A Review of Thermo-Oxidative Degradation of Food Lipids Studied by $^1$H NMR Spectroscopy: Influence of Degradative Conditions and Food Lipid Nature

Andrea Martínez-Yusta, Encarnación Goicoechea, and María D. Guillén

Abstract: This review summarizes present-day knowledge provided by proton nuclear magnetic resonance ($^1$H NMR) concerning food lipid thermo-oxidative degradation. The food lipids considered include edible oils and fats of animal and vegetable origin. The thermo-oxidation processes of food lipids of very different composition, occurring at low, intermediate, or high temperatures, with different food lipid surfaces exposed to oxygen, are reviewed. Mention is made of the influence of both food lipid nature and degradative conditions on the thermo-oxidation process. Interest is focused not only on the evolution of the compounds that degrade, but also on the intermediate or primary oxidation compounds formed, as well as on the secondary ones, from both qualitative and quantitative points of view. Very valuable qualitative and quantitative information is provided by $^1$H NMR, which can be useful for metabolomic and lipidomic studies. The chemical shift assignments of spectral signals of protons of primary (hydroperoxides and hydroxides associated with conjugated dienes) and secondary, or further (aldehydes, epoxides, among which 9,10-epoxy-12-octadecenoate [leukotoxin] can be cited, alcohols, ketones) oxidation compounds is summarized. It is worth noting the ability of $^1$H NMR to detect toxic oxygenated $\alpha,\beta$-unsaturated aldehydes, like 4-hydroperoxy-, 4,5-epoxy-, and 4-hydroxy-2-alkenals, which can be generated in the degradation of food lipids having omega-3 and omega-6 polyunsaturated groups in both biological systems and foodstuffs. They are considered as genotoxic and cytotoxic, and are potential causative agents of cancer, atherosclerosis, and Parkinson’s and Alzheimer’s diseases.

Keywords: characterization and quantification, food lipids, oxidation compounds, proton nuclear magnetic resonance ($^1$H NMR), thermo-oxidation

Introduction

Lipid oxidation involves a large group of complex reactions induced by oxygen in the presence of initiators, among which are heat, free radicals, light, photosensitizing pigments, and metal ions. It has been described that this process takes place through a sequential free radical chain reaction mechanism, which implies the well-known 3 stages of initiation, propagation, and termination (Frankel 2005).

In short, the initiation stage involves the homolytic breakdown of hydrogen in the $\alpha$-position in relation to the double bond in an unsaturated acyl group, forming unstable free radicals that stabilize by abstracting a hydrogen free radical from another chemical species. It has been postulated that hydroperoxides and hydroxides are formed at the propagation stage, and that they in turn generate free radicals; the formation of hydroperoxides or hydroxides can occur simultaneously with the formation of conjugated double bonds. The termination stage takes place when the collision of free radicals provokes the formation of stable molecules, thus making the free radicals disappear. The variety of compounds which may form in these reactions is great and their formation occurs sequentially; for this reason, some of them are called primary oxidation compounds, and those derived from them are called secondary oxidation compounds, and there may also be tertiary, or further, oxidation compounds formed.

Pioneer studies carried out with standard compounds made a very important contribution to our knowledge of these processes. In these studies, extraction and separation techniques, and in many cases proton nuclear magnetic resonance ($^1$H NMR) spectroscopy, were used to identify and quantify some of the compounds formed (Perkins and Anfinsen 1971 [using a Varian A-60 spectrometer]; Gardner and Weisleder 1972 [using a 100-MHz spectrometer]; Wineburg and Swern 1974 [using a Varian XL-100 spectrometer]; Neff and others 1981, 1988 [using a Bruker WH-90 spectrometer]; Neff and others 1983, 1988, 1990; Neff and Frankel 1984 [using a Bruker WM-300 spectrometer]; Frankel and others
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1982 [using a Bruker WH-90 spectrometer]; Frankel and others 1990 [using a Bruker WM-300 spectrometer]; Yamashita and others 1993 [using a Hitachi R-22 90 MHz spectrometer]; Coxon and others 1984 [using a 300-MHz spectrometer]; Porter and others 1994 [using a 300-MHz spectrometer]; Butovich 2006; Butovich and others 2006 [using a Varian 400-MHz spectrometer]; Dobson and others 2013 [using a Bruker Ascend 500 MHz or a JEOL Eclipse 400-MHz spectrometer]). In recent years, Hämäläinen and Kamal-Eldin (2005) have reviewed the $^1$H and $^{13}$C NMR spectral data of some of these standards of lipid oxidation compounds. Gas chromatography (GC) has also been essential in the study of the volatile compounds formed in thermo-oxidation processes of standard lipids (Thompson and others 1978; Frankel and others 1981, 1982, 1988), as has high-performance liquid chromatography (HPLC) in the study of the polymers, oligomers, and dimers formed in thermo-degradation processes (Dobarganes and Pérez-Camino 1987; Marquez-Ruiz and others 1995; Marquez-Ruiz and Dobarganes 2005; Dobarganes and Márquez-Ruiz 2007).

As it is known that certain compounds or functional groups can be formed in food lipid thermo-oxidation processes, many studies have been devoted to the determination of their concentrations. Thus, parameters such as peroxide value and conjugated dienes have been employed to evaluate the occurrence of primary oxidation compounds; however, it has been recently shown that certain secondary oxidation compounds can also support hydroperoxy groups and conjugated dienic systems (Guillén and Ruiz 2004b, 2005a, 2005b, 2005c). Similarly, anisidine value and thiobarbituric acid reactive substances (TBARS) tests have been used to evaluate the occurrence of secondary oxidation products; however, the usefulness of both methods has also been subject to criticism (Guillén-Sans and Guzman-Chozas 1998; Laguerre and others 2007). These classical methods give concentration values of certain compounds or functional groups in a generic way, but do not provide information about the specific nature of the compounds involved in each determination, which sometimes could give rise to inaccurate conclusions (Frankel 2005). Thus, these classical methods do not shed further light on the thermo-oxidative mechanisms of oils and caution is required in their interpretation.

Techniques that study food lipids as a whole have hardly ever been used in the analysis of food lipid thermo-oxidation. Two of the most important ones are Fourier transform infrared (FTIR) spectroscopy (Guillén and Cabo 1997, 1999, 2000) and NMR spectroscopy. However, as they are fast and do not require modification or special preparation of the sample, studies aimed at characterizing these food lipids by simple and direct observation of the spectral profile data in statistical analysis in order to extract information on processes or on samples under the light shed by the statistics. Nevertheless, to extract the relevant information contained in the $^1$H NMR spectra a correct assignment of the signals to the corresponding protons is essential. This assignment allows a correct identification of molecules or functional groups involved, and also the development of adequate quantitative approaches that permit one to obtain not only qualitative but also the quantitative information which is essential to draw sound conclusions.

In this context, a review of the use of $^1$H NMR spectroscopy in the study of food lipid thermo-oxidation is presented here. This review covers not only the influence of the food lipid nature and of the degradation conditions on the evolution of lipid thermo-oxidation processes, but also the nature and concentration of the compounds formed which may be detected by this technique. The aim of this review is to help in providing valuable tools to improve the conclusions in studies in which lipids and their thermo-oxidation processes are involved.

$^1$H NMR as a Tool for the Study of Food Lipid Thermo-Oxidation Processes

As mentioned, this spectroscopic technique is able to study the food lipid sample as a whole, before and after submission to any degradative process. For this reason, if the information provided is sound, it will be a very useful technique.

Characterization of food lipids before submission to oxidative conditions

Several studies have shown the usefulness of this technique in the characterization of nonoxidized food lipids. Since the pioneer studies of Hopkins and Bernstein (1959) and also of Johnson and Shoolery (1962) using equipment of 40 MHz and of 60 MHz, respectively, many advances have been made. In spite of the limited resolution of the spectra obtained with this equipment, the above-mentioned authors indicated that this technique could be useful not only for identification, but also for quantification of oil components.

Concerning food lipid main components.

Nowadays, due to several well-received contributions, the assignment of the signals of $^1$H NMR spectra to the protons of the main nonoxidized food lipid components, namely of triglycerides, and also of some minor food lipid components, is well established (Hopkins and Bernstein 1959; Johnson and Shoolery 1962; Gunstone 1990; Wollenberg 1991; Aursand and others 1993; Sacchi and others 1993, 1998; Segre and Mannina 1997; Igarashi and others 2000; Guillén and Ruiz 2001, 2003a; Siddiqui and others 2003; Guillén and others 2008; Mannina and others 2008; Vidal and others 2012). As an example, Figure 1 shows a region of the $^1$H NMR spectra, recorded with 400 MHz equipment, of lipids of animal origin such as pork adipose tissue (PAT), farmed sea bass (Dicentrarchus labrax; SB), and of 3 vegetable oils, namely virgin linseed (VL), extra-virgin olive (EVO), and sunflower (SF), dissolved in deuterated chloroform. All signals observable in this figure are due to protons of several triglycerides of food lipids. Table 1 gives their assignments to the corresponding protons, letters in Figure 1 and in Table 1 being in agreement. A thorough explanation of each of these signals and its assignment to the different kinds of protons of triglycerides has been carried out previously (Guillén and Ruiz 2001, 2003a; Guillén and others 2008; Vidal and others 2012).

