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Detection of Primary and Secondary Oxidation Products by Fourier Transform Infrared Spectroscopy (FTIR) and ^{1}H Nuclear Magnetic Resonance (NMR) in Sunflower Oil during Storage

MARÍA D. GUILLÉN* AND ENCARNACIÓN GOICOECHEA

Tecnología de Alimentos, Facultad de Farmacia, Universidad del Pais Vasco, Paseo de la Universidad Number 7, 01006 Vitoria, Spain

The oxidation of sunflower oil, stored in closed receptacles at room temperature for a period of 10 years, was monitored using Fourier transform infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (NMR). The objective was to understand the evolution of the oxidation process in sunflower oil under the conditions above mentioned. These techniques provide information about the oxidative status of several oil samples and the primary and some of the secondary oxidation products formed in the oxidation process. The results obtained show that, under these conditions, sunflower oxidation takes place in a different way to that at higher temperatures with aeration. The ^{1}H NMR spectra show that in the first oxidation stages of the process only hydroperoxides supporting *cis*,*trans*-conjugated double bonds are formed and that at more advanced stages hydroperoxides having *trans*,*trans*-conjugated double bonds are generated, with the latter always being in a smaller proportion than the former. In addition, the presence of hydroxy derivatives supporting *cis*,*trans*-conjugated double bonds among the primary oxidation compounds is shown for the first time. Also, from early oxidation stages onward and unlike the process at 70 °C with aeration, it is noticeable that 4-hydroxy-*trans*-2-alkenals are formed in much higher proportions than 4-hydroperoxy-*trans*-2-alkenals. This fact could be associated with the presence of hydroxy derivatives with *cis*,*trans*-conjugated double bonds among the primary oxidation products and the limited concentration of oxygen during the oxidation. Furthermore, relationships between some oxidation conditions and the oxidation level of the samples were statistically analyzed.

KEYWORDS: Fourier transform infrared spectroscopy; ^{1}H nuclear magnetic resonance; hydroperoxy and hydroxy dienic conjugated systems; 4-hydroperoxy-*trans*-2-alkenals; 4-hydroxy-*trans*-2-alkenals; oxidation process; principal component analysis; storage in closed receptacles; sunflower oil; room temperature

INTRODUCTION

The oxidation of edible oils and fats is a subject of concern both because this destructive process causes economic and nutritional losses and because it produces toxic compounds (*1–4*). Previous studies have shown the usefulness of spectroscopic techniques, including Fourier transform infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (^{1}H NMR), for the monitoring of oxidation of fats and oils (*5–14*). Using these techniques, the oxidation process provoked in oils of very different nature at 70 °C with aeration has been studied (*12, 14–19*). In these studies, these techniques have shown that under the oxidative conditions mentioned the process provokes the simultaneous generation of hydroperoxides having both *cis*,*trans*- and *trans*,*trans*-conjugated double bonds. The degradation of these primary oxidation products generates secondary oxidation products, including aldehydes. Under the oxidative conditions mentioned above, alkanals, *trans*-2-alkenals, *trans*,*trans*-2,4-alkadienals, 4,5-epoxy-*trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals, and 4-hydroperoxy-*trans*-2-alkenals are formed in different proportions depending upon the nature of the oil. Some of these geno- and cytotoxic oxygenated α,β-unsaturated aldehydes (O$\alpha\beta$UAs) are considered to be potential causal agents of degenerative diseases, such as cancer, atherosclerosis, and Alzheimer's, and can be absorbed through the diet (*20–29*).

It has been said that the oil oxidation process at room temperature is different from the accelerated process provoked at higher temperatures (*30*). However, not many studies have been published on edible oil oxidation processes at room temperature. This may be due to the slowness of this process (*31, 32*). Consequently, little is known about the primary and secondary oxidation products generated. For this reason, this paper reports the study by FTIR and ^{1}H NMR of the oxidation level of a significant number of samples of different

* To whom correspondence should be addressed. Telephone: 34-945-013081. Fax: 34-945-013014. E-mail: knpgulod@vf.ehu.es.

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sunflower oils. These had been stored at room temperature in closed receptacles, in the presence of limited amounts of air, over different periods of time. The aim of the study is to gain knowledge about this slow oxidation process in sunflower oil as well as the primary and secondary oxidation products formed. To the best of our knowledge, the oxidation of edible oils under these conditions has not been studied before by these methods.

MATERIALS AND METHODS

Samples and Oxidation Conditions. The study was carried out on 27 sunflower oil samples, acquired from local supermarkets over a period of 10 years, and stored at room temperature for different periods of time, under different air/oil volume ratios and different air/oil contact surfaces. The containers were maintained in the interior of a cupboard, receiving only a very weak ambient light; the room temperature was between 20 and 25 °C. In this group of samples, there were sunflower oils of the same brand and batch stored with different air/oil volume ratios and/or air/oil contact surfaces, oils of the same brand but from a different batch acquired 1 or more years later, and oils of different brands.

The compositions of these oils, when they were acquired, followed the legal requirements of the European Union for edible sunflower oils. Their acyl group proportions ranged between 12 and 13% by weight of saturated acyl groups, between 22 and 27% by weight of oleic acyl groups, and between 60 and 63% by weight of linoleic acyl groups; it should be taken into account that nowadays the sunflower oils on sale in the supermarkets are made of mixtures of oils coming from very different places in the world.

Under the above storage conditions, the sunflower oil samples underwent an oxidation process. **Table 1** gives the samples named from S1 to S27 (in order of successively higher oxidation stages) and storage conditions, such as storage time (ST), air/oil contact surface (CS), air volume (AV), oil volume (OV), air/oil volume ratio (AOVR), and the nature and concentration of the added antioxidants indicated in the label by the producers.

