform (purity 99.8%, acquired in Cortecnet, Paris, France) and a small proportion of tetramethylsilane as an internal reference (TMS, acquired in Cortecnet, Paris, France); this mixture was introduced into a 5 mm diameter tube. The acquisition parameters were spectral width 5000 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.744 s and pulse width 90°, with a total acquisition time of 12 min 54 s. The experiment was carried out at 25 °C. The assignment of the signals was made as in previous studies and is given in **Tables 1−4** (*2*−*4*, *13*−*17*). All figures of ^{1}H NMR spectra or of expanded regions were plotted at a fixed value of absolute intensity to be valid for comparative purposes. The molar concentrations (mmol/L oil) of some functional groups or of groups of compounds, such as hydroperoxides, *Z*,*E* and *E*,*E* conjugated dienic systems in chains having hydroperoxy groups, monoepoxides derived from oleic and linoleic acyl groups, diepoxides derived from linoleic acyl groups, alkanals, (*E*)-2-alkenals, 4-hydroperoxy-(*E*)-2-alkenals, 4-hydroxy-(*E*)-2-alkenals, (*E*,*E*)-2,4-alkadienals and 4,5-epoxy-(*E*)-2-alkenals, can be determined. For this, the nondeuterated chloroform (0.02%) present in the used deuterated chloroform, which gives a signal at 7.29 ppm and has the same concentration in all ^{1}H NMR experiments, was taken as standard compound for quantitative purposes, as in previous studies (*18*, *19*). These determinations are possible because the area of the ^{1}H NMR signal is proportional to the number of protons which generates the corresponding signal. Each sample was analyzed in triplicate, and data shown are average values.

Compounds, such as 4-hydroxy-(*E*)-2-nonenal, 4-hydroxy-(*E*)-2-hexenal, 4-hydroperoxy-(*E*)-2-nonenal and 4,5-epoxy-(*E*)-2-decenal, acquired from Cayman Chemical (Ann Arbor, MI), and heptanal, octanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal and 12,13-epoxy-Z,9-octadecenoic acid methyl ester (also called vernolic acid methyl ester or isoleukotoxin methyl ester) acquired from Sigma Aldrich, were used as standard compounds for identification purposes.

Statistic and Kinetic Studies. The change rate of the acyl groups' molar proportions in certain periods of time was inferred from the equations obtained by fitting the percentages of acyl groups in the oil samples and the corresponding period of time during which the oil was submitted either to 70 °C or to 100 °C; the statistical package SPSS (SPSS, Inc., Chicago, IL, 2004) was used for this purpose. Likewise, the relationships between the percentage of linoleic acyl groups and the iodine value were also studied by using the same software package mentioned above.

Table 1. Assignment of the Signals of the ^{1}H NMR Spectra of the Sunflower Oil[a]

signal	chemical shift (ppm)	functional group
A	0.83−0.93	−C*H*$_3$ (saturated, oleic and linoleic acyl group)
B	1.22−1.42	−(C*H*$_2$)$_n$− (acyl group)
C	1.52−1.70	−OCO−CH$_2$−C*H*$_2$− (acyl group)
D	1.94−2.14	−C*H*$_2$−CH=CH− (acyl group)
E	2.23−2.36	−OCO−C*H*$_2$− (acyl group)
F	2.70−2.84	=HC−C*H*$_2$−CH= (acyl group)
G	4.10−4.32	−C*H*$_2$OCOR (glyceryl group)
H	5.20−5.26	>C*H*OCOR (glyceryl group)
I	5.26−5.40	−C*H*=C*H*− (acyl group)

[a] The signal letters agree with those in **Figure 1**.

RESULTS AND DISCUSSION

This approach is based on the changes observed in the ^{1}H NMR spectrum of sunflower oil while the advance of thermo-oxidative processes, provoked with aeration at 100 °C or at 70 °C, takes place. Oil degradation causes changes in the original oil components, whose signals decrease in the spectrum, generating other new components with different kinds of protons, which give rise to new signals in the ^{1}H NMR spectrum whose intensity also changes as the degradation advances.

1. ^{1}H NMR Spectra Signals of the Original Sunflower Oil Main Components and Their Evolution throughout the Thermoxidation Processes. The spectrum of the original sunflower oil, before being submitted to thermo-oxidative conditions (see **Figure 1-I**, 0 h), shows nine well-known signals, with chemical shifts in the region between 0 and 5.5 ppm. These signals are generated by the different types of hydrogen atoms present in the main components of the sunflower oil, that is, in its triglycerides. Signals, named with different letters in **Figure 1**, are assigned to the different types of hydrogen atoms in the oil, which have been very broadly described before. Their assignments are indicated in **Table 1**, in agreement with previous studies (*13*). By using the intensity of some of these ^{1}H NMR signals, its composition in main components (11.8% of saturated acyl groups, 27.2% of oleic acyl groups, and 61.0% of linoleic acyl groups), expressed as molar percentages of the several kinds of acyl groups, was determined, as in previous papers (*19*, *20*).

Table 2. Chemical Shifts and Multiplicities of the ^{1}H NMR Signals of Protons of Hydroperoxide Groups, of Conjugated Dienic Systems Supported on Hydroperoxide Chains and of Other Related Unassigned Signals, Presumably Due to Secondary Oxidation Compounds, All of Them Generated in Both Oxidation Processes of Sunflower Oil

compounds and functional groups	chemical shift[a] (ppm)	functional group
hydroperoxy groups	8.2−8.9 (bs)	OO*H*−
unidentified signal 1	7.4−7.7 (bs)	not identified
unidentified signal 2	8.0−8.2 (bs)	not identified
hydroperoxy-(*Z*,*E*)-conjugated-dienic systems	6.58 (dddd), 6.00 (ddtd), 5.56 (ddm), 5.51 (dtm)	−C*H*=C*H*−C*H*=C*H*− (*Z*,*E*) conjugated double bond groups
hydroperoxy-(*E*,*E*)-conjugated-dienic systems	6.27 (ddm), 6.06 (ddtd), 5.76 (dtm), 5.47 (ddm)	−C*H*=C*H*−C*H*=C*H*− (*E*,*E*) conjugated double bond groups

[a] Abbreviations: bs, broad signal; m, multiplet; d, doublet; t, triplet.

Table 3. Chemical Shift Assignment of the ^{1}H NMR Signals of Some of the Secondary Oxidation Compounds (Aldehydes) Generated in the Oxidation Process of Sunflower Oil Samples, Together with their Multiplicities[a]