All spectra given in Figure 1 have been represented on the same scale. The different intensity of the common signals in all spectra given, and the presence or absence of others, allows an initial characterization of these food lipids by simple and direct observation; that is to say, without further enlargement of the spectra. This task is even easier when some of the signals are enlarged, as shown in Figure 2. Thus, the difference between PAT lipids and vegetable oils is clear, due to the high content of saturated (S) groups in the former, which is observable by the intensity of the signal at 1.259 ppm. It is also evident that SF oil does not contain linolenic (Ln) acyl groups (absence of signals at 0.972, 2.093, 2.111, and 2.801 ppm), but has a very important concentration of linoleic (L) acyl groups (intensity of signals at 0.889, 2.036, 2.056, and 2.765 ppm). Similarly, it can be seen without further enlargement that PAT and EVO oil have very small

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concentrations of Ln and L acyl groups, but important ones of oleic (O; intensity of signals at 0.879, 1.270, 2.002, and 2.020 ppm), this latter acyl group being higher in EVO oil than in the lipids of PAT. Finally, the abundance of ω-3 acyl groups in linseed oil and the lipids of sea bass is shown by the intensity of the signals at 0.972, 2.093, and 2.111 ppm and between 2.773 and 2.895 ppm. In addition, the high proportion of Ln acyl groups in linseed oil is seen by the intensity of the signal at 2.801 ppm, whereas in sea bass lipids that of eicosapentaenoic (EPA) plus arachidonic (ARA) and of docosahexaenoic (DHA) acyl groups is evidenced by the signals at 1.661 to 1.743 and at 2.364 to 2.421 ppm, respectively. It must be pointed out that farmed fish usually contains lower concentrations of ARA acyl groups than do wild specimens.

A much more accurate characterization can be carried out by the determination of the molar percentages of the different kinds of acyl groups. Taking into account that the area of the above-mentioned signals is proportional to the number of protons that generate them, and that the proportionality constant is the same in all the cases, several approaches have been developed to determine the molar percentage of several kinds of acyl groups in food lipids. These have been thoroughly explained in previous papers (Aursand and others 1993; Igarashi and others 2000; Guillén and Ruiz 2003b, 2004a, 2005a, 2005b, 2006, 2008; Guillén and Goicoechea 2007, 2009; Guillén and others 2008; Guillén and Uriarte 2009, 2012a, 2012b, 2012c; Goicoechea and Guillén 2010; Vidal and others 2012; Sopelana and others 2013; Martinez-Yusta and Guillén 2014a, 2014b). One of them, which is useful for vegetable oils and food lipids of animal origin, except fish lipids, involves the following equations:

\[
\text{Linolenic groups Ln} \% = 100 \left( \frac{A_J}{3 A_K} \right) \quad (1)
\]

\[
\text{Linoleic groups L} \% = 100 \left( \frac{2 A_I}{3 A_K} \right) \quad (2)
\]

\[
\text{Oleic (or monounsaturated) groups O (or MU)} \% = 100 \left[ \frac{A_F - 2 A_I - A_J}{3 A_K} \right] \quad (3)
\]

\[
\text{Saturated groups S} \% = 100 \left[ 1 - \frac{A_F}{3 A_K} \right] \quad (4)
\]

In these equations, \( A_J \), \( A_I \), and \( A_F \) are the area of the signal \( J \) (bis-allylic protons of Ln groups), signal \( I \) (bis-allylic protons of L groups), and signal \( F \) (mono-allylic protons of all unsaturated acyl groups), respectively, indicated in Table 1 and Figure 1, and \( A_K \) is the area of signal K due to the protons of carbons 1 and 3 of the glycerol backbone of triglycerides. Using these equations, the molar percentages of the above-mentioned acyl groups in the several lipids were determined and these are given in Table 2; the results are in agreement with those expected for oils and fats of this nature. The accuracy of these determinations made from \(^1\)H NMR spectral data has been demonstrated by using standard mixtures of triglycerides, giving satisfactory results (Guillén and Ruiz 2003b).

Similarly, the molar percentage of certain acyl groups of fish lipids can also be determined from \(^1\)H NMR spectra using different approaches, which have been very thoroughly explained in previous papers (Guillén and Ruiz 2004a; Guillén and others 2008; Vidal and others 2012); one of these possible approaches involves these equations:

\[
\text{Total } \omega - 3 \text{ groups } \omega - 3 \% = 100 \left( \frac{4 A_H}{3 (A_H + 2 A_K)} \right) \quad (5)
\]

\[
\text{Docosahexaenoic groups DHA} \% = 100 \frac{A_H}{(A_H + 2 A_K)} \quad (6)
\]
Table 1—Chemical shift assignments and multiplicities of the $^1$H NMR signals in CDCl₃ of the main acyl groups and minor compounds of food lipids. The signal letters agree with those given in Figures 1 to 4.

<table>
<thead>
<tr>
<th>Signal</th>
<th>Chemical shift (ppm)</th>
<th>Multiplicity</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main acyl groups</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.879</td>
<td>t</td>
<td>Saturated, monounsaturated $\omega$-9 and/or $\omega$-7 acyl groups</td>
</tr>
<tr>
<td>A</td>
<td>0.889</td>
<td>t</td>
<td>Unsaturated $\omega$-6 acyl groups</td>
</tr>
<tr>
<td>B</td>
<td>0.972</td>
<td>t</td>
<td>Unsaturated $\omega$-3 acyl groups</td>
</tr>
<tr>
<td>C</td>
<td>1.221 to 1.419</td>
<td>m</td>
<td>Acyl groups except for DHA, EPA and ARA acyl groups</td>
</tr>
<tr>
<td>E</td>
<td>1.522 to 1.700</td>
<td>m</td>
<td>Acyl groups except for DHA acyl groups</td>
</tr>
<tr>
<td>F</td>
<td>1.941 to 2.139</td>
<td>m</td>
<td>Acyl groups for CH$_2$ of DHA acyl group in $\beta$-position in relation to carbonyl group</td>
</tr>
<tr>
<td>G</td>
<td>2.305</td>
<td>dt</td>
<td>Acyl groups except for DHA acyl groups</td>
</tr>
<tr>
<td>H</td>
<td>2.364 to 2.421</td>
<td>m</td>
<td>DHA acyl groups</td>
</tr>
<tr>
<td>J</td>
<td>2.765</td>
<td>t</td>
<td>Dihunsaturated $\omega$-6 acyl groups</td>
</tr>
<tr>
<td>J'</td>
<td>2.773 to 2.895</td>
<td>m</td>
<td>Triunsaturated $\omega$-3 acyl groups</td>
</tr>
<tr>
<td>K</td>
<td>4.139, 4.303</td>
<td>dd</td>
<td>DHA, EPA, ARA and other polyunsaturated $\omega$-3 acyl groups</td>
</tr>
<tr>
<td>L</td>
<td>5.225 to 5.296</td>
<td>m</td>
<td>Glycerol groups</td>
</tr>
<tr>
<td>M</td>
<td>5.296 to 5.470</td>
<td>m</td>
<td>Glycerol groups</td>
</tr>
<tr>
<td><strong>Minor compounds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0.540</td>
<td>s</td>
<td>$\Delta$7-avenasterol</td>
</tr>
<tr>
<td>O</td>
<td>0.681</td>
<td>s</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>P</td>
<td>0.684</td>
<td>s</td>
<td>$\Delta$5-stigmastanol and $\Delta$5-campsterol</td>
</tr>
<tr>
<td>Q</td>
<td>0.704</td>
<td>s</td>
<td>$\Delta$5-stigmasterol and brassicasterol</td>
</tr>
<tr>
<td>R</td>
<td>1.670</td>
<td>s</td>
<td>Squalene</td>
</tr>
<tr>
<td>S</td>
<td>3.350</td>
<td>s</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>T</td>
<td>3.725</td>
<td>ts</td>
<td>1,2-diglycerides</td>
</tr>
<tr>
<td>U</td>
<td>4.949 to 5.072</td>
<td>dq,dq</td>
<td>Ununsaturated $\omega$-1 acyl groups</td>
</tr>
<tr>
<td>V</td>
<td>5.749 to 5.858</td>
<td>s</td>
<td>Ununsaturated $\omega$-1 acyl groups</td>
</tr>
</tbody>
</table>

$^a$Overlapping of multiplets of methylenic protons in the different acyl groups either in $\beta$-position, or further, in relation to double bonds, or in $\gamma$-position, or further, in relation to the carbonyl group.

$^b$Overlapping of multiplets of the $\alpha$-methylene protons in relation to a single double bond of the different unsaturated acyl groups.

$^c$Standard compounds (dioleoin and dipalmitin) give a triplet centered at 3.73 ppm, and in complex mixtures the signal generated is a doublet centered at 3.79 ppm instead of a triplet (Sopelana and others 2013).

$^d$Triplet, m; multiplet, d; DHA, docoahexaenoic acid groups; EPA, eicosapentaenoic acid groups; ARA, arachidonic acid groups; d, doublet; s, singlet; q, quartet.

Eicosapentaenoic plus arachidonic groups EPA + ARA (%) = 100(2A) / (A + 2A$^c$) (7)

Diunsaturated $\omega$-6 (mainly linoleic) groups DU$\omega$ - 6 (%) = 100(A$^c$) / (A + 2A$^c$) (8)

Total unsaturated groups U (%) = 100(A + A$^c$) / (2A + 4A$^c$) (9)

In these equations $A_1$ is the area of signal B due to methylic protons of $\omega$-3 acyl groups, $A_1$ is the area of signal H due to methylenic protons in $\alpha$- and $\beta$-positions in relation to the carbonyl group of DHA groups, $A_1$ is the area of the signal G related to the methylenic protons in the $\alpha$-position in relation to carbonyl groups, except those of DHA groups, and finally $A_1$ and $A_2$ were as defined. All these signals are indicated in Figure 1 and in Table 1.