Furthermore, for comparative purposes, as in previous studies (*12, 13, 15, 18*), 10 g of a sunflower oil, acquired in a local supermarket, were weighed in crystal Petri dishes of 80 mm in diameter and 15 mm high and placed in a *Selecta* convection oven, with circulating air, whose temperature was maintained at 70 °C with a stability of ±0.5%. The Petri dishes were introduced into the oven without their lids to facilitate the exposure of the sample to the circulating air. This degradation process was carried out in duplicate and monitored daily by FTIR.

Infrared Spectra Acquisition. The infrared spectra were recorded on a FTIR Bruker Vector 33 (Bruker Optic Gmbh) interfaced to a personal computer operating under Opus NT software (version 2.0). As in previous studies (*12*), a film of a small amount of sample (approximately 2 μL) was deposited between two disks of KBr, avoiding the presence of air, and screwing the screws of the sample holder as far as possible so that the path length was constant for all of the samples. All spectra were recorded from 4000 to 500 cm^{-1}, with a resolution of 4 cm^{-1}. For each spectrum, 32 interferograms were co-added before Fourier transformation and zero-filled to give a data point spacing of approximately 1.9 cm^{-1}. The measurement accuracy in the frequency data is better than 0.01 cm^{-1} because of the laser He–Ne internal reference of the instrument. The frequency value for each band was obtained automatically by the equipment software. The assignment of the bands to the specific functional group vibration mode was made by a comparison to previous studies of edible fats and oils (*12*). All figures of FTIR spectra or expanded FTIR spectra regions were plotted at a fixed value of absolute intensity to be valid for comparative purposes.

^{1}H NMR Spectra Acquisition. The ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. Each oil sample, weighing 0.2 g, was mixed with 400 μL of deuterated chloroform and a small proportion of tetramethylsilane (TMS) as an internal reference; 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 and 54 s. The experiment was carried out at 25 °C. The assignment of the signals was made as in previous studies (*14*). All figures of ^{1}H NMR spectra or expanded ^{1}H NMR spectra regions were plotted at a fixed value of absolute intensity to be valid for comparative purposes. The relative molar proportions of hydroperoxides and aldehydes in each sample were determined from these spectra by the integration of the signals between 8.4 and 8.9 ppm and between 9.4 and 9.8 ppm, respectively; these determinations were carried out assigning the unity to the area of the chloroform protons, which has the same concentration in all ^{1}H NMR experiments. This determination is possible because the area of the ^{1}H NMR signal is proportional to the number of protons, which generates the corresponding signal. The values obtained are given in **Table 1**.

Table 1. Some of the Oxidation Conditions during Storage, such as Storage Time (ST), Air/Oil Contact Surface (CS), Air Volume (AV), Oil Volume (OV), Air/Oil Volume Ratio (AOVR), and Added Antioxidants, Together with the Relative Molar Proportions of Hydroperoxides (HY) and Aldehydes (AL) Present in the Sunflower Oil Samples

sample	ST (months)	CS (cm^2)	AV (cm^3)	OV (cm^3)	AOVR	added antioxidants	HY	AL
S1	40	11.3	4.1	828.6	0.005		0.065	0.000
S2	63	38.5	100.5	769.7	0.131		0.134	0.000
S3	90	36.3	118.8	1700.3	0.070		0.257	0.000
S4	4	15.2	10.9	860.1	0.013	αT[a]	0.096	0.000
S5	4	15.2	10.9	898.6	0.012		0.075	0.000
S6	4	15.2	10.9	898.6	0.012		0.075	0.000
S7	63	32.2	45.3	729.6	0.062		0.270	0.000
S8	64	38.5	254.5	615.7	0.413	αT[a]	0.316	0.000
S9	40	15.2	10.9	840.8	0.013	αT[a]	0.152	0.000
S10	53	34.2	34.2	581.6	0.059		0.195	0.000
S11	4	15.2	10.9	860.1	0.013		0.250	0.000
S12	53	38.5	119.8	731.2	0.164	αT[a]	0.273	0.000
S13	112	69.4	328.7	1492.4	0.220	BHT[b] plus BHA[c]	0.372	0.000
S14	6	15.2	10.9	860.1	0.013	αT[a]	0.313	0.000
S15	71	38.5	312.2	596.5	0.523	αT[a]	0.296	0.000
S16	105	38.5	466.1	423.3	1.101	αT[a]	0.697	0.000
S17	90	38.5	697.0	192.4	3.622	αT[a]	0.782	0.170
S18	106	12.6	12.6	62.8	0.200		1.391	0.226
S19	77	38.5	754.8	134.7	5.604	αT[a]	1.854	0.287
S20	101	12.6	6.3	69.2	0.091	αT[a]	2.802	0.338
S21	112	12.6	50.3	25.1	2.000		2.446	0.487
S22	112	12.6	37.7	37.7	1.000	BHT[b] plus BHA[c]	2.044	0.779
S23	106	12.6	37.7	37.7	1.000		2.091	0.751
S24	112	12.6	56.5	18.8	3.000	BHT[b] plus BHA[c]	1.138	0.694
S25	112	12.6	56.5	18.8	3.000	αT[a]	1.013	0.695
S26	112	12.6	56.5	18.8	3.000		0.965	0.762
S27	106	12.6	62.8	12.6	5.000		0.322	1.489

[a] α-Tocopherol (αT) concentration ranges from 600 to 720 mg/kg of oil. [b] 2,6-Ditert-butyl-4-hydroxytoluene (BHT) concentration ranges from 25 to 75 mg/kg of oil. [c] 2(3)-Tert-butyl-4-hydroxyanisole (BHA) concentration ranges from 125 to 175 mg/kg of oil.