compounds and functional groups	chemical shift[b] (ppm)	functional group
(**a**) (*E*)-2-alkenals	9.49 (d)	C*H*O−
	6.85 (tt)	CHO−CH=C*H*−
	6.11 (dd)	CHO−C*H*=CH−
	2.32 (q)	CHO−CH=CH−CH_2−
	1.69−1.19 (bs)	CHO−CH=CH−CH$_2$−(CH_2)$_n$−
	0.89 (t)	CHO−CH=CH−CH$_2$−(CH$_2$)$_n$−CH_3
(**b**) 4-hydroperoxy-(*E*)-2-alkenals	9.58 (d)	C*H*O−
	8.20 (d)	CHO−CH=CH−CHOO*H*−
	6.80 (dd)	CHO−CH=C*H*−
	6.33 (m)	CHO−C*H*=CH−
	4.66 (dd)	CHO−CH=CH−C*H*OOH−
	1.73−1.21 (m)	CHO−CH=CH−CHOOH−(CH_2)$_n$−
	0.89 (t)	CHO−CH=CH−CHOOH−(CH$_2$)$_n$−CH_3
(**c**) alkanals	9.75 (t)	C*H*O−
	2.40 (dt)	CHO−CH_2−
	1.61 (m)	CHO−CH$_2$−CH_2
	1.32−1.27 (bs)	CHO−CH$_2$−CH$_2$−(CH_2)$_n$−
	0.88 (t)	CHO−CH$_2$−CH$_2$−(CH$_2$)$_n$−CH_3
(**d**) (*E*,*E*)-2,4-alkadienals	9.53 (d)	C*H*O−
	7.09 (m)	CHO−CH=C*H*−
	6.30 (m)	CHO−CH=CH−C*H*=C*H*−
	6.08 (dd)	CHO−C*H*=CH−
	2.22(m)	CHO−CH=CH−CH=CH−CH_2−
	1.47−1.3 (m)	CHO−CH=CH−CH=CH−CH$_2$−(CH_2)$_n$−
	0.90 (t)	CHO−CH=CH−CH=CH−CH$_2$−(CH$_2$)$_n$−CH_3
(**e**) 4-hydroxy-(*E*)-2-alkenals	9.57 (d)	C*H*O−
	6.84 (dd)	CHO−CH=C*H*−
	6.33(ddd)	CHO−C*H*=CH−
	4.43 (m)	CHO−CH=CH−C*H*OH−
	1.30−1.70 (m)	CHO−CH=CH−CHOH− (CH_2)$_n$−
	0.90 (t)	CHO−CH=CH−CHOH−(CH$_2$)$_n$−CH_3
(**f**) 4,5-epoxy-(*E*)-2-alkenals	9.54 (d)	C*H*O−
	6.56 (dd)	CHO−CH=C*H*−
	6.40 (dd)	CHO−C*H*=CH−
	3.33 (dd)	CHO−CH=CH−C*H*O
	2.96 (td)	CHO−CH=CH−CHOC*H*−
	1.65−1.33 (m)	CHO−CH=CH−CHOCH−(CH_2)$_n$−
	0.91 (t)	CHO−CH=CH−CHOCH−(CH$_2$)$_n$−CH_3

[a] The letters are in agreement with those in **Figure 3**. [b] Abbreviations: bs, broad signal; m, multiplet; d, doublet; t, triplet; q, quadruplet.

As mentioned before, when the original oil is kept under thermo-oxidative conditions at 100 °C or at 70 °C, it suffers degradation, its composition changes, and as expected, its ^{1}H NMR spectra change too. **Figure 1-I** shows the ^{1}H NMR spectra of the oil after being submitted to 100 °C for 22 and 51 h respectively. It can be observed in this figure that as the oil degradation advances, the intensity of some signals decreases, due to the concentrations and proportions of some hydrogen atoms decrease. The heating time of 55 h was considered to be the end point of the process because the sample was practically solid due to its polymerization. It is worth noting the lowering of the proportions of olefinic (signal **I**), bis-allylic (signal **F**), and allylic protons (signal **D**), in relation to the proportions of methylic protons (signal **A**), of glyceryl structure protons (signals **H** and **G**), and of methylenic protons in α or β positions in relation to the carbonyl group (signals **E** and **C** respectively).

The enlargement of some of these signals permits one to observe some features of the composition of sunflower oil and its evolution under these thermo-oxidative conditions. **Figure 1-II** shows the signals **A**, **B** and **D** of sunflower oil at 0 h, 22 and 51 h after being submitted to 100 °C with aeration. Looking at the figures it is evident that the degradation suffered by this oil affects linoleic groups to a higher degree than it does monounsaturated or saturated acyl groups. Thus in signal **A**, as heating time increases, the intensity of the triplet signal of the linoleic acyl group methylic protons centered at 0.889 ppm becomes smaller than that of monounsaturated plus saturated acyl groups (triplet centered at 0.873 ppm) after 51 h, showing the preferential degradation of the first. The same can be deduced from the observation of the enlarged signal **B**, due to the methylene protons being in β-position or further in relation to double bonds, or in γ-position or further in relation to the carbonyl group; in this case, the intensity of the signal near 1.302 ppm (of the methylene protons of linoleic plus oleic groups) decreases in comparison with the intensity of the signal near 1.257 ppm (of saturated acyl groups) and with that of the shoulder near 1.275 ppm (of oleic groups), becoming smaller as the heating process advances. The preferential degradation of linoleic groups is also observable in signal **D** of the allylic protons (α-methylenic protons in relation to only one double bond); it is evident that, as the heating time increases, the intensity of the signal of linoleic group (mainly 2.038 and 2.055 ppm) decreases in comparison with the intensity of the signal of oleic group (mainly 2.021 and 2.004 ppm). This degradation is also clearly shown in the diminution of the bis-allylic protons of linoleic acyl group (α-methylenic protons in relation to two double bonds) throughout the degradation process (see triplet centered at 2.769 ppm, signal **F** in **Figure 1-I**). A similar evolution is observed in the spectral signals of the main

Table 4. Chemical Shift Assignment of Certain ^{1}H NMR Signals of Some Epoxy Acyl Groups Able To Be Generated in the Oxidation Process of Sunflower Oil Samples, Together with Their Multiplicities[a]

Compound	Chemical Shift (ppm)	Functional Group
9,10-epoxy-octadecanoate	1.5	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-$(CH_2)_6$-CH_3
	(**a**) 2.9 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-$(CH_2)_6$-CH_3
9,10-epoxy-12-octadecenoate (leukotoxin)	(**a**) 2.9 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH=CH-CH_2-$(CH_2)_3$-CH_3
	1.5* (dd)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH=CH-CH_2-$(CH_2)_3$-CH_3
	2.3** (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH=CH-CH_2-$(CH_2)_3$-CH_3
	2.0	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH=CH-CH_2-$(CH_2)_3$-CH_3
12,13-epoxy-9-octadecenoate (isoleukotoxin)	(**a**) 2.9 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH=CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
	1.5 (dd)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH=CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
	2.3 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH=CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
	2.0	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH=CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
9,10-12,13-diepoxy-octadecanoate	(**a**) 2.9 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
	1.7	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
	(**b**) 3.1 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
	1.5	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH-CH-CH_2-CH-CH-CH_2-$(CH_2)_3$-CH_3
9,10-dihydroxy-12-octadecenoate (leukotoxindiol)	(**c**) 3.4 (m)	R-O-CO-$(CH_2)_5$-CH_2-CH_2-CH(OH)-CH(OH)-CH_2-CH=CH-CH_2-$(CH_2)_3$-CH_3

[a] The letters **a**, **b** and **c** are in agreement with those in **Figure 4**. Abbreviations: m, multiplet; d, doublet. *Some authors indicate that the chemical shift of these protons is 2.0 (*15*). **Some authors indicate that the chemical shift of these protons is 1.2−1.4 (*15*).