Concerning food lipid minor components. In addition to main components, nonoxidized food lipids also contain minor components, which are also important because some of them can show antioxidant ability or interesting nutritional or health-related properties. If these compounds are present in high enough concentrations for them to be detected by $^1$H NMR, and if the signals of their protons do not overlap with those of the main lipid components, then this technique provides information about them. Some of these detected in the above-mentioned food lipids are shown in Figure 3. The assignment of these signals is also given in Table 1. It has been found that food lipids of animal origin, like those of PAT and sea bass, contain cholesterol (signal O) containing approximate concentrations near 93 and 734 mg/100 g, respectively (Chizzolini and others 1999; Orban and others 2003). Vegetable oils contain sterols, with $\beta$-sitosterol plus $\Delta$5-campsterol (signal P), $\Delta$5-stigmastanol plus brassicasterol (signal Q) being present, as well as $\Delta$7-avenasterol (signal N) in SF oil (Kurata and others 2005; Zhang and others 2006a; Sopelana and others 2013). The contents of sterols in vegetable oils can be very varied, even in those of the same vegetable origin; concentrations ranging from 0 to 260 mg/100 g of individual sterols have been found in different vegetable oils (Schwartz and others 2008). However, it must be taken into account that when operating at 400 MHz there is an overlap between the signal of $\Delta$5-stigmastanol and brassicasterol and the side band of the methylic protons of linoleic, oleic, and saturated acyl groups.

Other very interesting minor components are squalene (signal R), present mainly in EVO oil (Mannina and others 2003, 2008), and phosphatidylcholine (signal S), present in many lipids of animal origin like sea bass lipids (Siddiqui and others 2003). Furthermore, some lipids, such as VL, EVO, and SF oils, as well as PAT, also contain 1,2-diglycerides (signal T; Sacchi and others 1996; Segre and Mannina 1997). In addition, it is worth mentioning the occurrence in sea bass lipids of $\omega$-1 acyl groups which give typical signals U and V. Although these had been already observed in previous studies on salmon lipids (Guillén and Ruiz 2004a), they have recently been identified (Shimizu and others 1991; Fiori and others 2012; Vidal and others 2012).

The concentration of these compounds in the corresponding food lipids can be determined from the area of the above-mentioned signals. It is possible to do this determination either in an absolute way, using a standard compound, or in relation to other food lipid components present in the same sample.

Characterization of food lipids after submission to thermo-oxidative conditions. During thermo-oxidative processes changes are produced in the original food lipid components and, as mentioned before,
new compounds are generated. Those formed in the 1st step are named primary oxidation compounds and the ones derived from them are called secondary or further oxidation compounds. First, the evolution of the original food lipid components will be commented on, followed by the formation and evolution of the different oxidation compounds.

**Evolution of main and minor original lipid components.** Under thermo-oxidative conditions both main and minor components can evolve to give new compounds, and this is reflected in the intensity of the signals of their protons, which undergo changes, which are observable in the region of the spectra in which these signals appear. Figure 4(a), as an example, shows the spectral regions of the original SF oil and of the same oil after submission, for different heating times, to 2 different temperatures (70 °C and 190 °C) under different processing conditions (10 g of oil in Petri dishes and 4 L of oil in a deep-fryer, respectively; Goicoechea and Guillén 2010; Guillén and Uriarte 2012c). It can be observed in the spectrum of SF oil submitted to 70 °C for 264 h (11 d), under certain oxidative conditions, that the intensity of the signals of linoleic (L) protons (signals at 0.889, 2.036, 2.056, and 2.765 ppm) have drastically decreased; however, in the spectrum of SF oil submitted to 190 °C for 40 h under other thermo-oxidative conditions, although the intensity of the same signals due to linoleic (L) groups has been reduced in relation to its original intensity, they have not disappeared from the spectra. As Figure 4(a) shows, the evolution of the different acyl groups throughout the processing time, under any processing conditions, can be observed directly in the 1H NMR spectra. Furthermore, this evolution can also be quantified using the appropriate equations, developed for each specific case on the basis of the same principles as mentioned for nonoxidized lipids (Guillén and Uriarte 2012a, 2012b, 2012c). Figure 4(b) shows the quantification of the evolution throughout processing time of the molar percentage of the several kinds of acyl groups in SF oil submitted to the above-mentioned thermo-oxidative conditions. It can be observed that great differences are found, depending on the thermo-oxidation conditions.

Similarly, if minor lipid components are affected during the thermo-oxidative degradation, the intensity of their proton
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Table 2–Composition of food lipid samples shown in Figures 1 to 3, obtained from $^1$H NMR data and expressed as molar percentage of the different kinds of acyl groups (data taken from Guillén and Uriarte 2012a, 2012b, 2012c; Vidal and others 2012; Martínez-Yusta and Guillén 2014a, 2014b).

<table>
<thead>
<tr>
<th>Food lipid</th>
<th>Ln or $\omega$-3 (%)</th>
<th>L (%)</th>
<th>O or MU (%)</th>
<th>S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork adipose tissue lipids (PAT)</td>
<td>1.67 ± 0.08</td>
<td>10.21 ± 0.02</td>
<td>46.83 ± 0.31</td>
<td>41.29 ± 0.41</td>
</tr>
<tr>
<td>Sea bass lipids (SB)</td>
<td>22.10 ± 1.60</td>
<td>14.28 ± 2.92</td>
<td>36.36 ± 2.97</td>
<td>26.46 ± 2.16</td>
</tr>
<tr>
<td>(DHA 8.09 ± 1.45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(EPA 9.49 ± 1.59)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virgin linseed oil (VL)</td>
<td>50.37 ± 0.50</td>
<td>17.85 ± 0.03</td>
<td>22.56 ± 0.37</td>
<td>9.20 ± 0.19</td>
</tr>
<tr>
<td>Extra-virgin olive oil (EVO)</td>
<td>0.49 ± 0.02</td>
<td>4.49 ± 0.28</td>
<td>82.06 ± 0.98</td>
<td>12.93 ± 0.65</td>
</tr>
<tr>
<td>Sunflower oil (SF)</td>
<td></td>
<td>55.05 ± 4.31</td>
<td>34.80 ± 2.83</td>
<td>10.03 ± 0.95</td>
</tr>
</tbody>
</table>

Acyl groups: Ln, linolenic; $\omega$-3, omega-3; L, linoleic; O or MU, oleic or monounsaturated; S, saturated; DHA, docosahexaenoic; EPA, eicosapentaenoic.

Figure 3—Enlargement of $^1$H NMR spectral signals related to minor compounds present in pork adipose tissue lipids (PAT), farmed sea bass lipids (SB), virgin linseed oil (VL), extra-virgin olive oil (EVO), and sunflower oil (SF). These spectra were acquired in a Bruker Avance 400 spectrometer operating at 400 MHz. Signal letters agree with those in Table 1.

signals in the spectra decreases or increases with the processing time, and the evolution of their concentration can also be determined, if the signals of their protons do not overlap with other signals.

Primary oxidation compounds and their evolution throughout the thermo-oxidation process. The degradation of the acyl groups of food lipids provokes the formation of new compounds, the ones formed at the 1st step being named primary oxidation compounds. Their natures can vary, and among them some acyl chains supporting hydroperoxy or hydroxy groups have been found, as well as conjugated dienic systems, which can also have either $Z,E$ or $E,E$ isomerism. The possibility that some of the hydroperoxy groups can be supported on saturated and unsaturated acyl chains without conjugated dienic systems exists, as well as the possibility of 2 hydroperoxy groups being supported in the same acyl chain, that is to say the occurrence of di-hydroperoxides in the same acyl chain (Coxon and others 1984; Neff and others 1990; Frankel and others, 1990; Porter and others 1994; Neff and El-Agaimy 1996; Kuklev and others 1997; Jie and Lam 2004; Lin and others 2007; Pajunen and others 2008). The formation of each of these functional groups depends on both the food lipid nature and the thermo-oxidation conditions. In fact, under certain degradative conditions the occurrence of these primary oxidation compounds is not detected by this technique, which indicates either that they are not formed or that, if they are formed, their degradation rate is too high for them to be detected by $^1$H NMR (Guillén and Uriarte 2009, 2012a, 2012b, 2012c).

Some of the protons of these compounds give signals in the $^1$H NMR spectra that do not overlap with those of acyl groups, thus making their identification and also their quantification possible. The identification of these signals has been based on previous studies carried out with standard compounds (Neff and others 1990; Frankel and others, 1990; Porter and others 1994; Neff and El-Agaimy 1996; Kuklev and others 1997; Jie and Lam 2004; Lin and others 2007; Pajunen and others 2008). Table 3 gives the

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Food lipid thermo-oxidation studied by \(^1\text{H}\) NMR...

Figure 4–(a) \(^1\text{H}\) NMR spectra of sunflower oil (SF) submitted to 70 °C (10 g oil, Petri dish) and 190 °C (4 L oil, deep-fryer) for different periods. These spectra were acquired in a Bruker Avance 400 spectrometer operating at 400 MHz. (b) Evolution in both processes of the molar percentages of linoleic (L), monounsaturated (MU), and saturated + modified (S+M) acyl groups along the heating time (Goicoechea and Guillén 2010; Guillén and Uriarte 2012c).

The evolution of these primary oxidation compounds or functional groups throughout a thermo-oxidative process can be observed directly in the spectra and quantified, as mentioned before. This quantification can be carried out either in an absolute way by using a standard compound or in relation to main functional groups present in the food lipids, such as triglycerides. As will be seen in subsequent sections, great differences have been found in relation to the formation, or not, of primary oxidation compounds in high enough concentrations for detection by this technique, in relation to their nature and also to their formation and degradation rates. These differences between primary oxidation compounds are functions of both the food lipid nature and of the conditions under which the thermo-oxidation occurs.