Statistical Study. First, a preliminary principal component analysis of seven variables in **Table 1** was performed. Furthermore, the principal component analysis of some of the variables associated with oxidation conditions (OC), such as storage time (ST), air/oil contact surface (CS), air volume (AV), oil volume (OV), and air/oil volume ratio (AOVR), was performed; likewise, the principal component analysis of the variables associated with the oxidation level (OL) reached by the oil samples, such as relative proportions of hydroperoxydes (HY) and aldehydes (AL) determined from ^{1}H NMR spectral data, was also carried out. Multiple linear regression between the principal component of the variables associated with the oxidation level (OL) and the two principal components of the variables associated with the oxidation conditions (OC) was also performed. These studies were carried out using the statistical package SPSS, Inc. (Chicago, IL).

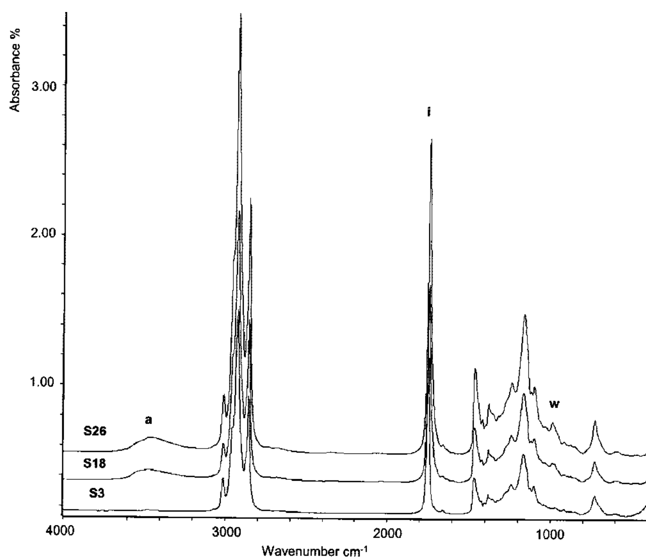

Figure 1. Fourier transform infrared spectra of the sunflower oil samples S3, S18, and S26 stored at room temperature.

RESULTS AND DISCUSSION

The detailed observation of the infrared spectra of the 27 sunflower oil samples reveals that many of them are at different oxidation stages and that they cover a broad range of oxidation states. **Figure 1** gives the infrared spectra of three sunflower oil samples (samples S3, S18, and S26), each having a different oxidation level, as a simple observation of the bands near 3471 cm^{-1} (band "a") and near 988 cm^{-1} (band "w") shows.

As mentioned in previous papers (*12, 13, 15, 16*), a great deal of information about the oxidative status of an oil can be provided by the frequency and absorbance values of the bands of the infrared spectra. Of these, bands "a", "i", and "w" are of special significance. It was previously shown that during the oxidation of sunflower oil submitted to 70 °C with aeration, the band near 3471 cm^{-1} (band "a") (see **Figure 2A**), assigned in non-oxidized oils to the overtone of the glyceride ester carbonyl group, widens and intensifies as the oxidation degree of the oil increases. This is due to the overlapping of the original band with new absorptions caused by hydroperoxides generated in the oxidation process, and at more advanced oxidation stages, this band shows a shoulder near 3530 cm^{-1} associated with the presence of hydroxy groups (see day 6 in **Figure 2A**) (*12, 15*). Furthermore, it was observed that the frequency value of this band "a" in non-oxidized oils is near 3471 cm^{-1}, but the presence of hydroperoxides lowers this frequency value toward smaller values. As the oxidation process advances, the degradation of hydroperoxides occurs, giving secondary oxidation products, some of which contain hydroxy groups; the appearance of the latter shifts the frequency value of this band back to near the original (see **Figure 2C**) (*15*). This evolution of the band "a" throughout the oxidation process was observed in all edible oils studied at 70 °C with aeration, with only small variations related to the specific nature of each type of oil (*12, 15*).

Among the 27 sunflower oil samples studied here, it has been found that the Fourier transform infrared spectra of samples from S1 to S17 show a band "a" typical of an oil with little or no concentrations of hydroperoxides. This can be observed in **Figure 2B** in samples S4 and S15. However, from samples S17 to S20 this band becomes wider and more intense, showing a shoulder near 3530 cm^{-1}, indicating a progressive increase in oxidation, which is more pronounced from sample S21 onward, as can be observed in **Figure 2B**. In addition, **Figure 2D** shows the frequency values of this band in the infrared spectra of the different sunflower oil samples. It is evident that the values of the frequency of this band in spectra of samples from S1 to S9 are close to the originals, decreasing slowly from samples S10 to S17 and more sharply from this latter sample to S20. This may be due to the presence of hydroperoxides, in agreement with that observed in **Figure 2B**. Finally, the frequency value of this band "a" shifts back near the original one from the S21 sample onward. This may be due to the progressive degradation of hydroperoxides and/or the progressive generation of products with absorptions near 3530 cm^{-1}, also in agreement with that observed in **Figure 2B**.