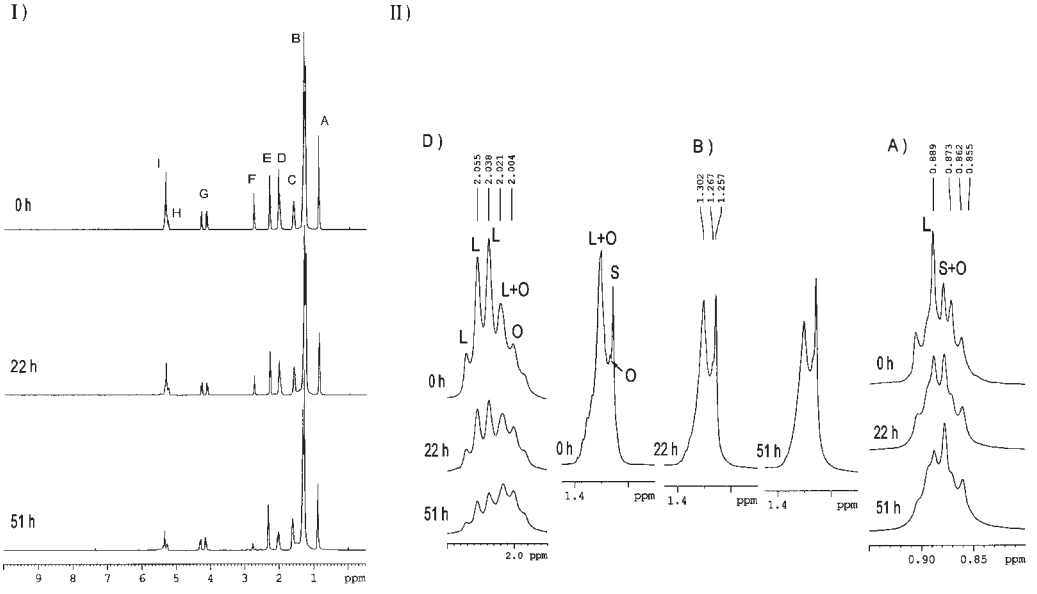

Figure 1. ^{1}H NMR spectra of sunflower oil samples submitted to 100 °C with aeration, during different periods of time indicated in hours: (**I**) Region between 0 and 10 ppm; (**II**) Expanded signals **A**, **B**, and **D**. The signal letters agree with those in **Table 1**.

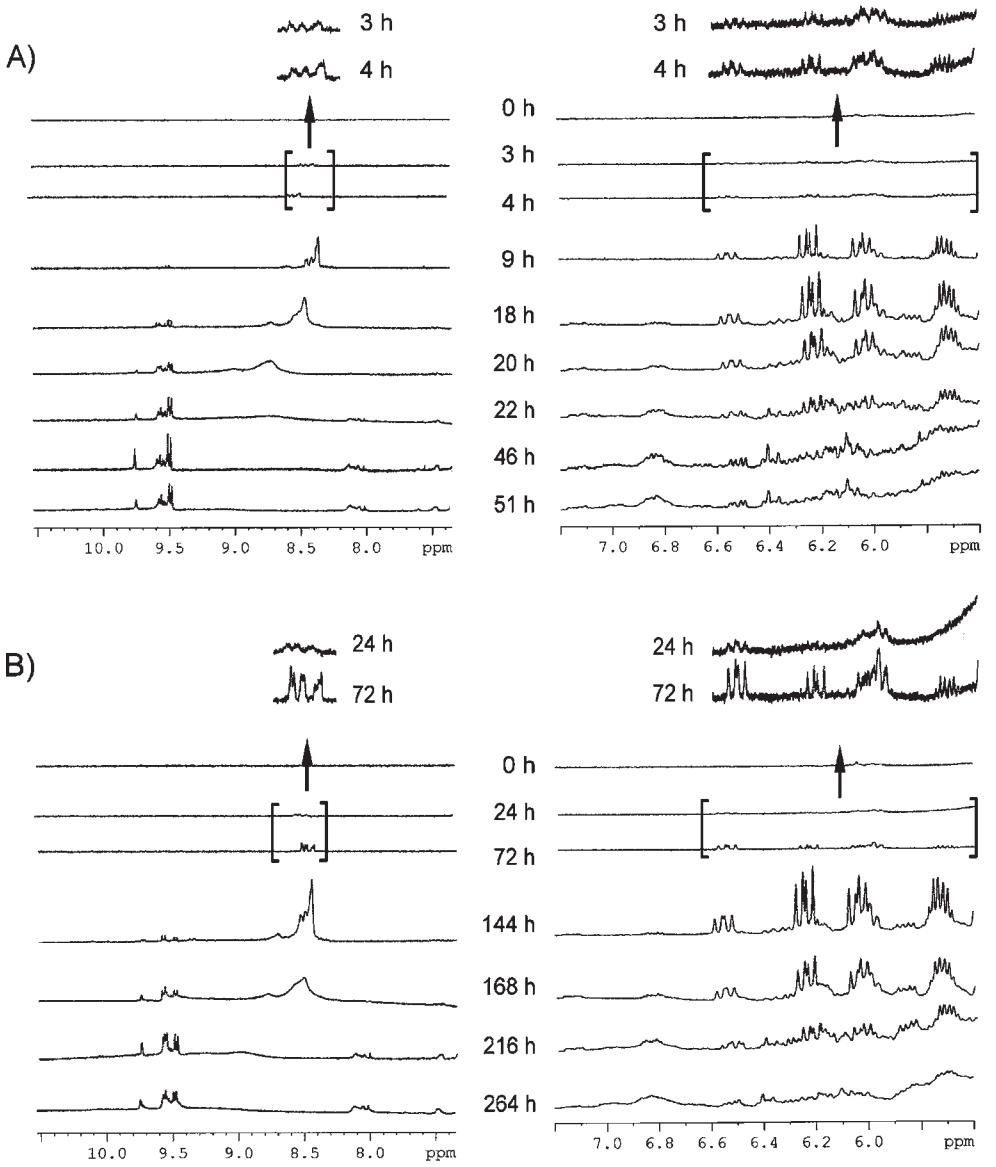

Figure 2. Expanded regions between 5.6 and 7.2 ppm and between 7.4 and 10.3 ppm of the ^{1}H NMR spectra of some sunflower oil samples: (**A**) kept at 100 °C with aeration during different periods of time indicated in hours; (**B**) kept at 70 °C with aeration, during different periods of time indicated in hours. See the corresponding chemical shift assignments in **Tables 2** and **3**.

components of this sunflower oil submitted to 70 °C until sample solidification (polymerization), with clear differences in timing (figures not shown).

It is well-known that during oil thermoxidation the linoleic acyl group chains may evolve in different ways such as breaking it up into two parts, one of small molecular weight and the other one remaining bonded to the triglyceride structure; being modified without breaking, by incorporation of oxygen atoms in their structure; or forming carbon−carbon bonds with other acyl group chains, modified or not, of the same or of different triglyceride molecule, giving rise to monounsaturated or saturated chains. The formation of dimers, oligomers or polymers is also possible by these means. The possibility of the formation of ether bridges between acyl group chains cannot be discarded (*21*). All these possibilities have as their consequence the destruction of the linoleic acyl group chains to form different kinds of structures. The same would be possible in the case of monounsaturated acyl group chains, which could evolve to give saturated acyl groups if carbon−carbon linkages are formed. The formation of dimers, oligomers, polymers, and of modified acyl group chains of the same length as the original acyl group, or shorter, can also occur by means of this mechanism. The formation of small molecules and of ether bridges with other unsaturated acyl groups may also be possible.

2. Primary Oxidation Compounds Formed. In relation to the changes mentioned above, the enlargement of some regions of the ^{1}H NMR spectra of the sunflower oil throughout the thermoxidative processes provides information on some of the compounds formed. Focusing attention on the spectral region between 5.5 and 10.0 ppm, it can be observed in **Figure 2** that, in both processes, the formation of primary oxidation compounds, having hydroperoxy groups and *Z,E* or *E,E* conjugated dienic systems supported on chains having hydroperoxy groups, occurs. These *Z,E* conjugated dienic systems give four signals in the spectrum whose chemical shifts are given in **Table 2**. Although previously published data indicated that *E,E* conjugated dienic systems supported on hydroperoxy acyl group chains only give two signals at 6.25 and 5.75 ppm (*22*), results recently published show that these systems give the four signals in the ^{1}H NMR spectra, as is indicated in **Table 2** (*17*).