Secondary or further oxidation compounds and evolution throughout the thermo-oxidation process. Primary oxidation compounds are unstable and degrade to generate secondary oxidation compounds, which can have very varied structures. Some of them have protons whose signals in the \(^1\text{H}\) NMR spectra do not overlap with any other, making it possible to identify and quantify them. Table 3 gives the chemical shifts of some of the protons of different secondary oxidation compounds or functional groups which can be present in oxidized food lipids. Among them, there are different kinds of aldehydes, alcohols, epoxides, and ketones. The formation of these compounds also depends on both the original food lipid composition and the conditions of the thermo-oxidation process. Thus, aldehydes including n-alkanals, (E)-2-alkenals, (E,E)-2,4-alkadienals, (Z,E)-2,4-alkadienals, 4,5-epoxy-2-alkenals, 4-hydroxy-(E)-alkenals, 4-hydroperoxy-(E)-alkenals, as well as 4-oxo-alkanals can be found (Guillén and Ruiz 2004a, 2004b, 2005a, 2005b, 2005c, 2006, 2008; Guillén and Goicoechea 2007, 2009; Guillén and Uriarte 2009, 2012a, 2012b, 2012c; Goicoechea and Guillén 2010; Martínez-Yusta and Guillén 2014a, 2014b). It should be noticed that although aldehydes are generally considered as secondary oxidation compounds, all \(\alpha,\beta\)-unsaturated aldehydes have conjugated chemical shifts of the signals of the protons of the above-mentioned primary oxidation compounds. In addition, Figure 5(a) gives the \(^1\text{H}\) NMR spectral region of nonoxidized corn oil and of this same oil after storage at room temperature in a closed receptacle for 121 mo (Guillén and Goicoechea 2009), of SF oil submitted to 70 °C for 72 h and to 100 °C for 9 h (Goicoechea and Guillén 2010). It can be observed that, depending on the degradative conditions, different kinds of primary oxidation compounds or functional groups can be formed or not.
Concerning alcohols, primary and secondary ones, together with dialcohols, have been found (Guillén and Uriarte 2009, 2012a, 2012b, 2012c; Goicoechea and Guillén 2010; Martínez-Yusta and Guillén 2014a, 2014b). With regard to epoxides, those derived from oleic and from linoleic acyl groups have been detected. The latter may be in the form of not only mono- but also of diepoxides (Goicoechea and Guillén 2010; Guillén and Uriarte 2012a; Martínez-Yusta and Guillén 2014a). And finally, ketones having a conjugated dienic system (keto group conjugated with a double bond) derived from oleic acyl groups have also been found. Although signals of these latter ketones were observed previously (Guillén and Ruiz 2005a, 2005c), this review is where they have been assigned for the first time on the basis of data for pure compounds provided by other authors (Kuklev and others 1997; Jie and Lam 2004; Lin and others 2007). However, it should be Remembered that none of these compounds can be formed in the oxidation of every food lipid, or under every thermo-oxidative condition. Figure 5(b) shows the spectral regions in which some...
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Figure 5–(a) $^1$H NMR spectra of primary oxidation compounds formed in corn oil (CO), nonoxidized (0 h), and stored at room temperature in closed receptacles for 121 mo, sunflower oil (SF) submitted (10 g oil, Petri dish) to 70 °C for 72 h and to 100 °C for 9 h. (b) $^1$H NMR spectra of secondary or further oxidation compounds formed in fish lipids extracted from salmon (SA) submitted to 50 °C for 768 h, sunflower oil (SF), and extra-virgin olive oil (EVO) submitted (10 g oil, Petri dish) to 70 °C for 216 h and 1560 h, respectively, and to 190 °C (4 L oil, deep-fryer) for 32.5 h (Guillén and Ruiz 2004a, 2005a; Guillén and Goicoechea 2009; Goicoechea and Guillén 2010; Guillén and Uriarte 2012a, 2012c). These spectra were acquired in a Bruker Avance 400 spectrometer operating at 400 MHz. Signal letters agree with those in Table 3.

of these compounds appear in salmon lipids from fillets submitted to 50 °C for 768 h (extraction using CS$_2$ as solvent and assisted by an ultrasound bath), SF oil and EVO oil submitted to 70 °C, and to deep-frying conditions at 190 °C for different periods of time (Guillén and Ruiz 2004a, 2005a; Goicoechea and Guillén 2010; Guillén and Uriarte 2012a, 2012c).

The formation of all these compounds and the evolution of their concentration throughout the thermo-oxidative degradation process can be observed directly in the $^1$H NMR spectra and can be quantified either in an absolute way, in relation to a standard compound or in relation to a main functional group such as triglycerides, this latter method being much easier and faster (Guillén and Goicoechea 2007, 2009; Guillén and Uriarte 2009, 2012a, 2012b, 2012c; Goicoechea and Guillén 2010; Martinez-Yusta and Guillén 2014a, 2014b).

It is worth noting the capacity for $^1$H NMR spectroscopy to detect and quantify oxygenated $\alpha,\beta$-unsaturated aldehydes, such as 4,5-epoxy-$, 4$-hydroperoxy-$, and 4-hydroxy-(E)-2-alkenals, which are considered as genotoxic and cytotoxic, and potential causal agents of cancer, atherosclerosis, Parkinson’s disease, or Alzheimer’s disease, among others (Esterbauer and others 1991; Guillén and Goicoechea 2008). They are generated in the degradation of $\omega$-3 and $\omega$-6 polyunsaturated acyl groups, fatty acids, and esters, both in biological systems and foodstuffs, and can be absorbed through the diet. Similarly, it should be noticed that this technique permits detection and quantification of 9,10-epoxy-12-octadecenoate (leukotoxin) and 12,13-epoxy-9-octadecenoate (isoleukotoxin) groups, derived from linoleic acyl groups and bonded to triglyceride backbones (Goicoechea and Guillén 2010). The former is thus named as it can be formed endogenously, 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 (Markaverich and others 2005; Thompson and Hammock 2007).

In the thermo-oxidation of food lipids of a specific composition, the types and concentrations of primary and also of secondary oxidation compounds formed, and also the timing of their formation, depend on the conditions under which the process takes place. It is also true that under the same thermo-oxidative conditions the types and concentrations of primary and also of secondary...
Food lipid thermo-oxidation studied by $^1$H NMR . . .

oxidation compounds formed and also the timing of their formation depend on the food lipid nature. Both food lipid composition and conditions of the thermo-oxidation process govern the physico-chemical rules under which the process evolves. It is obvious that not all intermediate or all final oxidation compounds mentioned are formed in any particular thermo-oxidation process or from any food lipid.

Evolution of food lipid oxidation under various degradative conditions as seen in terms of $^1$H NMR

The first studies by $^1$H NMR (400 and 600 MHz) on the oxidation process of food lipids, and not on pure standard compounds, were carried out by Grootveld and coworkers, who submitted 10 to 25 g of oil samples to 180 °C for 90 min, 20 to 60 mL of oil samples to pan-frying conditions with food (180 °C, 30 min), and also analyzed several repeatedly used culinary frying oils, among them lamb, beef fat, ghee, corn, SF soybean, grape seed, coconut, rapsseed, peanut, and EVO oils (Claxson and others 1994; Sheerin and others 1997; Silwood and Grootveld 1999). In short, they concluded that thermal stressing of culinary oils rich in polyunsaturated acyl groups generated high levels of $n$-alkanals, ($E$)-2-alkenals, and ($E,E$)-2,4-alkadienals, and the tentatively identified 4-hydroxy-($E$)-2-alkenals, via decomposition of their precursor hydroperoxides associated with conjugated dienes. When oils with a low unsaturation degree and lard were subjected to the above-mentioned heat treatments, only low concentrations of these aldehydes were produced. The authors also discussed the dietary, physiological, and toxicological significance of these results regarding frying practices. Furthermore, using 1,3,5-trichlorobenzene as an external standard, the concentration (mol/kg oil) of total unsaturated and saturated aldehydes generated in corn oil, submitted to 180 °C for 90 min in vessels of different size, was determined. It was concluded that, as expected, the longer the heating time and the bigger the vessel size (and therefore the exposure to oxygen), the higher the concentration of both saturated and unsaturated aldehydes (Haywood and others 1995).

In this section the information obtained by $^1$H NMR on the mechanisms of lipid oxidation that takes place under different conditions is reviewed, with regard to degradation of main components, namely the acyl groups of triglycerides, and also to the formation of oxidation compounds. Special attention is paid to the influence of both degradative conditions and food lipid nature. Table 4 summarizes the different food lipid oxidation processes studied by $^1$H NMR and reviewed in the following sections.

Evolution of the oxidation of refined SF and corn oils at room temperature in closed receptacles.

As for the experimental conditions of these studies, different amounts of refined SF and corn oils, both rich in linoleic acyl groups, were stored separately in closed receptacles, in the presence of different air volumes and having different air/oil contact surfaces. These oil samples were maintained at room temperature for different periods of time up to 10 y (Guillén and Goicoechea 2007, 2009). Under these conditions the oils underwent oxidation very slowly.