In general, the evolution of band "a" of the infrared spectra of the sunflower oils studied, in order of increasing oxidation level, is in agreement with the evolution of the same band of sunflower oil submitted to more extreme oxidative conditions (70 °C with aeration), as **Figure 2** shows. However, some differences are also observed. One is that the relative intensity reached by the band "a" in this latter oxidative process is much higher than that reached by any of the samples of sunflower oil oxidized at room temperature in closed receptacles (see parts **A** and **B** of **Figure 2**). This could indicate that the concentration of hydroperoxides in the oil samples in the first case (70 °C with aeration) is higher than in the second (room temperature and closed containers). Furthermore, the lowest frequency value reached by band "a" (near 3464 cm^{-1}, as can be seen in **Figure 2D**) in the spectra of sunflower oil samples stored at room temperature is never as low as that reached (near 3430 cm^{-1}) by sunflower oil submitted to 70 °C with aeration (see **Figure 2C**) (*12, 15*). These facts could indicate that in the oxidation process at room temperature in the presence of limited amounts of air the generation of hydroperoxides is less than under stronger oxidative conditions. Alternatively, it may indicate that the hydroperoxides formed do not accumulate in such great amounts. It is also possible that both of these occur. In addition, in the sunflower oil samples studied here, the appearance of the shoulder near 3530 cm^{-1} is observed almost simultaneously with the widening of the band "a" (see **Figure 2B**), indicating either the very rapid degradation of hydroperoxy derivatives to give secondary oxidation compounds having hydroxy groups or the presence of primary oxidation compounds having hydroxy groups or both.

The different oxidation degrees of several sunflower oil samples are also shown by the frequency values of the band near 1746.4 cm^{-1}, named band "i". This band in non-oxidized oils is solely due to the ester carbonyl functional group of triglycerides. In oxidized oils, it overlaps with that of aldehydes, causing a decrease in the frequency value of the resulting band in relation to the first (see **Figure 3A**) (*12, 15*); this decrease is higher when the oxidation degree of the sample is more advanced. As can be observed in **Figure 3B**, the frequency value of the band "i" varies between 1746.4 and 1746.2 cm^{-1} from samples S1 to S17, indicating the absence or very slight presence of aldehydes. However, from samples S19 to S27, the frequency of this band decreases to reach values near 1745.2 cm^{-1}, because of an increase in the concentration of aldehydes as a result of the higher oxidation degree of these latter oil samples. However, in none of these samples does the frequency of this band reach such low values as it does in the oxidation of sunflower oil at 70 °C with aeration (near 1744 cm^{-1}, see **Figure 3A**) (*12, 15*).

It is known that *trans*,*trans*-conjugated dienic systems absorb in the infrared spectrum near 988 cm^{-1} and *cis*,*trans*-conjugated dienic systems absorb near 988 and 950 cm^{-1}, with this second

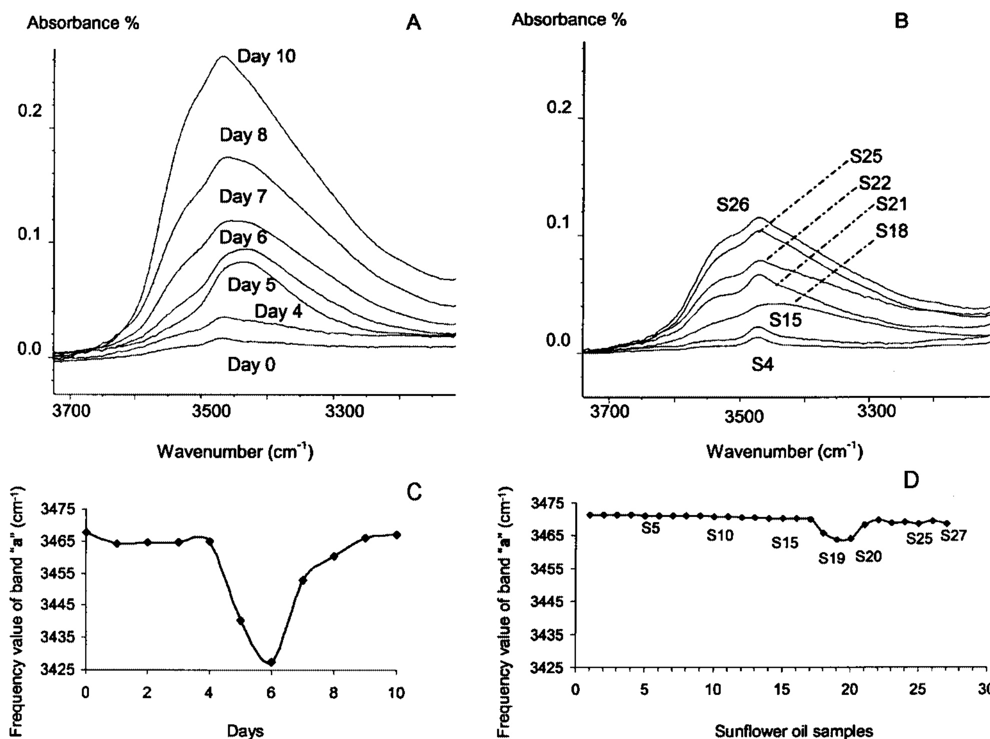

Figure 2. Region between 3360 and 3560 cm^{-1} (band "a") of the Fourier transform infrared spectra of (A) sunflower oil submitted to 70 °C with aeration and (B) some sunflower oil samples stored at room temperature in closed receptacles. Frequency values of the band near 3470 cm^{-1} ("a") of (C) sunflower oil submitted to 70 °C with aeration and (D) several sunflower oil samples stored at room temperature in closed receptacles.