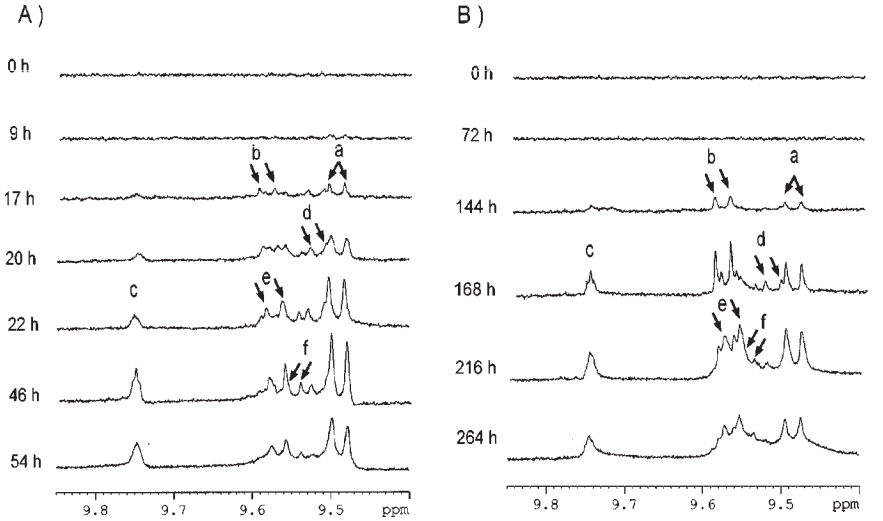

Figure 3. Enlargement of the region between 9.3 and 10.0 ppm of the ^{1}H NMR spectra of some of the sunflower oil samples submitted to thermo-oxidative conditions at 100 °C (**A**) and at 70 °C (**B**): (**a**) doublet signal of (*E*)-2-alkenals; (**b**) doublet signal of 4-hydroperoxy-(*E*)-2-alkenals; (**c**) triplet signal of *n*-alkanals; (**d**) doublet signal of (*E*,*E*)-2,4-alkadienals; (**e**) doublet signal of 4-hydroxy-(*E*)-2-alkenals; and (**f**) signal of 4,5-epoxy-(*E*)-2-alkenals. See the corresponding chemical shift assignments in **Table 3**.

Figure 2A shows the expanded ^{1}H NMR spectral region between 5.6 and 7.2 ppm and between 7.9 and 10.3 ppm of sunflower oil submitted to 100 °C with aeration. Thus at 0 h these regions are free of signals, but at 3 h a very small signal near 8.4 ppm due to the presence of hydroperoxides can be observed, and at the same time, incipient signals centered at 5.76, 6.00, 6.06, 6.27, and 6.58 ppm due to *E*,*E* and *Z*,*E* conjugated diene structures present in hydroperoxide chains are also observed; these signals show their highest intensity at 18−20 h, after which they decrease owing to the degradation of these intermediate compounds. In the process that takes place at 70 °C, hydroperoxides and their associated *E*,*E* and *Z*,*E* conjugated dienic systems are detected from 24 h onward showing their maximum intensity at 144 h and their decrease from then onward.

The differences between both processes in terms of primary oxidation compounds can be stated as follows: first, the timing at which the formation of primary oxidation compounds occurs; second, in the 70 °C process initially (from the beginning to 120 h), the formation of *Z*,*E* conjugated dienic groups is favored versus that of *E*,*E* and this trend changes from 130 h onward (see **Figure 2B**), whereas in the 100 °C process the formation of *E*,*E* conjugated dienic systems is favored versus that of *Z*,*E* throughout all the process (see **Figure 2A**). The spectra of both processes in the early degradation steps (from 0 to 120 h in the process at 70 °C; and from 0 to 12 h in the process at 100 °C) suggest that hydroperoxide signal near 8.46 ppm is associated with *E*,*E* dienes and those at 8.50 ppm and at 8.53 are associated with *Z*,*E* dienes.

This initial preferential formation of *Z*,*E* hydroperoxides versus *E*,*E* hydroperoxides in the 70 °C process is in agreement with what is observed in oxidation processes at room temperature (*18*), in which the formation of *E*,*E* conjugated dienic systems was only observed at advanced oxidation stages; this may be due to the higher activation energy required for the formation of *E*,*E* than for the formation of *Z*,*E* conjugated systems. From these results it could be concluded that the predominant reactions, and thus the global mechanisms, are different initially and for a certain period of time in the processes at 70 and 100 °C.

3. Secondary Oxidation Compounds Formed. As both processes evolve, in addition to signals of protons of primary oxidation compounds, signals of protons of some secondary oxidation compounds, such as aldehydes, also appear in the spectral region comprised between 5.5 ppm and 10.0 ppm (**Figures 2A** and **2B**); the enlargement of the spectral region between 9.4 and 9.8 in which the signal of the aldehydic protons appears is shown in **Figures 3A** and **3B**. The aldehydic functional groups formed in the oil thermoxidation processes can be supported either on small molecules and/or on triglycerides having truncated chains; these latter are included in the previously mentioned modified acyl group chains. The assignment of the signals in **Figures 2** and **3** to the different kinds of aldehydes are given in **Table 3**, in which the chemical shifts of all protons of these compounds are also indicated (*13*, *14*, *18*).

The same kinds of aldehydes are formed at 100 °C and at 70 °C, that is alkanals, (*E*)-2-alkenals, (*E*,*E*)-2,4-alkadienals, and the genotoxic and cytotoxic oxygenated alpha,beta-unsaturated aldehydes 4-hydroxy-(*E*)-2-alkenals, 4-hydroperoxy-(*E*)-2-alkenals and 4,5-epoxy-(*E*)-2-alkenals, all of them previously found in studies of oil degradation (*4*, *5*, *14*, *18*, *19*). A second similarity is that in both processes 4-hydroperoxy-(*E*)-2-alkenals are formed before 4-hydroxy-(*E*)-2-alkenals, the former showing initially higher concentration than the latter. However, as the processes evolve, the concentration of 4-hydroxy-(*E*)-2-alkenals becomes higher than that of 4-hydroperoxy-(*E*)-2-alkenals (after 20 h at 100 °C and after 192 h at 70 °C with aeration). These results are in agreement with other authors, which suggest that 4-hydroperoxy-(*E*)-2-alkenals are precursors of 4-hydroxy-(*E*)-2-alkenals (*23*). And finally, a similar relation is found between (*E*,*E*)-2,4-alkadienals and 4,5-epoxy-(*E*)-2-alkenals, because in both processes the results suggest that (*E*,*E*)-2,4-alkadienals are precursors of 4,5-epoxy-(*E*)-2-alkenals, in agreement with other authors (*24*). Differences found regarding quantitative data will be commented on later.