A study by $^1$H NMR of the different samples evidenced that some of them were nonoxidized, others were at incipient or intermediate oxidation stages, and some others at very advanced oxidation stages. The $^1$H NMR spectra showed that under these conditions the oxidation process of the above-mentioned oils has a prolonged period of initiation (induction period), after which the formation of primary oxidation compounds begins as a consequence of the degradation of the acyl groups, in this case mainly linoleic. Figure 6(a) shows the enlarged regions of the $^1$H NMR spectra of several SF oil samples in different oxidation stages (samples S6, S7, S17, S18, and S23), in which the evolution of the primary oxidation compounds is shown. It can be observed that this oxidation process evolves, forming primary oxidation compounds having initially hydroperoxy groups as well as ($Z,E$)-conjugated dienic systems. At more advanced oxidation stages, hydroperoxy groups associated with ($E,E$)-conjugated dienic systems and hydroxy groups supported on chains also having ($Z,E$)-conjugated dienic systems appear, both in smaller concentrations than hydroperoxy-($Z,E$)-conjugated dienic systems. According to other authors, hydroperoxy-($Z,E$)-conjugated dienic systems are considered to be formed under kinetic control at low temperatures, because they are formed earlier (Schneider 2009). However, with prolonged autooxidation or at higher temperatures, the double-bond configuration of the conjugated hydroperoxides rearranges from ($Z,E$) to ($E,E$). The apparent driving force for this transformation is the greater thermodynamic stability of the ($E,E$)-conjugated double bond in comparison with the ($Z,E$) arrangement. Hydroperoxy-($E,E$)-conjugated dienic systems could be formed at the expense of the ($Z,E$), and therefore be considered to form under thermodynamic control, because they are formed later and are more stable. Many different molecular structures can be associated with the above-mentioned functional groups. Figure 6(b) shows some of the structures, which are present in oxidation compounds, for example, 13-hydroperoxyoctadeca-9,11-dienoate, 9-hydroperoxy-, and 9-hydroxyoctadeca-10,12-dienoates, all derived from linoleic acyl groups and able to be formed in this process.

It is noteworthy that, of all the oxidation processes analyzed, only in those that occur under these conditions (room temperature and closed receptacles) has the formation of hydroxy groups supported on long acyl chains having a conjugated dienic system been observed; this could be related to the limited concentration of oxygen in the closed receptacles. As far as we know, only 1 previous paper has reported the formation, in SF oil after prolonged storage, of ($Z,E$)-conjugated diene primary oxidation products having hydroxy groups (Mikolajczak and others 1968). The formation of this kind of compounds is known to occur in vivo oxidation processes, and, in fact, they are considered as clinical markers of lipid peroxidation and oxidative stress, usually detected in urine and serum (Spindler and others 1997; Browne and Armstrong 2000). Similarly, the formation of either ($Z,E$)- or ($E,E$)-conjugated dienic systems related to hydroperoxy groups is highly dependent on the conditions to which the oil is submitted.

Very different situations are possible, as the following sections will show.

All the above-mentioned compounds are usually called primary oxidation products; however, as Figure 6(a) shows (see S17 and S18 samples), the formation of the hydroxy-($Z,E$)-conjugated dienic systems, and of hydroperoxy-($E,E$)-conjugated dienic systems, occurs simultaneously with that of aldehydes (signals between 9.4 and 9.8 ppm). It can be observed in Figure 6(a) that the appearance of these 3 kinds of groups is incipient in S17 sample and that their signals are clearly observable in the S18 sample and onwards.

The conditions under which these oils are stored affect not only the nature and concentration of the hydroxy and hydroperoxy compounds mentioned above, but also those derived from them, like aldehydes and epoxides. As Figure 7(a) shows, the main aldehydes formed were $n$-alkanals, ($E$)-2-alkenals, and 4-hydroxy-($E$)-2-alkenals. In smaller concentrations ($E,E$)-2,4-alkadienals and 4-hydroperoxy-($E$)-2-alkenals and, in very small proportions, 4,5-epoxy-2-alkenals were generated. Molecular structures of some of
Table 4—Summary of studies on food lipid oxidation processes carried out by $^1$H NMR.

<table>
<thead>
<tr>
<th>Food lipids</th>
<th>Temperature (°C)</th>
<th>Time under oxidative conditions</th>
<th>Receptacle</th>
<th>Oil volume</th>
<th>Air/oil contact surface</th>
<th>Heating source</th>
<th>Aeration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower</td>
<td>Room temperature</td>
<td>Up to 10 y</td>
<td>Closed receptacles</td>
<td>Varied</td>
<td>Undetermined</td>
<td>—</td>
<td>Not</td>
<td>Guillen and Goicoechea (2007)</td>
</tr>
<tr>
<td>Corn</td>
<td>Room temperature</td>
<td>Up to 10 y</td>
<td>Closed receptacles</td>
<td>Varied</td>
<td>Undetermined</td>
<td>—</td>
<td>Not</td>
<td>Guillen and Goicoechea (2009)</td>
</tr>
<tr>
<td>Fish lipids (Salmo salar)</td>
<td>50</td>
<td>Up to 32 d</td>
<td>Salmon tissue</td>
<td>—</td>
<td>Heated air</td>
<td>Forced</td>
<td>Guillen and Ruiz (2004a)</td>
<td></td>
</tr>
<tr>
<td>Sesame</td>
<td>70</td>
<td>Until polymerization (20 d)</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Heated air</td>
<td>Guillen and Ruiz (2004b)</td>
<td></td>
</tr>
<tr>
<td>Virgin olive, hazelnut, and peanut</td>
<td>70</td>
<td>Until polymerization (72, 20, and 51 d)</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Heated air</td>
<td>Guillen and Ruiz (2005a)</td>
<td></td>
</tr>
<tr>
<td>Corn, and sunflower</td>
<td>70</td>
<td>Until polymerization (11 and 12 d)</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Heated air</td>
<td>Guillen and Ruiz (2005b)</td>
<td></td>
</tr>
<tr>
<td>Rapeseed, walnut, and linseed</td>
<td>70</td>
<td>Until polymerization (13, 5, and 4 d)</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Heated air</td>
<td>Guillen and Ruiz (2005c)</td>
<td></td>
</tr>
<tr>
<td>Sunflower</td>
<td>100</td>
<td>Until polymerization (55 h)</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Heated air</td>
<td>Guillen and Ruiz (2010)</td>
<td></td>
</tr>
<tr>
<td>Virgin olive, olive, hazelnut, corn, sunflower, soybean, and linseed</td>
<td>190</td>
<td>Until polymerization (linseed 7 h and the others 12 h)</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Heated air</td>
<td>Guillen and Ruiz (2008)</td>
<td></td>
</tr>
<tr>
<td>Virgin olive, corn, and linseed</td>
<td>190</td>
<td>Up to 4 h</td>
<td>Open Petri dishes</td>
<td>10 g</td>
<td>50.3 cm$^2$</td>
<td>Microwave</td>
<td>Natural</td>
<td>Guillen and Ruiz (2006)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>190</td>
<td>Up to 32 h</td>
<td>Domestic fryer (20.5 x 23.5 x 13.5 cm)</td>
<td>2 L</td>
<td>481.7 cm$^2$</td>
<td>Electric resistance</td>
<td>Natural</td>
<td>Guillen and Uriarte (2009)</td>
</tr>
<tr>
<td>Extra-virgin olive</td>
<td>190</td>
<td>Up to 40 h</td>
<td>Industrial fryer (12 x 30 x 17 cm)</td>
<td>4 L</td>
<td>360 cm$^2$</td>
<td>Electric resistance</td>
<td>Natural</td>
<td>Guillen and Uriarte (2012a)</td>
</tr>
<tr>
<td>Virgin linseed</td>
<td>190</td>
<td>Up to 20 h</td>
<td>Industrial fryer (12 x 30 x 17 cm)</td>
<td>4 L</td>
<td>360 cm$^2$</td>
<td>Electric resistance</td>
<td>Natural</td>
<td>Guillen and Uriarte (2012b)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>190</td>
<td>Up to 40 h</td>
<td>Industrial fryer (12 x 30 x 17 cm)</td>
<td>4 L</td>
<td>360 cm$^2$</td>
<td>Electric resistance</td>
<td>Natural</td>
<td>Guillen and Uriarte (2012c)</td>
</tr>
</tbody>
</table>
Food lipid thermo-oxidation studied by $^1$H NMR.

These compounds are given in Figure 7(b). It should be commented on that both the nature and concentration of aldehydes formed from the same oil are very different depending on the conditions under which the oxidation process takes place. It is noticeable that under the conditions of this study (room temperature and limited amount of air in closed receptacles) 4-hydroxy-(E)-2-alkenals are formed in significant concentration and that 4-hydroperoxy-(E)-2-alkenals are also formed, but in much smaller concentrations than the former, both known to be genotoxic and cytotoxic compounds. The fact that the concentration of 4-hydroxy-(E)-2-alkenals is higher than that of 4-hydroperoxy-(E)-2-alkenals could be due to either the limited concentration of oxygen in this oxidation process, which could favor the formation of hydroxy over that of hydroperoxy aldehydes, or to the presence of hydroxy-(Z,E)-conjugated diene systems among the intermediate oxidation products, which can evolve directly to give 4-hydroxy-(E)-2-alkenals, as has been described by Schneider and others (2004).

It is also worth mentioning that together with the formation of aldehydes and of the other compounds mentioned, the generation of epoxy groups supported on acyl chains also occurs. 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 (Lercker and others 2003). The protons of the carbon atoms bonded to the epoxy group give signals in the spectral region between 2.8 and 3.2 ppm, being clearly observable in increasing concentration in S17, S18, and S23 samples (see Figure 7a). They are derived from linoleic groups and are composed not only of monoepoxides (signals at 2.9 ppm) but also of diepoxides (signals at 2.9 and 3.1 ppm), these latter being in the samples at the most advanced oxidation stages. A wide variety of structures can be found among epoxides derived from linoleic acyl groups; it is worth noting the monoepoxide 9,10-epoxy-12-octadecenoate, whose hydrolysis gives leukotoxin, and its isomer, the monoepoxide 12,13-epoxy-9-octadecenoate whose hydrolysis gives isoleukotoxin. As mentioned before, in recent years a great deal of attention has been paid to these 2 compounds as a result of their biological activity (Markaverich and others 2005; Thompson and Hammock 2007). According to $^1$H NMR data provided by other authors, the 2 protons corresponding to the carbon atoms in positions 9 and 10 of leukotoxin diol give a multiplet signal at 3.43 ppm (Aerts and Jacobs 2004); in S23 spectra a broad signal can be found there (see Figure 7a) that appears simultaneously with that of epoxy groups, and that could be assigned to the dihydroxyleukotoxin derivative, as Table 3 shows. Like aldehydes, the formation of epoxides, their nature, and concentration are highly dependent on the oil nature and degradation conditions, as will be seen later.