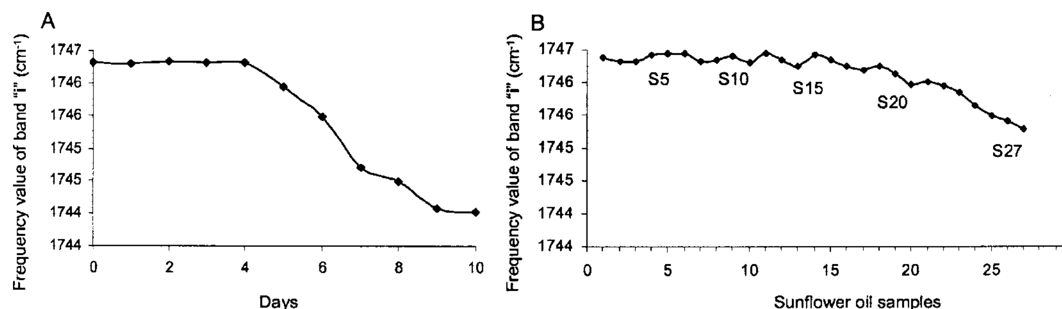

Figure 3. Frequency values of the band near 1746 cm^{-1} ("i") of (A) sunflower oil submitted to 70 °C with aeration and (B) several sunflower oil samples stored at room temperature in closed receptacles.

absorption being less intense than the first (*33–35*). The Fourier transform infrared spectra of edible oils show a band "w" near 988 cm^{-1}. This may indicate the presence of *trans*,*trans*- and/or of *cis*,*trans*-conjugated double bonds associated with primary oxidation products. A second absorption of *cis*,*trans*-conjugated double bonds near 950 cm^{-1} is not observed in oxidized sunflower oil infrared spectra, probably because of its overlapping with the band near 967 cm^{-1} of the isolated *trans*-olefins (*12, 15*). As shown in previous studies, the band at 988 cm^{-1} is either not appreciable or is totally absent in the spectra of non-oxidized sunflower oils. When these are submitted to 70 °C with aeration, this band "w" appears at the same time as the band of hydroperoxide groups (see day 4 in **Figure 4A**). It reaches its highest intensity when the frequency of band "a" shows the lowest value (day 6). It disappears from the spectrum when the frequency value of band "a" returns to around its original value (days 9–10), because of both the progressive degradation of hydroperoxides and the growing presence of significant concentrations of secondary oxidation products with hydroxy groups (*12, 13, 15*). **Figure 4B** shows band "w" of the infrared spectra of sunflower oils stored at room temperature in closed containers. It is absent in the samples from S1 to S15, appearing with an increasing intensity in the spectra of samples from S18 to S26. This indicates the presence in these latter samples of *trans*,*trans*- and/or *cis*,*trans*-conjugated double bonds associated with primary oxidation compounds; however, in this case, the band "w" does not disappear when band "a" returns to near its original frequency value (see from S21 onward, **Figures 2D** and **4B**). These results indicate that the oxidative conditions under which these oxidation processes are produced influence the reactions, rate, and mechanisms involved in each process.

To summarize, the Fourier transform infrared spectra of sunflower oils stored at room temperature in the presence of a limited amount of air provide information about the oxidation degree reached by these oil samples. These data provide fragments of the oxidation process at room temperature that could be used to reconstruct in an approximate way how this oxidative process takes place. However, it is not easy to find direct relationships between the oxidation level reached by each sample expressed with FTIR spectral data and only one of the storage conditions given in **Table 1**; it is evident that the oxidation level reached by each sample depends upon the combination of several factors. Among these factors, those included in **Table 1** are important, in addition to their composi-

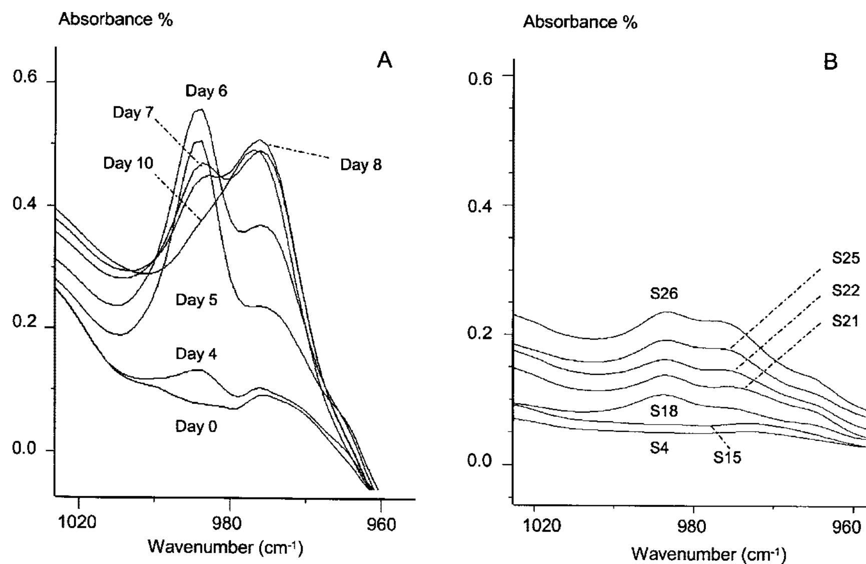

Figure 4. Region between 950 and 1020 cm^{-1} (band "w") of the Fourier transform infrared spectra of (A) sunflower oil submitted to 70 °C with aeration and (B) some sunflower oil samples stored at room temperature in closed receptacles.

tions in main and minor components. Because of the different storage times, different air/oil volume ratios and/or air/oil contact surface, different composition in main and minor components, as well as different content and nature of added antioxidants, the oxidation degree reached by each sunflower oil sample varies greatly from non-oxidized oils to oils in very advanced oxidation stages, covering a broad range of oxidative states. Their FTIR study shows that primary oxidation products, containing *cis*, *trans*- and/or *trans*,*trans*-conjugated double bonds, are formed in this oxidation process, although in smaller proportions than in the oxidation process provoked at higher temperatures with aeration. They may be of a different nature because the frequency of the infrared band "a" does not reach such low values as in the above-mentioned oxidation process. Furthermore, it is worth noting that the shoulder near 3530 cm^{-1} in band "a" is present in the spectrum of samples, which are not at very advanced oxidation stages, and that band "w" continues to be present in the most oxidized samples, in comparison to the oxidation process at 70 °C with aeration (see **Figures 2** and **4**). These differences shown by FTIR spectra prove that the oxidation process at room temperature in the presence of a limited amount of air evolves differently to that provoked at 70 °C with aeration.