As mentioned in the Introduction, it has been postulated that epoxy derivatives from hydroperoxy derivatives are formed in the thermoxidation process. These epoxy groups could be derived not only from oleic but also from linoleic acyl groups; in addition, these latter could evolve to give not only mono- but also diepoxy derivatives. Some theoretical mechanisms proposed for the formation of epoxides involve the participation of hydroperoxy groups and of double bonds and entail the disappearance of both groups (*25*). The protons of the carbon atoms bonded to the epoxy group give signals in observable regions of the ^{1}H NMR

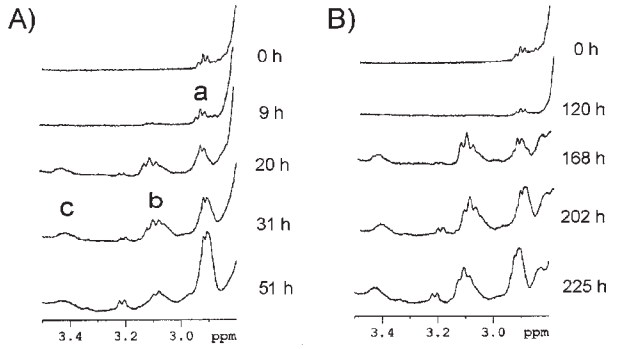

Figure 4. Expanded region between 2.8 and 3.5 ppm of the ^{1}H NMR spectra of sunflower oil submitted to thermo-oxidative conditions, at 100 °C (**A**) and at 70 °C (**B**), during different periods of time. In agreement with chemical shift assignments given in **Table 4**: (**a**) signal of protons belonging to mono- or/and to diepoxy acyl group derivatives; (**b**) signal of protons belonging to diepoxy acyl group derivatives; (**c**) signal tentatively assigned to protons of dihydroxy acyl group of derivatives.

spectrum, thus making their identification possible. **Table 4** gives some structures of epoxy derivatives which could be formed during the thermoxidation of sunflower oil maintained at 70 or 100 °C with aeration, together with the chemical shifts of some of their protons taken from the literature (*15*, *16*), which are coincident for *Z* and *E* epoxy groups. As can be observed in **Table 4**, the protons of the carbon atoms bonded to epoxy groups in monoepoxides derived from oleic or from linoleic groups give signals at 2.9 ppm (two protons of this type per mol); however, the protons of the carbon atoms bonded to epoxy groups in diepoxides derived from linoleic groups give signals at 2.9 ppm and at 3.1 ppm (two protons of each type per mol). **Figure 4** shows the signals assigned to epoxy group protons and their evolution in both processes.

Assuming that these signals belong exclusively to epoxides, the spectra of the sunflower oil throughout the thermoxidation at 70 and 100 °C indicate that initially and for a certain period of time under thermo-degradative conditions there are no signals either at 2.9 ppm or at 3.1 ppm (see **Figure 4**). However, from 120 h onward in the 70 °C process and from 9 h onward in the 100 °C process, signals at the above-mentioned ppm appear, which could be assigned to protons of epoxy groups (associated to the formation of epoxy acyl groups). The appearance of these signals is coincident in time with the appearance of the aldehydic signals, showing that both aldehydes and epoxy acyl groups are formed as soon as hydroperoxides degrade and are detected by this technique when they reach a certain concentration in the sample.

As far as we know this is the first time that these compounds have been detected by ^{1}H NMR in the liquid phase of thermodegraded oils. Other authors have only detected monoepoxides in several thermodegraded oils at frying temperatures using a quite laborious method based on derivatization of the epoxy acyl groups to methyl esters followed by GC/MS (*26*), and as far as we know there is only one paper in which mono- and diepoxy acyl groups were detected at the same time, using derivatization to form methyl esters followed by LC−GC/FID (*27*); in this latter case the vegetable origin of the oils and the degradation conditions were not specified.

A great variety of structures can be found among epoxy acyl groups (see **Table 4**); it is worth noting the monoepoxide 9,10-epoxy-12-octadecenoate, derived from linoleic acyl groups, whose hydrolysis gives leukotoxin, and its isomer the monoepoxide 12,13-epoxy-9-octadecenoate, also derived from linoleic acyl groups, whose hydrolysis gives isoleukotoxin. In recent years a great deal of attention has been paid to these two compounds, as a result of their biological activity (*10*). According to ^{1}H NMR data provided by other authors the two protons corresponding to the carbon atoms in positions 9 and 10 of this leukotoxin diol give a multiplet signal at 3.45 ppm (*15*); in our spectra a broad signal can be found there (see **Figure 4**) that appears simultaneously with that of epoxy groups, and that could be assigned to the dihydroxyleukotoxin derivative, as **Table 4** shows.

Furthermore, as can be seen in **Figure 2**, in the ^{1}H NMR spectra of more oxidized samples at 100 °C (from 22 h onward) and at 70 °C with aeration (from 168 h onward) two groups of unidentified signals centered near 7.46 ppm and near 8.1 ppm are present (see unidentified 1 and 2 signals in **Table 2**). The assignment of these signals is difficult, although the first one could be assigned to keto acyl groups, which will be studied in the future, and the second one could be due to hydroperoxy or hydroxy protons and includes the hydroperoxy group of 4-hydroperoxy-(*E*)-2-alkenals near 8.2 ppm, as **Table 3** indicates.

4. Quantitative Analysis of Acyl Groups and Iodine Value. As mentioned before, the study of these ^{1}H NMR spectra was used to monitor sunflower oil thermoxidation processes at 70 °C and at 100 °C, not only qualitatively but also quantitatively. It is known that the area (*A*) of these signals is directly proportional to the number of hydrogen atoms that generate them, and the proportionality constant is the same for all types of hydrogen atoms. For this reason, and taking into account the nature of the acyl groups in the sunflower oil triglycerides, several approaches have been described to determine the percentages of the different kinds of acyl group chains in the sunflower oil (*18*, *19*). The approach used here was developed considering that the percentage of free fatty acids present in the oil is very small, and the equations involved are the following (*19*):

$$\text{linoleic (\%)} = 100(2A_F/3A_G) \quad (1)$$

$$\text{oleic (monounsaturated) (\%)} = 100(A_D - 2A_F)/3A_G \text{ (in degraded oils)} \quad (2)$$

$$\text{saturated (saturated + modified) (\%)} = 100[1 - (A_D/3A_G)] \text{ (in degraded oils)} \quad (3)$$

The signals involved in these determinations are as follows: signal **F** due to bis-allylic protons (or protons in α position in relation to two double bonds); signal **D** due to allylic protons (or protons in α position in relation to one double bond); and signal **G** due to protons of CH_2 groups of the glyceryl structure. These equations permit the determination of the molar percentages of the several kinds of acyl groups (saturated, oleic and linoleic) of the original sunflower oil, as well as of the linoleic, monounsaturated and saturated plus modified acyl groups in sunflower oil having different degradation levels. These latter determinations were possible because the assignment of the signals **F**, **D** and **G** above is also valid in the thermo-oxidized oil. It should be mentioned that regarding monounsaturated epoxy acyl groups derived from linoleic groups, half of them are included in the percentage of monounsaturated acyl groups and the other half are included in the percentage of saturated plus modified acyl groups. This is so because the signals of the protons in α position in relation to both the double bond and the epoxy group in monounsaturated epoxy acyl groups appear at 2.3 ppm, overlapped with the signal of the protons in α position in relation to a carbonyl group (signal **E** at 2.23−2.36 ppm in **Figure 1** and **Tables 1** and **4**), instead of between 1.94 and 2.14 ppm, where the protons in α position to a double bond usually appear (signal **D** at 1.94−2.14 ppm in **Figure 1** and **Table 1**).