It is also remarkable that in these polyunsaturated oils (corn and SF) at room temperature all kinds of oxidation-derived compounds are present simultaneously for a very prolonged period, except at the early stages, when only hydroperoxy groups and (Z,E)-conjugated dienic systems are present (see Figure 6a and 7a). This may be because at low temperatures the hydroperoxides have longer average lives than at higher temperatures.

Evolution of the thermo-oxidation of fish lipids contained in dry-salted and unsalted salmon (Salmo salar) fillets submitted to 50 °C with aeration in a convection oven. This study was carried out on lipids contained in fillets of dry-salted and unsalted salmon (Salmo salar) submitted to thermo-oxidative conditions at 50 °C with aeration in a convection oven (Guillén and Ruiz 2004a). The lipids were extracted periodically after submitting fillets to the above-mentioned degradative conditions for different periods...
and were studied by $^1$H NMR; the extraction was carried out at low temperature in order to avoid additional oxidation. Regarding salmon lipid nature, their important content in ω-3 acyl groups (around 25% molar percentage), mainly DHA and EPA groups, and their low oxidative stability are well known. Nevertheless, their high carotenoid content, which is able to exhibit antioxidant ability, is also well known.

In this case the oxidation process was fairly different to that occurring in the previous room temperature studies, not only regarding the conditions to which the lipids were submitted, but also their special composition and the environment in which these lipids were surrounded by water, proteins, and so on. Figure 8 shows the spectral regions of the unsalted samples in which the $^1$H NMR signals of the protons of primary and secondary oxidation compounds appear. It is noteworthy that in spite of the low temperature, neither the signals of hydroperoxides nor those of the conjugated double bonds are clearly observed. This could be due to the high degree of unsaturation of salmon lipids, which generate very reactive and unstable primary oxidation compounds, which disappear very quickly. After 17 d under degradative conditions very weak signals of saturated aldehydic protons around 9.75 ppm appear. These have greater intensity in the spectra of more oxidized samples, signals of saturated aldehydes of small size like ethanal and propanal at 9.79 ppm also being observable, as well as other less well-resolved signals between 9.48 and 9.60 ppm. As mentioned before, the signals of protons of oxygenated and nonoxygenated α,β-unsaturated aldehydes appear in this latter region. A poor resolution of signals in this region has also been observed in other lipids rich in ω-3 acyl groups oxidized at intermediate temperatures, as will be commented on later.

In summary, the oxidation process in this case displayed a very prolonged period of initiation, probably due to the high content of carotenoids and other compounds with antioxidant ability in the salmon. In spite of these, it was demonstrated that although the dry-salting process does not provoke any immediate oxidation of fish lipids, it reduces fish oxidative stability in an important way, with the lipids of salted fillets being oxidized more quickly than those of unsalted fillets. Signals of protons of hydroperoxides or of conjugated dienic system were not observed before the appearance of signals of aldehydic protons in any of them; this could be due to the fact that they are very unstable and disappear very quickly. The aldehydes formed were n-alkanals of various sizes and nonoxygenated and oxygenated α,β-unsaturated aldehydes; the signals of these latter appeared overlapped. Moreover, the presence of amino groups of proteins or of other compounds of similar nature to the compounds formed in fish lipid oxidation, either primary or secondary, can provoke the establishment of reactions that reduce their presence in the system, such as the Maillard reaction. However, it should be mentioned that both facts, the nondetection of hydroperoxides or conjugated dienic systems and the overlapping of oxygenated α,β-unsaturated aldehydes, are also observed in studies of the oxidation process...
In these studies vegetable oils rich in oleic (olive, hazelnut, peanut), linoleic (sesame, SF, corn), and linolenic (rapeseed, walnut, linseed) acyl groups were submitted to accelerated storage conditions at 70 °C with aeration (10 g of the oil in Petri dishes; Guillén and Ruiz 2004b, 2005a, 2005b, 2005c; Goicoechea and Guillén 2010). In these studies the thermo-oxidative conditions were the same for all the oils; the only difference was the oil composition. The processes were studied until the samples were totally polymerized and it was not possible to take samples to acquire the \(^1\text{H}\) NMR spectra. In all the oils studied the signals of the protons of polyunsaturated (linoleic and linolenic) groups disappeared at the end of the process.

Food lipid thermo-oxidation studied by \(^1\text{H}\) NMR: Under these degradative conditions the evolution of oils rich in \(\omega-3\) groups (Guillén and Ruiz 2005c).

Evolution of the thermo-oxidation of edible oils of very different composition submitted to 70 °C with aeration in a convection oven. In these studies vegetable oils rich in oleic (olive, hazelnut, peanut), linoleic (sesame, SF, corn), and linolenic (rapeseed, walnut, linseed) acyl groups were submitted to accelerated storage conditions at 70 °C with aeration (10 g of the oil in Petri dishes; Guillén and Ruiz 2004b, 2005a, 2005b, 2005c; Goicoechea and Guillén 2010). In these studies the thermo-oxidative conditions were the same for all the oils; the only difference was the oil composition. The processes were studied until the samples were totally polymerized and it was not possible to take samples to acquire the \(^1\text{H}\) NMR spectra. In all the oils studied the signals of the protons of polyunsaturated (linoleic and linolenic) groups disappeared at the end of the process.

Figure 8 shows some enlarged regions of the \(^1\text{H}\) NMR spectra of the lipids extracted from unsalted salmon fillets that were submitted to 50 °C for days (Guillén and Ruiz 2004a). These spectra were acquired in a Varian Plus spectrometer operating at 300 MHz. Signal letters agree with those in Table 3.

Evolution of the thermo-oxidation of edible oils of very different composition submitted to 70 °C with aeration in a convection oven.

Oils rich in linoleic acyl groups: Under these degradative conditions the evolution of oils rich in linoleic groups, such as refined SF, is fairly different from that occurring at room temperature in closed receptacles, although the nature of the compounds formed is relatively similar, with certain exceptions. After 11 d under these degradative conditions this oil is totally polymerized.

As Figure 9 shows, the concentration of these primary oxidation compounds, especially the hydroperoxy-\((E,E)\)-conjugated dienic systems, increases as the process advances, reaching a maximum value after 7 d under degradative conditions; afterwards their sharp degradation occurs. Like at room temperature, the degradation of primary oxidation compounds provokes the formation of aldehydes, epoxides, and alcohols. Nevertheless, some differences may be observed. Thus, in relation to aldehydes it is particularly worth mentioning that the main aldehydes initially formed are 4-hydroperoxy-\((E,E)\)-2-alkenals, with \(n\)-alkenals and \((E)\)-2-alkenals in smaller concentrations. As the process advances \((E,E)\)-2,4-alkadienals and 4-hydroxy-\((E)\)-alkenals appear: the concentration of \((E,E)\)-2,4-alkadienals remains small throughout the entire degradation process, whereas that of 4-hydroxy-\((E)\)-alkenals increases significantly and becomes higher than that of 4-hydroperoxy-\((E)\)-alkenals at the end of the process; it seems that the formation of the former involves the disappearance of the latter. The concentration of 4,5-epoxy-\(2\)-alkenals is even smaller than that of \((E,E)\)-2,4-alkadienals and they are only observable at advanced oxidation stages.

As mentioned, epoxides are also formed in the oxidation of SF oil at 70 °C. A comparison of Figure 9(a) and 7(a) indicates that at 70 °C the same epoxides and dialcohols, as at room temperature in closed receptacles, are formed but their concentrations are different. It is clear that the formation of diepoxides and also of dialcohols is more favored at 70 °C, probably due to the greater presence of oxygen in the system. The same can be said of aldehydes, which give proton signals at 3.59 and 3.62 ppm.

In summary, in comparison with room temperature the effect of the temperature (70 °C) in this process provokes the formation of greater concentrations of hydroperoxides in chains of \((E,E)\)-conjugated dienic systems and the higher availability of oxygen gives rise to the formation of greater concentrations of the most oxygenated final compounds in the groups of aldehydes (the toxic and reactive 4-hydroperoxy-\((E,E)\)-alkenals, 4-hydroxy-\((E,E)\)-alkenals, epoxides (diepoxides), and alcohols. This higher temperature also significantly affects the rate of the formation of all of these compounds.

Oils rich in oleic acyl groups: The evolution of oils rich in oleic acyl groups, such as EVO oil, under these same degradative conditions, shares some features with the above-mentioned linoleic-rich oils, but great differences also exist. This is summarized in Figure 9(b). First, the induction period is much more prolonged (day 26) than in SF oil, and after 72 d under these degradative conditions, EVO oil polymerizes totally; and the main compounds formed, even though some of them are the same, are somewhat different. As can be observed in Figure 9(b), the formation of hydroperoxy groups begins after a prolonged period under degradative conditions. The antioxidant compounds present...
Food lipid thermo-oxidation studied by $^1$H NMR...