In addition to the Fourier transform infrared spectroscopic study, ^{1}H NMR spectra of the same 27 sunflower oil samples were acquired. **Figure 5A** shows the spectral region comprised between 0 and 5.5 ppm, of three samples (S3, S18, and S26) chosen as representative of the evolution of the sunflower oxidation process at room temperature; the observation of this figure indicates that these three samples are in different oxidation stages, as shown by the intensity of the signals of bis-allylic (signal G) or allylic (signal E) protons compared to the intensity of the signals of methylenic protons in α (signal F) or β (signal D) positions in relation to the carbonyl group (*17*). These differences of intensity are due to the degradation of significant proportions of unsaturated acyl groups in those samples having a higher oxidation degree.

Parts **B** and **C** of **Figure 5** show the ^{1}H NMR spectral region between 5.5 and 7.2 ppm and between 7.9 and 10.0 ppm, of some sunflower oil samples, enlarged properly. In sample S6, almost undetectable signals of hydroperoxyde protons and conjugated dienic systems are observed. However, from samples S8 to S17, the presence of two clearly defined signals of protons of hydroperoxides, between 8.4 and 8.6 ppm (**Figure 5C**), are observable, in increasing intensity from samples S8 to S17. In addition, two multiplet signals of *cis*,*trans*-conjugated dienic protons, also of increasing intensity, centered near 6.0 and 6.5 ppm approximately (**Figure 5B**), can be seen. These results show that ^{1}H NMR detects the presence of small proportions of hydroperoxides more clearly than FTIR. This latter method only indicates very small changes in the frequency of band "a", as observed in **Figure 2B** from samples S8 to S17.

It is worth noting the difference between the nature of the hydroperoxides present in the sunflower oil samples at early stages of oxidation at room temperature and the nature of those produced in the early stages of the oxidation of sunflower oil submitted to higher temperatures with aeration (*19*). In the first process, only *cis*,*trans*-conjugated hydroperoxides are generated, while in the second process, *cis*,*trans*- and *trans*,*trans*-conjugated hydroperoxides in similar proportions are generated from the beginning of the process. This indicates that the intermediate compounds through which both processes evolve are different at these early stages.

It is also noteworthy that in the spectral region comprised between 5.5 and 7.2 ppm, from sample S17 onward, two multiplet signals near 6.48 and 5.98 ppm, respectively, are observed. These are assignable to the hydrogen atoms of *cis*,*trans*-conjugated double bonds of hydroxy primary intermediate derivatives, in agreement with data provided by other authors (*36*). The signal of the hydroxy proton of these primary intermediate compounds is difficult to assign because it can appear in very different spectral regions. However, from sample S17 onward, several signals between 8.0 and 8.2 ppm that could be tentatively assigned to protons of hydroperoxy or hydroxy groups appear. The concentration of these latter intermediate derivatives increases with the oxidation degree of the sample but is always smaller than that of hydroperoxides bonded to *cis*,*trans*-conjugated systems. As far as we know, only one previous paper has reported the formation, in sunflower oil after prolonged storage, of *cis*,*trans*-conjugated diene primary oxidation products having hydroxy groups (*37*). The formation of these compounds could be related to the limited amount of oxygen in contact with the oil in this process in comparison to the process at 70 °C with aeration, in which only hydroperoxides were detected. The formation of hydroxy primary oxidation compounds in endogenous oxidation processes of lipids, detected