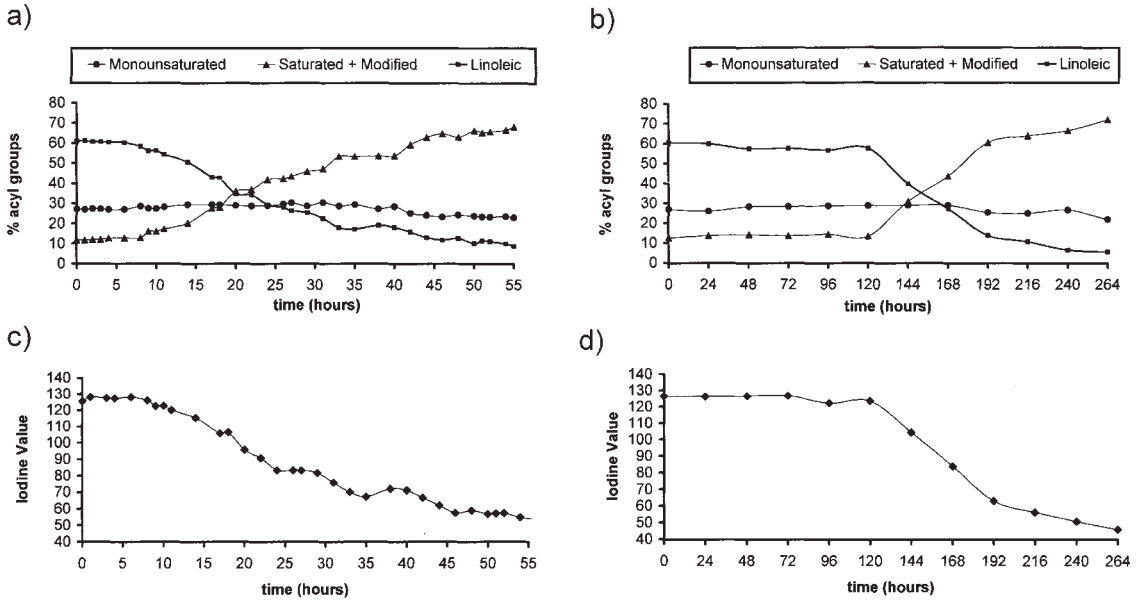

Figure 5. Molar proportions of acyl groups in sunflower oil throughout the oxidation process provoked at 100 °C (**a**) and at 70 °C (**b**), and evolution of the iodine values at 100 °C (**c**) and at 70 °C (**d**).

The data thus obtained were represented versus heating time in **Figures 5a** and **5b**. These figures show that in both processes the evolution of the molar percentage of the different kinds of acyl groups is as follows: first, there is a period of time in which the percentage of the different kinds of acyl group chains remains almost constant; second, there is a period of time in which the diminution of the molar percentage of linoleic and the increase of the molar percentage of saturated plus modified acyl group chains is very pronounced; and finally, there is a third period of time in which the molar percentages of the above-mentioned acyl group chains vary very slowly. The molar percentage of linoleic acyl group chains diminishes from near 61.0% to 5.8% and to 8.8% in the processes at 70 and 100 °C respectively; these decreases coincide with similar increases in the molar percentage of saturated plus modified acyl group chains. Nevertheless, only very small variations are found in the molar percentage of monounsaturated acyl groups; at first there is a very slight increase in this group, probably due to the conversion of linoleic acyl groups into monounsaturated acyl groups. Finally, there is a slight decrease probably due to their degradation into saturated or modified acyl groups.

The differences found in these processes, related to the evolution of acyl group chains, are first in the length of the period in which the percentages of linoleic and of saturated plus modified acyl group chains remain almost constant; this period of time could be considered as around 120 h at 70 °C and as around 6 h at 100 °C, showing a diminution in the length of this period of 3.8 h per each °C of increase in the temperature.

In addition, both processes also differ in the second period in terms of the rate at which the diminution of the molar percentage of linoleic or the increase of the molar percentage of saturated plus modified acyl group chains is produced. The equations that fit the percentages of these acyl group chains and the heating time in this second period of time for the 70 °C process, from 120 to 192 h of heating, are the following:

$$\text{linoleic (L)}\ \% = 128.294 - 0.60t\ \text{(hours)}, R = 0.997,\ n = 4 \quad (4)$$

$$\text{saturated} + \text{modified (S + Mo)}\ \% = -62.904 + 0.64t\ \text{(hours)}, R = 0.998,\ n = 4 \quad (5)$$

The same kind of equations can be obtained for the 100 °C process, from 6 to 35 h of heating:

$$\text{linoleic (L)}\ \% = 70.69 - 1.60t\ \text{(hours)}, R = 0.993,\ n = 17 \quad (6)$$

$$\text{saturated} + \text{modified (S + Mo)}\ \% = 1.77 + 1.53t\ \text{(hours)}, R = 0.992,\ n = 17 \quad (7)$$

These equations indicate that in the 70 °C process the rate of diminutions of the molar percentage of linoleic acyl groups is 0.60% per hour, a value very close to the rate of increase of the molar percentage of saturated plus modified acyl groups (0.64% per hour). At 100 °C these changes are produced at a higher rate; so the diminution rate of the molar percentage of linoleic acyl groups is 1.60% per hour, also very near to the increase rate of the molar percentage of saturated plus modified acyl groups (1.53% per hour). Differences between both processes are also observed in the third period, in which the molar percentages of the above-mentioned acyl group chains vary very slowly.

In a previous paper a very close relationship was found between iodine value (IV), determined by conventional wet methods, and the percentage of olefinic protons (OP) present in the oil determined from its area in the ^{1}H NMR spectra, in such a way that both sets of data were related by the equation IV = 10.54 + 13.39 OP (*19*). The percentage of olefinic protons in the oil is determined from the area of signal **I** in **Figure 1-I** and the area of total protons in each spectrum. Using this latter equation the iodine values of sunflower oil throughout both thermooxidative processes were determined, and they were represented versus heating time in **Figures 5c** and **5d**. It can be observed that the evolution of the iodine values in both processes agrees, basically, with that of linoleic acyl group chains, showing the same three periods mentioned above and having also the same rate of change (or slope of the fitted lines) (at 70 °C correlation coefficient = 0.997 and at 100 °C correlation coefficient = 0.998). The great concordance between IV and percentage of linoleic acyl group chains indicates that the disappearance of double bonds during these processes takes place mainly in linoleic acyl group chains, in agreement with the previous comments in relation to