Figure 9—Expanded regions of the $^1$H NMR spectra of oils submitted to accelerated storage conditions in Petri dishes (10 g) in a convection oven at 70 °C with aeration until polymerization: (a) refined sunflower (SF), (b) extra-virgin olive (EVO), and (c) virgin linseed (VL; Guillén and Ruiz 2005a, 2005b; Goicoechea and Guillén 2010). These spectra were acquired in a Bruker Avance 400 spectrometer operating at 400 MHz. Signal letters agree with those in Table 3.

in the oil and the lower unsaturation degree of the acyl groups are the reason for this. Among the hydroperoxy groups formed, some are derived from linoleic groups, which are the same as those generated in SF oil; however, in this case their concentration is very small. In addition, there are other ones in higher concentrations derived from oleic, some of whose structures are given in Figure 10; they can be distinguished from the ones derived from linoleic by the signal of their olefinic protons at 5.72 ppm (Porter and others 1994). In any case, under the same degradative conditions the formation of hydroperoxides in EVO oil is much smaller than in SF oil.

The difference between the compounds formed from olive oil and SF oil is even more noticeable in the groups of aldehydes, epoxides, and ketones. Thus, regarding aldehydes, at 70 °C with aeration in EVO oil practically only n-alkanals and (E)-2-alkenals are formed, with 4-hydroperoxy- and 4-hydroxy-(E)-2-alkenals in very small concentrations, although these were the main ones formed in SF oil. As can be observed in Figure 9(b), at advanced oxidation stages these oxygenated aldehydes are almost unappreciable in the spectra.

From these results it is evident that the determination of the concentration of the aldehydic functional group, in a global way, in different oils submitted to degradation is a very crude way to define or to establish oil safety.

As in SF oil, epoxy groups are also formed in EVO oil; nevertheless, in this latter case the main ones are those derived from oleic groups, namely (E)-9,10-epoxystearate groups (signal at 2.63 ppm) and (Z)-9,10-epoxystearate groups (signal at 2.88 ppm; see Figure 9b and 10). By contrast, in SF oil leukotoxin (9,10-epoxy-12-octadecenoate) and/or isoleukotoxin (12,13-epoxy-9-octadecenoate) were formed, in addition to diepoxides, all of them derived from linoleic acyl groups.

Another distinctive characteristic of oils rich in oleic groups oxidized at intermediate temperatures with aeration is the formation, in important concentrations, of keto groups conjugated with a double bond (Gibson 1948), this latter giving the characteristic signals at 6.08 and 6.82 ppm in the spectral region of protons supported on conjugated systems (see Figure 9b and 10). These signals, which had been observed in previous studies (Guillén and Ruiz 2005a, 2005c) and were unidentified at the time, can now...
be identified. This identification has been carried out by comparison with data on similar oxidation compounds provided by other authors (Kuklev and others 1997; Jie and Lam 2004; Lin and others 2007); these keto groups, like epoxides, are derived from the hydroperoxides of oleic groups.

Again, this shows that the determination, in a global way, of conjugated dienic systems is a very crude way of establishing the oxidation degree, because not only primary but also secondary compounds, these of very different natures, have conjugated dienic systems.

In summary, under the same conditions of accelerated storage, the different composition of EVO oil and SF oil determine that: the rate of oxidation in the former is much smaller than in the latter; the main hydroperoxides and epoxides formed in the former are those derived from oleic acyl groups, whereas in the latter they are those derived from linoleic, with leukotoxin (9,10-epoxy-12-octadecenoate), isoleukotoxin (12,13-epoxy-9-octadecenoate), and diepoxides in important concentrations; the main aldehydes formed in EVO oil are n-alkanals and (E)-2-alkenals, with very small amounts of the genotoxic and cytotoxic 4-hydroperoxy-(E)-2-alkenals and 4-hydroxy-(E)-2-alkenals, which are the main ones in oxidized SF oil; and finally that in the oxidized EVO oil, by contrast with oxidized SF oil, chains having keto groups conjugated with a double bond are present in significant concentrations.

Oils rich in linolenic acyl groups: The evolution of the thermo-oxidative process of oils rich in linolenic groups, such as VL oil submitted to 70 °C with aeration, is also very different from that of the previously mentioned oils, as may be expected. The rate of degradation is very high and in 4 d under these degradative conditions linseed oil is totally polymerized.

As Figure 9(c) shows, the spectra of linseed oil do not show well-resolved signals either of protons of hydroperoxides or of those of conjugated dienic systems related to them, these signals being of low intensity in comparison with those observed in SF oil (see Figure 9a). Their low concentration could be due to the instability of these hydroperoxides, which do not accumulate and evolve very quickly to give other compounds. It must be remembered that in the thermo-oxidation at 50 °C of fish lipids, which are also rich in ω-3 groups, hydroperoxides, and the dienic conjugated systems associated with them, were not clearly observed (see Figure 8; Guillén and Ruiz 2004a).

The low resolution of these signals could be due to the overlapping of those of several different structures of hydroperoxides and conjugated dienic systems, supported by octadecadienoates and octadecatrienoates, derived from linoleic and linolenic acyl groups, respectively. Frankel and others (1990) submitted pure trilinolenin to 40 °C and studied the primary oxidation products generated by different techniques, among them 1H NMR. The main compounds identified were several hydroperoxy-octadecatrienoates showing 2 of the double bonds conjugated (see Figure 10b). The characteristic proton signals of these (Z,E)-conjugated dienes of octadecatrienoates appeared at 6.55, 6.00, 5.56, and 5.42 ppm, with chemical shifts that were very similar to those of the (Z,E)-conjugated dienes present in hydroperoxides derived from linoleic (see Table 3).

It is noteworthy that many other different structures of oxidation products derived from pure linolenic acid or ester were identified by 1H NMR, such as dihydroperoxides or dihydroxides associated with conjugated diene-triene systems, hydroxy or hydroperoxy-epidioxides (also called hydroxy or hydroperoxy-cyclic peroxides), and so on (Gardner and Weisleder 1972; Neff and others 1981;
Coxon and others 1984; Frankel and others 1990). This great complexity of structures could explain the above-mentioned low-signal resolution in certain regions of the spectra.

Regarding secondary oxidation compounds, as in SF oil, 4-hydroperoxy- and 4-hydroxy-(E)-2-alkenals, are formed in important concentrations, in addition to n-alkanals, (E)-2-alkenals, (Z,E)-2,4-alkadienals, 4,5-epoxy-2-alkenals, and their precursors (E,E)-2,4-alkadienals. The signals of these aldehydes greatly overlap, as was observed in the oxidation of the fish lipids mentioned before. It is remarkable that from 2.75 d onwards, when VL oil started to polymerize, some signals appeared at 9.38 to 9.42 ppm. Although these signals were previously considered as unknown (Guillén and Ruiz 2005c), now they can be tentatively attributed to the hydroperoxy proton (OOH) present in the above-mentioned hydroperoxy-cyclic peroxides, which are secondary or further oxidation compounds derived from linolenate (Neff and others 1981).

As far as epoxides are concerned, mono- and diepoxides can be tentatively identified, because proton signals near 3 ppm are highly overlapped, probably due to the great variety of structures mentioned before. However, they are in lower concentrations than in SF oil submitted to similar conditions (see Figure 9a and c).

In summary, under the same thermo-oxidative conditions (10 g of oil at 70 °C with aeration) oil composition decisively influences not only the rate of the oxidation process, but also the compounds formed, which are very different from 1 oil to another. The oils taken as examples in the previous paragraphs show significant abundances of the 3 kinds of acyl groups: linoleic, oleic, and linolenic. The performance in the thermo-oxidation processes of other oils having intermediate acyl group composition is also intermediate, in comparison with that exhibited by the above-mentioned oils.

**Evolution of the thermo-oxidation process of refined SF oil submitted to 100 °C with aeration in a convection oven.** In this study, as above, samples of 10 g of SF oil placed in open Petri dishes were submitted to 100 °C with aeration in a convection oven and the evolution of their composition was studied by 1H NMR (Goicoechea and Guillén 2010). As expected, under these conditions the induction period was shorter than in SF oil submitted to 70 °C, and the formation of hydroperoxides associated with (E,E)-conjugated dienic systems predominated from the beginning of the process, attributable to the increase of the temperature by 30 °C (Schneider 2009).

In relation to the generation of aldehydes, it can be seen that the same kinds as those previously detected at 70 °C are also formed at 100 °C; however, the formation of oxygenated α,β-unsaturated aldehydes is less favored than at 70 °C. It is worth mentioning that in the process at 100 °C the detection of aldehydes is produced at lower concentrations of hydroperoxides (77.2 mmol/L at 9 h) than in the process at 70 °C (346.8 mmol/L at 144 h); this proves that the degradation of hydroperoxides at higher temperatures is faster than at lower temperatures. This may be one of the reasons for discrepancies when classical methods are used to evaluate the oxidation level of edible oils. Furthermore, the sequential formation of 4,5-epoxy-2-alkenals and of 4-hydroxy-(E)-2-alkenals after (E,E)-2,4-alkadienals and 4-hydroperoxy-(E)-2-alkenals, respectively, also observed at 70 °C is maintained, together with the diminution in the concentration of the 2 latter types of aldehydes as the concentration of the 2 former increases.

Temperature also affects epoxide formation, favoring that of monoepoxides (leukotoxin [9,10-epoxy-12-octadecenoate] and isoleukotoxin [12,13-epoxy-9-octadecenoate]) over that of diepoxides and their diol derivatives, unlike what was seen at 70 °C.