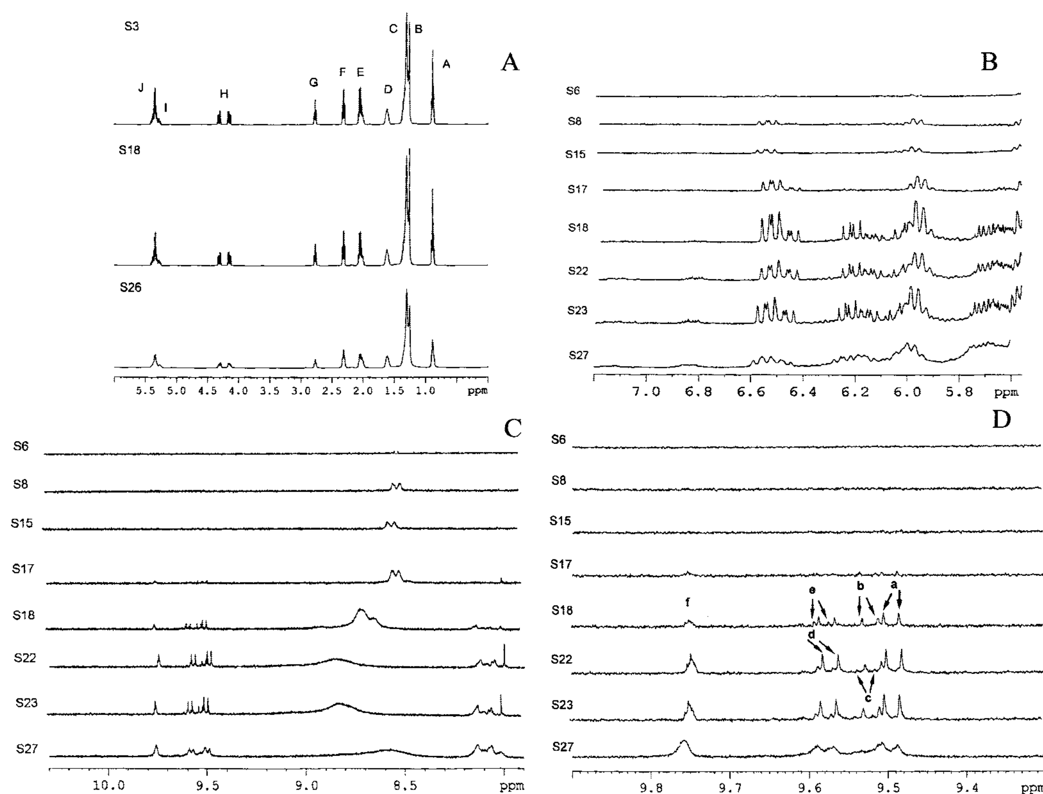

Figure 5. (A) ^{1}H NMR spectra of S3, S18, and S26 sunflower oil samples. Expanded regions of the ^{1}H NMR spectra of some of the sunflower oil samples stored at room temperature in closed receptacles: (B) region between 5.7 and 7.2 ppm and (C) region between 7.9 and 10.3 ppm. D) Enlargement of the region between 9.3 and 10.0 ppm of the ^{1}H NMR spectra of some of the sunflower oil samples stored at room temperature in closed receptacles: (a) doublet signal of *trans*-2-alkenals, (b) doublet signal of *trans*,*trans*-2,4-alkadienals, (c) signal attributable to 4,5-epoxy-*trans*-2-alkenals, (d) doublet signal of 4-hydroxy-*trans*-2-alkenal, (e) doublet signal of 4-hydroperoxy-*trans*-2-alkenals, and (f) triplet signal of *n*-alkanals.

in urine and serum (*38, 39*), is known. It is considered as a clinical marker of lipid peroxidation and oxidative stress.

Furthermore, in the ^{1}H NMR spectra from sample S17 (see **Figure 5B**) onward two additional multiplet signals also appear, centered near 6.2 and 5.7 ppm, corresponding to hydroperoxides having *trans*,*trans*-conjugated double bonds. Their intensity increases throughout the process, being in the most oxidized samples of a smaller order to that of the hydroperoxy derivatives with *cis*,*trans*-conjugated systems.

The results obtained from the ^{1}H NMR data coincide with those obtained from FTIR spectral data. Nevertheless, the first technique can be said to be more sensitive when detecting small changes produced by oxidation. In addition, it provides detailed information on the nature of some of the intermediate compounds generated.

In addition to primary oxidation products, some of the samples contain secondary oxidation products, such as aldehydes (see parts **C** and **D** of **Figure 5**). The ^{1}H NMR spectra of the samples from S1 to S16 do not show signals of aldehydes. However, the spectrum of sample S17 shows incipient signals of these secondary oxidation products between 9.4 and 9.8 ppm. Samples from S18 onward show very clear signals of aldehydes. In the ^{1}H NMR spectra of the samples at their most advanced oxidation stages, these signals undergo a broadening produced by the high viscosity of the oils caused by the polymers formed in the oxidation process (see spectrum of sample S27 in **Figure 5D**). It could be said that the presence of aldehydes in the oxidized samples is detected simultaneously with that of hydroperoxides supporting *trans*,*trans*-conjugated double bonds and that of hydroxy derivatives having *cis*,*trans*-conjugated double bonds. The different kinds of aldehydes formed, as **Figure 5D** shows, are alkanals giving a triplet signal (signal f) centered at 9.748 ppm, *trans*-2-alkenals having a doublet signal (signal a) centered at 9.491 ppm, *trans*,*trans*-2,4-alkadienals exhibiting a doublet signal (signal b) centered at 9.527 ppm, the genotoxic and cytotoxic 4-hydroxy-*trans*-2-alkenals having a doublet signal (signal d) centered at 9.577 ppm, and in very small proportion, 4-hydroperoxy-*trans*-2-alkenals, which show a doublet signal (signal e) centered at 9.582 ppm. Finally, in the spectra of oil samples with a certain oxidation degree there is also a doublet signal (signal c) centered at 9.536 ppm, assignable to 4,5-epoxy-*trans*-2-alkenals. These aldehydes were also all detected in the oxidation of sunflower oil at higher temperature (70 °C) with aeration (*18*). However, there are significant differences. In the high-temperature process (70 °C with aeration) 4-hydroperoxy-*trans*-2-alkenals are formed before 4-hydroxy-*trans*-2-alkenals and the concentration of the latter increases gradually, reaching both kinds of aldehydes similar concentrations in advanced oxidation stages (*18*). However, in the samples oxidized at room temperature, both types of aldehydes are detected at the same time (see sample S18 in **Figure 5D**), with 4-hydroxy-*trans*-2-alkenals in a much higher proportion than 4-hydroperoxy-*trans*-2-alkenals. This latter fact could be due to either the presence of *cis*,*trans*-conjugated diene hydroxy derivatives among the primary oxidation products, which can evolve to give 4-hydroxy-*trans*-2-alkenals directly, as has been described (*40*), or the limitation in the concentration of oxygen in the oxidation process, which could favor the formation of hydroxy over that of hydroperoxy aldehydes.