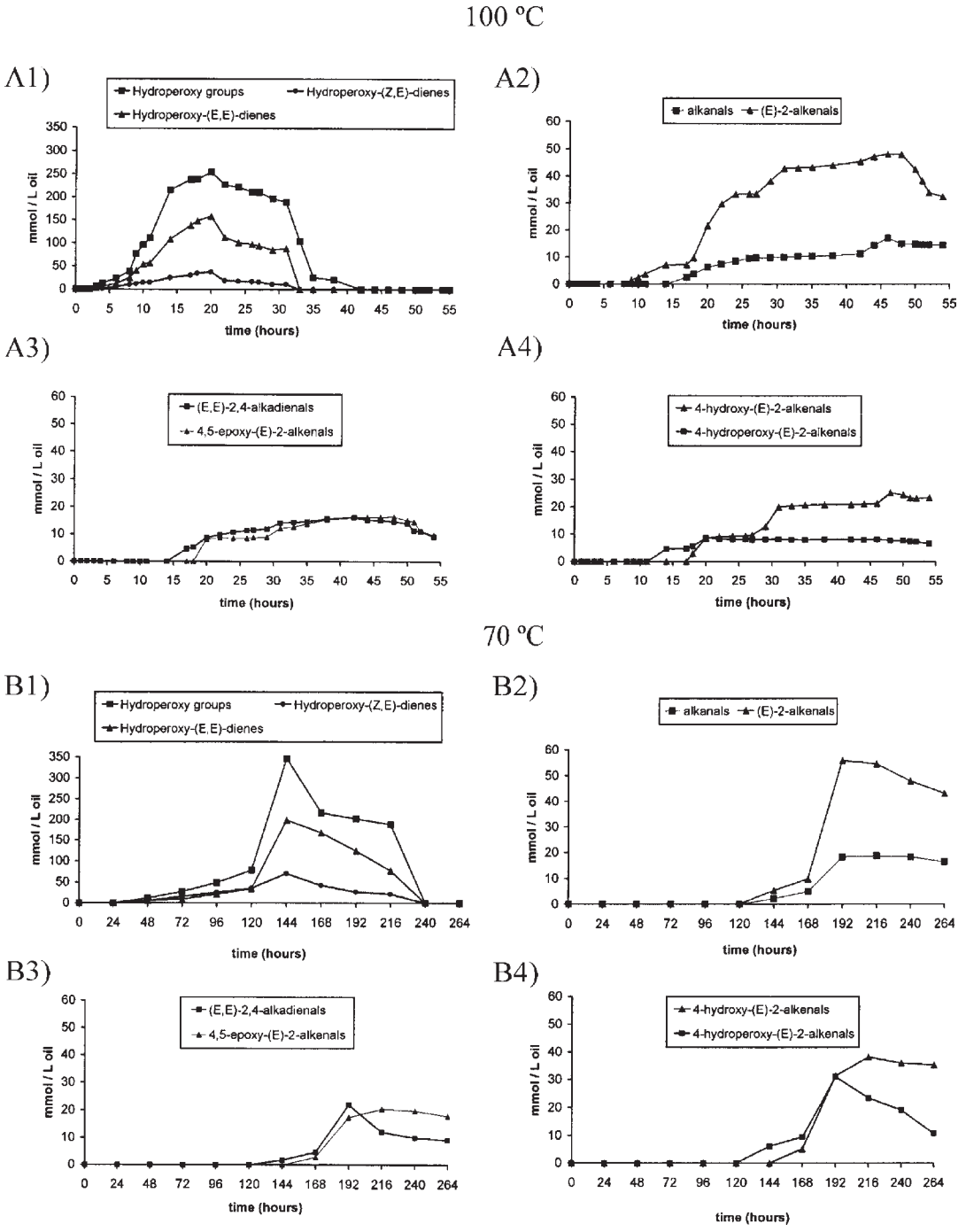

Figure 6. Concentrations (mmol/L oil), calculated from ^{1}H NMR data, of some primary (**A1**) and secondary (**A2**, **A3**, **A4**) oxidation compounds generated in sunflower oil submitted to 100 °C with aeration, and of some primary (**B1**) and secondary (**B2**, **B3**, **B4**) oxidation compounds generated in sunflower oil submitted to 70 °C with aeration.

Figure 1, and that the contribution of monounsaturated acyl group chains to the unsaturation degree of the oil, that is to the iodine value, seems to be fairly constant throughout these oxidation processes.

5. Quantification of Primary Oxidation Compounds. Regarding primary oxidation compounds, **Figure 6** shows the evolution of the concentration, given in mmol/L oil of hydroperoxides, and of *Z*,*E*- and of *E*,*E*-conjugated dienic systems supported on hydroperoxide acyl chains in sunflower oil throughout the degradation processes at 100 °C (see **Figure 6A1**) and at 70 °C (see **Figure 6B1**). These were determined as indicated in Materials and Methods. Similarities between both processes can be cited (see **Figure 6**, **A1**,**B1**): first, that the molar concentration of hydroperoxy groups is higher than that of the sum of *Z*,*E*- and *E*,*E*-conjugated dienic systems; second, that the highest concentration of hydroperoxy groups coincides with the maximum concentration in dienic conjugated systems supported on hydroperoxide acyl chains, in agreement with the above-mentioned; third, that when the concentration of these functional groups is maximum, the ratio between the concentration of hydroperoxy groups and the sum of the concentration of (*Z*,*E*)- plus (*E*,*E*)-conjugated dienic systems is of a similar order in both processes (near 1.3 in both processes); and fourth, that in both processes either there is more than one hydroperoxy group in chains supporting one conjugated dienic system or that some hydroperoxy groups are not in chains supporting conjugated dienic

systems, in agreement with pathways proposed previously by other authors (*23*, *28*, *29*), or both.

Nevertheless, as for the concentrations of primary oxidation compounds in both processes, not only similarities but also differences were found: first, in the 100 °C process the concentration not only of hydroperoxy but also of total conjugated dienic groups (maximum concentrations: total hydroperoxy groups 253.9 mmol/L oil, hydroperoxy-(*Z*,*E*)-conjugated dienic systems 36.9 mmol/L oil and hydroperoxy-(*E*,*E*)-conjugated dienic systems 157.8 mmol/L oil) is smaller than at 70 °C (maximum concentrations: hydroperoxy groups 346.8 mmol/L oil, hydroperoxy-(*Z*,*E*)-conjugated dienic systems 71.0 mmol/L oil and hydroperoxy-(*E*,*E*)-conjugated dienic systems 198.6 mmol/L oil); and second, the ratio between the concentration of hydroperoxy-(*E*,*E*)-conjugated dienic systems and of hydroperoxy-(*Z*,*E*)-conjugated dienic systems, in the maximum of concentration of primary oxidation compounds and onward, is much higher in the 100 °C process (4.3 when maximum concentration of primary compounds) than in the 70 °C process (2.8 when maximum concentration of primary compound).

From these results, it is evident that the increase of temperature from 70 to 100 °C does not favor the formation of hydroperoxides and dienic conjugated systems. In addition, it is also evident that although the formation of hydroperoxy-conjugated dienic systems, in general, is not favored by the increase in temperature, this increase favored the formation of hydroperoxy-(*E*,*E*)-conjugated dienic systems over that of hydroperoxy-(*Z*,*E*)-conjugated dienic systems.

6. Quantification of Secondary Oxidation Compounds. Regarding the secondary oxidation compounds, **Figure 6** gives the evolution of the concentration, determined from ^{1}H NMR spectral data, of the different kinds of aldehydes throughout both thermo-oxidative processes. Some differences are found in these processes; so, in the 100 °C process the detection of aldehydes is produced at lower concentration of hydroperoxides (77.2 mmol/L oil at 9 h) than in the 70 °C process (346.8 mmol/L oil at 144 h); it seems that the degradation of primary oxidation compounds is produced earlier at higher temperature or that hydroperoxides in the process at lower temperature have a longer average life. This may be one of the reasons for discrepancies when classical methods are used to evaluate the oxidation level of edible oils (*1*).

In addition, the rate of formation of different kinds of aldehydes is also a function of the temperature of the process. Thus, in the 100 °C process the first aldehydes detected were (*E*)-2-alkenals (**Figure 6A2**, detected at 9 h of thermal treatment, when the concentration of hydroperoxy groups was near 77.2 mmol/L oil) and 4-hydroperoxy-(*E*)-2-alkenals (**Figure 6A4**, detected at around 15 h of thermal treatment), followed by alkanals (**Figure 6A2**) and (*E*,*E*)-2,4-alkadienals (**Figure 6A3**), being 4-hydroxy-(*E*)-2-alkenals (**Figure 6A4** at 18 h) and 4,5-epoxy-(*E*)-2-alkenals (**Figure 6A3** at 18 h) the last detected; this latter period of time is shorter than the one required for the occurrence of the maximum concentration of hydroperoxides (**Figure 6A1** at 20 h). However, in the 70 °C process different types of aldehydes such as alkanals, (*E*)-2-alkenals (**Figure 6B2**), (*E*,*E*)-2,4-alkadienals (**Figure 6B3**) and 4-hydroperoxy-(*E*)-2-alkenals (**Figure 6B4**) were detected simultaneously (at 144 h of thermal treatment), whereas 4,5-epoxy-(*E*)-2-alkenals (**Figure 6B3** at 168 h) and 4-hydroxy-(*E*)-2-alkenals (**Figure 6B4** at 168 h) were detected later, coinciding with the peak concentration of hydroperoxides.