As said before, **Table 1** gives the relative proportion of hydroperoxides and aldehydes in the different samples determined from the ^{1}H NMR spectra. As expected, the relative proportions of hydroperoxides are null or very small in

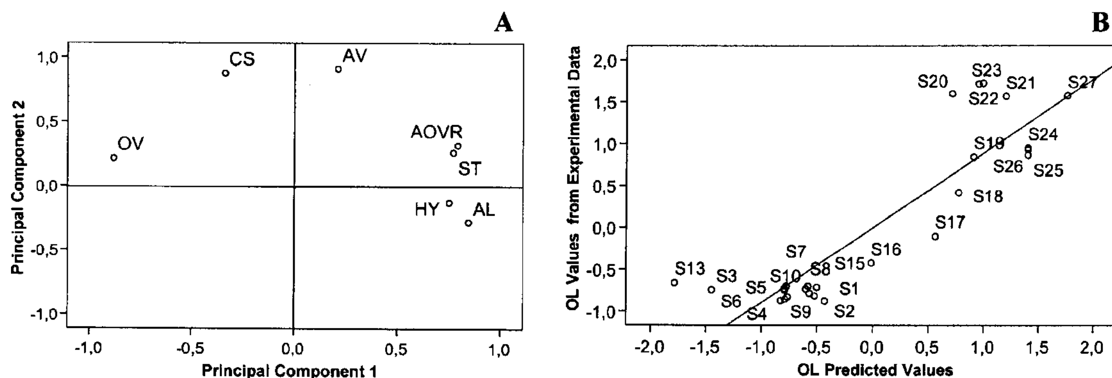

Figure 6. (A) PC1 (49.4%) versus PC2 (27.1%) of the variables ST, CS, AV, OV, AOVR, HY, and AL. (B) Oxidation level values (OL) defined as the principal component of variables HY and AL versus predicted values of the oxidation level from the multiple linear regression equation of OL, OC1, and OC2 principal components.

unoxidized samples. They increase with the oxidation level of the sample to reach certain values, after which they decrease until they are small again in the most oxidized samples. Likewise, as expected, the relative proportion of aldehydes is null in unoxidized samples and rises with the oxidation level of the sample.

First, to study relationships between the oxidation level of the samples and some of the oxidation conditions given in **Table 1**, an exploratory principal component analysis of the variables HY and AL, both representative of oxidation level (OL) and the variables ST, CS, AV, OV, and AOVR representative of the oxidation conditions (OC), was performed. The seven variables considered in this analysis are reduced to two principal components PC1 and PC2. These explain 79% of the total variance. The first component explains 49.4% of the total variance and is given by the expression PC1 = −0.254(OV) –0.074(CS) + 0.078(AV) + 0.229(ST) + 0.235(AOVR) + 0.219(HY) + 0.242(AL); it is closely related, in a positive way, with the variables ST, AOVR, HY, and AL, and in a negative way to the variable OV. The second component explains 27.1% of the total variance and is given by the expression PC2 = 0.090(OV) + 0.460(CS) + 0.481(AV) + 0.154(ST) + 0.178(AOVR) – 0.043(HY) – 0.128(AL); this principal component is closely related, in a positive way, with the variables CS and AV. **Figure 6A** shows these relations graphically. From this preliminary study, it seems evident that the oxidation level, represented by the variables HY and AL, is more closely related to oxidation conditions, such as storage time (ST), air/oil volume ratio (AOVR), and oil volume (OV) than the other two, air/oil contact surface (CS) and air volume (AV).

The principal component analysis of the five variables associated with oxidation conditions (OC) was performed, and these were reduced to two principal components, which were named OC1 and OC2. They explain 81% of the total variance. The first component, OC1, explains 46% of the total variance and is given by the expression OC1 = −0.395(OV) – 0.107(CS) + 0.149(AV) + 0.328(ST) + 0.381(AOVR); the second component, OC2, explains 35% of the total variance and is given by the expression OC2 = 0.229(OV) + 0.535(CS) + 0.461(AV) + 0.075(ST) + 0.071(AOVR). Likewise, the principal component analysis of the two variables associated with the oxidation level of the samples was performed, and these were reduced to one component, which was named OL; this principal component OL explains 74% of the variance and is given by the expression OL = 0.581(HY) + 0.581(AL). To find relationships between the oxidation level and oxidation conditions, multiple linear regression between the principal components OL, OC1, and OC2 was performed; the correlation coefficient ($R = 0.890$) of the equation obtained, OL = 0.823(OC1) – 0.337(OC2), shows that a close relation exists between the oxidation level of the samples defined in this way and the two new variables associated with the oxidation conditions, as could be expected. It can be observed that the OC1 variable is more closely related to OL than OC2. This is in agreement with the above. **Figure 6B** represents OL values obtained from the principal component analysis of HY and AL variables versus OL predicted values from the regression equation given above. Despite the fact that important factors, such as main and minor components and the concentration and nature of the antioxidant added to the original oils, have not been included in this statistical study, a close relationship has been found to exist between the oxidation level of the samples and oxidation conditions, especially ST, AOVR, and OV.

In short, the study of the results obtained by using ^{1}H NMR spectroscopy reveals that samples with a low level of oxidation contain only hydroperoxides having *cis*,*trans*-conjugated double bonds; this indicates that, at the early oxidation stages produced at room temperature, only these types of hydroperoxides are formed. In samples whose oxidation state is more advanced, from sample S17 onward, in addition to the above-mentioned hydroperoxides, signals of aldehydes, hydroperoxides having *trans*,*trans*-conjugated double bonds, and hydroxy derivatives having *cis*,*trans*-conjugated dienic systems also appear. Among aldehydes, the main are alkanals, *trans*-2-alkenals, and 4-hydroxy-*trans*-2-alkenals; in addition, there are *trans*,*trans*-2,4-alkadienals and, in very small proportions, 4-hydroperoxy-*trans*-2-alkenals. The results obtained by ^{1}H NMR agree with those obtained by FTIR. They indicate that the oxidation process that takes place at room temperature in closed recipients is somewhat different to that provoked at 70 °C with aeration. The most important differences found between both processes are referred to the nature and proportions of the intermediate compounds formed, the timing of their formation, and the relative proportions of the secondary oxidation products generated. It is particularly worth noting the formation of hydroxy derivatives with *cis*,*trans*-conjugated double bonds among the primary oxidation products.

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