From these results it could be concluded that in the higher temperature process the reactions that lead to the formation of (*E*)-2-alkenals and 4-hydroperoxy-(*E*)-2-alkenals are favored against those that lead to the formation of the other types of aldehydes; however, at lower temperatures all types of aldehydes show similar rates of formation except for 4,5-epoxy-(*E*)-2-alkenals and 4-hydroxy-(*E*)-2-alkenals. Moreover, in both processes the sequence is the formation of 4,5-epoxy-(*E*)-2-alkenals and of 4-hydroxy-(*E*)-2-alkenals after (*E*,*E*)-2,4-alkadienals and 4-hydroperoxy-(*E*)-2-alkenals, respectively, together with the diminution in the concentration of the two latter types of aldehydes as the increase in the concentration of the two former is produced (see **Figure 6 A3**,**A4** and **B3**,**B4**). This is in agreement with the pathways of formation proposed by some authors (*23*, *24*), in which these latter are precursors of the former.

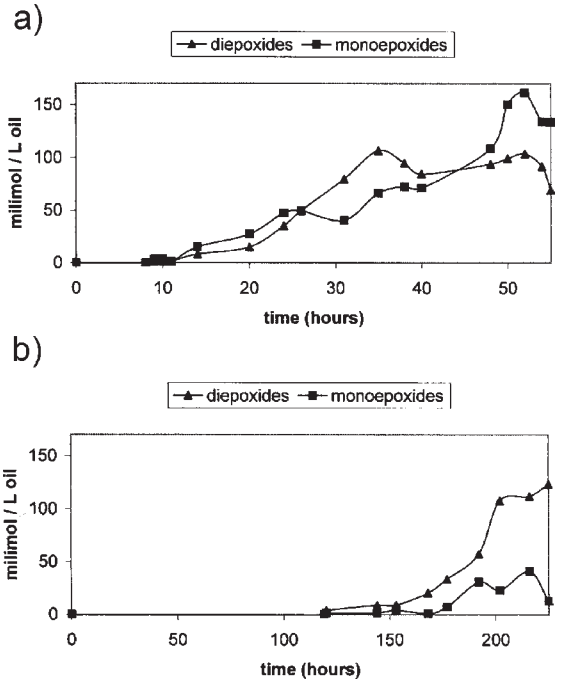

Figure 7. Concentrations (mmol/L oil), calculated from ^{1}H NMR data, of some mono- and diepoxy acyl groups present in sunflower oil throughout the thermo-oxidation process provoked at 100 °C with aeration (**a**) and at 70 °C with aeration (**b**).

Another difference between both processes lies in the maximum concentration found for the different types of aldehydes. Some of them, such as (*E*)-2-alkenals (maximum concentration: 48.1 mmol/L oil at 100 °C; 54.5 mmol/L oil at 70 °C) and alkanals (maximum concentration: 17.2 mmol/L oil at 100 °C; 18.7 mmol/L oil at 70 °C), reach a maximum concentration of similar order in both processes (see **Figure 6 A2**,**B2**). However, the other aldehydes reach lower concentrations in the 100 °C process than in the 70 °C process, this being specially significant in the case of the toxic oxygenated alpha,beta-unsaturated aldehydes 4-hydroperoxy-(*E*)-2-alkenals (maximum concentrations: 8.6 mmol/L oil at 100 °C; 31.0 mmol/L oil at 70 °C) and 4-hydroxy-(*E*)-2-alkenals, (maximum concentrations: 25.2 mmol/L oil at 100 °C; 38.1 mmol/L oil at 70 °C) (see **Figure 6 A4**,**B4**). This fact is in agreement with the higher concentration of primary oxidation compounds found in the oil submitted to 70 °C, indicating that although the 70 °C process is much slower, the amount of the toxic oxygenated alpha,beta-unsaturated aldehydes formed is much higher than at 100 °C.

Other secondary oxidation products are epoxides. In these, the evolution of the concentrations of mono- and diepoxy acyl groups has been determined, from the signals at 2.9 ppm and at 3.1 ppm. **Figure 7** gives this evolution showing that in both processes mono- and diepoxides are formed at the same time and are detected simultaneously with the first aldehydes. In spite of this, some differences in their quantitative evolution are also

found. In sunflower oil thermoxidation at 100 °C (see **Figure 7a**) monoepoxides show initially higher concentrations than diepoxides; however, from 26 h onward, and coinciding with the sharp diminution in the concentration of hydroperoxides (see **Figure 6A1**), diepoxides show higher concentrations than monoepoxides, the former reaching their maximum concentration (107 mmol/L oil) after 35 h of heating; from 45 h onward it is just the opposite, monoepoxides reaching their maximum concentration (161 mmol/L oil) after 52 h of heating.

In sunflower oil thermoxidation at 70 °C both kinds of epoxides are detected from 120 h onward (see **Figure 7b**), just the moment when the first aldehyde signals appear in the spectrum. In this process diepoxides always show higher concentrations than monoepoxides, their maximum concentration found being 123 mmol/L oil, which is of a similar order to the maximum concentration found in the 100 °C process. However, monoepoxides show much lower concentrations than diepoxides in this process and much lower also than in the 100 °C process, their maximum concentration being near 41 mmol/L oil.

These results indicate that the increase of the temperature in the oxidation process from 70 to 100 °C, in addition to the increase in the epoxide formation rate, favors the formation of monoepoxides and hinders that of diepoxides. The formation of these latter is much more favored at lower temperatures, at which the process is also much slower. Perhaps this is the reason why diepoxides were not detected in the few studies published on epoxides in oxidized edible oils, because the oils were submitted in all cases to frying temperatures (*26*). To the best of our knowledge this is the first time that the influence of temperature on the formation of epoxy acyl groups in edible oils groups is considered.

The great influence of the thermoxidation conditions on the mechanisms and reactions that take place in the edible oil degradation processes makes it difficult to compare the quantitative results obtained here with others published, which in all cases were obtained after chemical changes in the degraded oil subject of study.

In summary, as far as we know this is the first time that the progress of the thermoxidation process of sunflower oil is monitored, using ^{1}H NMR, through the quantitative simultaneous evaluation of primary and secondary oxidation compounds, such as hydroperoxides, *Z*,*E* and *E*,*E* conjugated dienic systems supported on hydroperoxy acyl groups, different kinds of aldehydes, and epoxy acyl groups including mono- and diepoxides. The influence of the increase of temperature from 70 to 100 °C on the evolution of the oxidation of sunflower oil has been shown. Differences regarding not only the rate of degradation of acyl groups and of formation of the several compounds but also the evolution of their concentrations could explain the inconsistent results obtained by some authors in the monitoring of oxidative stability with some accelerated stability methods, such as Schaal oven test (65 °C) and active oxygen method (AOM) (98 °C) (*30*). The results of this study evidenced that detailed information is needed for an accurate description of the oxidation level of edible oils, which cannot be properly obtained by classical methods. For the first time, the formation of diepoxides in oxidation processes of edible oils is reported. This study has shown that the level of toxic compounds reached by this edible oil in the oxidation process depends greatly on the degradation conditions; thus, O$\alpha\beta$UAs are generated in higher amounts in the sunflower oil degradation at lower temperatures, whereas toxic monoepoxy unsaturated acyl chains related to leukotoxin and isoleukotoxin are generated in higher amounts in the sunflower oil degradation at higher temperatures. Both kinds of toxic compounds can be formed not only endogenously in cells and tissues but also in degradation processes of edible oils, and could be absorbed through the diet. Taking this into account it seems evident that further research on edible oil oxidation could be very helpful to understand better the endogenous oxidation processes, as well as to the suitable use of edible oils in industrial and culinary treatments in order to safeguard human health.

ABBREVIATIONS USED

AOM, active oxygen method; ^{1}H NMR, ^{1}H nuclear magnetic resonance; O$\alpha\beta$UAs, oxygenated alpha,beta-unsaturated aldehydes; TMS, tetramethylsilane.

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