In short, the increase of the temperature from 70 to 100 °C in the SF oil thermo-oxidative process favors, on the one hand, the formation of hydroperoxy groups supported by chains having (E,E)-conjugated dienic systems versus those supported by (Z,E)-dienic systems, and, on the other hand, reduces the formation of oxygenated α,β-unsaturated aldehydes, as well as of diepoxides versus monoeopoxides.

**Evolution of the thermo-oxidation of edible oils of very different compositions submitted at 190 °C with aeration in a convection oven.** In this study, samples of 10 g of oil put in open Petri dishes were also submitted to 190 °C with aeration in a convection oven until total polymerization; the oils in this study were rich in oleic (virgin olive, olive, hazelnut), linoleic (corn, SF), and linolenic acyl groups (soybean, linseed; Guillén and Ruiz 2008). The decreasing order of resistance to degradation was as follows: virgin-olive oil > olive oil > hazelnut oil > corn oil > SF oil > soybean oil > linseed oil, in agreement with the studies carried out at 70 °C with aeration (Guillén and Ruiz 2005a, 2005b, 2005c). In general, the lower the content in polyunsaturated groups in the oil, the higher its resistance to degradation; however, other factors, such as the presence of minor antioxidant components, play a very important role in the degradation rate of the oils, including when the oil is submitted to high temperatures. So, the virgin olive oil studied contains similar proportions of oleic and linoleic groups to olive oil, but its degradation rate is lower, making it evident that the differences found in the resistance against degradation among the different oils studied are not only due to the structure and composition of the main components.

Regarding the formation of oxidation compounds, neither protons of hydroperoxy groups nor protons of conjugated dienic systems bonded to hydroperoxy or hydroxy derivatives were observed in the 1H NMR spectra of the oils kept under these degradative conditions, while aldehydic signals were the first to be observed. The signals observed between 6.08 and 7.09 ppm are related to the conjugated double bonds present in aldehydes (Goicoechea and Guillén 2010). This fact indicates that, if primary oxidation compounds are formed, they degrade at very high rates and do not accumulate in sufficient amounts to be detected by 1H NMR.

It is noteworthy that oils of different compositions generated aldehydes of different natures and also in different proportions. Thus, in virgin olive, olive, and hazelnut oil, only significant concentrations of (E)-2-alkenals and of n-alkanals are generated. However, in oils rich in polyunsaturated groups, in addition to n-alkanals and (E)-2-alkenals, significant proportions of (E,E)-2,4-alkadienals are also formed, as well as 4-hydroxy-(E)-2-alkenals, those tentatively identified as (Z,E)-2,4-alkadienals, and also small proportions of 4,5-epoxy-2-alkenals. In all the oils, 4-oxo-alkanals, a kind of aldehyde typical of frying temperatures, were tentatively identified (Takeoka and others 1995). In none of the oils were 4-hydroperoxy-(E)-2-alkenals detected. In comparison with the thermo-oxidation process of these oils at 70 °C with aeration (Guillén and Ruiz 2005a, 2005b, 2005c), the increase of the temperature to 190 °C provoked a lesser generation of oxygenated α,β-unsaturated aldehydes. It is also remarkable that oils rich in oleic acyl groups were not only more resistant to degradation than oils rich in linoleic and linolenic acyl groups, but also produced lower proportions of toxic aldehydes.

Regarding other secondary or further oxidation compounds, small proton signals at 6.08 ppm and 6.82 ppm related to keto groups conjugated with a double bond were observed in oils rich in...
oleic acyl groups; as mentioned before, these signals also appeared when these oils were submitted to 70 °C with aeration, but in much greater intensities.

In short, if primary oxidation compounds were formed at 190 °C with aeration, they degraded so fast that they cannot be observed in the ¹H NMR spectra. As for aldehydes, in comparison with the thermo-oxidation with aeration at lower temperatures, smaller concentrations of oxygenated α,β-unsaturated aldehydes were generated; 4-hydroperoxy-(E)-2-alkenals were not detected, and 4-oxo-alkanals were.

Evolution of the thermo-oxidation process of virgin olive, refined corn, and VL oils submitted to microwave action at 190 °C. In this study, virgin olive, refined corn, and VL oils (10 g of oil in Petri dishes) were repeatedly submitted to the effect of microwave heating at 190 °C without exceeding this temperature (Guillén and Ruiz 2006) for 240 min. In virgin olive oil the highest level of degradation was undergone by the oleic acyl groups, in corn oil by the linoleic, and in linseed oil by the linolenic acyl groups. In contrast with observations made at lower temperatures, none of these acyl groups was totally degraded in any of the 3 oils, with a very small remaining molar percentage of linoleic in virgin olive and in corn oils after 240 min.

The times at which the initiation of the degradation was detected in these 3 oils were very similar in this process and when submitted to 190 °C with aeration in a convection oven (Guillén and Ruiz 2008), suggesting that the heat supplied to the oil affects the rate of its degradation to a higher degree than the means by which the heat is produced.

Regarding the formation and occurrence of primary oxidation compounds, only very slight and occasional hydroperoxide proton signals appear between 8.4 and 8.6 ppm in the ¹H NMR spectra (in virgin olive oil at minute 70 and in corn oil at minute 40), which were not accompanied by signals of protons of associated dienic conjugated systems. Signals of aldehydes were detected at the same time as the appearance of these rare incipient signals of hydroperoxides. In VL oil, hydroperoxides were not clearly detected. The absence of significant concentrations of hydroperoxides in the oils submitted to microwave-heating is remarkable, along with the absence of related dienic systems, and the rapid generation of aldehydes. These results are in agreement with those obtained in oils submitted to 190 °C with aeration, but in such a way that they are not present in the sample in significant concentrations at any time. This is in agreement with the absence of significant changes in the classical hydroperoxide value assay of oils submitted to frying temperature (Doubarges and Pérez-Camino 1988), and with the observations by other authors concerning the formation of aldehydes in oils heated at high temperature without previous induction periods (Silvagni and others 2010). Moreover, the classical parameter, conjugated dienes, associated with the concentration of acyl group chains having conjugated dienic systems in the oils, in other words, primary oxidation compounds, which is determined by absorption at 232 nm in ultraviolet (UV) spectroscopy, is not useful for this purpose in oils submitted to these conditions (190 °C). This is so because, as Figure 11 shows, the only signals present at this temperature, in the spectra of protons belonging to conjugated dienic systems, are those of unsaturated aldehydes and of other secondary oxidation compounds.

With regard to the formation of aldehydes under deep-frying conditions, there are very important differences between oils. Thus, in EVO oil, as Figure 11(b) shows, the most abundant aldehydes are the least reactive, that is (E)-2-alkenals and n-alkanals, whereas those generated from corn and VL oils are (E,E)-2,4-alkadienals. Furthermore, virgin olive oil is the only one that does not produce 4-hydroxy-(E)-2-alkenals. These facts are very important because of the different reactivity and toxicity of the several kinds of aldehydes.

In short, the results obtained after submitting the oils to microwave action at 190 °C are in agreement with those obtained at 190 °C with aeration in a convection oven. The absence of significant concentrations of primary oxidation compounds and the rapid generation of aldehydes, especially nonoxygenated ones, is noteworthy.

Evolution of EVO, refined SF, and VL oils under deep-frying conditions at 190 °C in the absence of food. EVO, refined SF, and VL oils were submitted to deep-frying conditions (4 L oil) without food at 190 °C for a prolonged period of time (Guillén and Uriarte 2012a, 2012b, 2012c). Regarding the degradation of the acyl groups, it can be observed that the highest rate occurred in the main unsaturated acyl group in the oil, independently of its unsaturation degree. Furthermore, it can also be seen that the molar percentages of the acyl groups, which are less saturated than the main one, decreased with heating time, whereas those more saturated either increased or remained unchanged. Thus, the highest decrease per hour in VL oil was produced in the molar percentage of linoleic acyl groups, in SF oil in the molar percentage of linoleic acyl groups, and in EVO oil in the molar percentage of oleic acyl groups; these results indicate that the rate of degradation of the various acyl groups, at frying temperature, depends more on their concentrations in the oil than on their unsaturation degree, this latter associated with their facility or tendency to oxidize.

Under these conditions formation of primary oxidation compounds was not observed. As can be observed in Figure 11, proton signals of hydroperoxy groups, as well as of conjugated dienes, were not detected, in agreement with what was observed during thermo-oxidation at 190 °C in a convection oven (Guillén and Ruiz 2008) and at 190 °C under microwave action (Guillén and Ruiz 2006). This fact indicates that in oils submitted to high temperatures if hydroperoxides are formed, they decompose very quickly, independently of the heating method, in such a way that they are not present in the sample in significant concentrations at any time. This is in agreement with the absence of significant changes in the classical hydroperoxide value assay of oils submitted to frying temperature (Doubarges and Pérez-Camino 1988), and with the observations by other authors concerning the formation of aldehydes in oils heated at high temperature without previous induction periods (Silvagni and others 2010). Moreover, the classical parameter, conjugated dienes, associated with the concentration of acyl group chains having conjugated dienic systems in the oils, in other words, primary oxidation compounds, which is determined by absorption at 232 nm in ultraviolet (UV) spectroscopy, is not useful for this purpose in oils submitted to these conditions (190 °C). This is so because, as Figure 11 shows, the only signals present at this temperature, in the spectra of protons belonging to conjugated dienic systems, are those of unsaturated aldehydes and of other secondary oxidation compounds.
and Uriarte 2012d), which has a lower boiling point (177 °C) than the frying temperature (190 °C), and for that reason a large quantity of this aldehyde escapes into the atmosphere. However, in SF oil the (E,E)-2,4-alkadienals formed were mainly (E,E)-2,4-decadienal and (Z,E)-2,4-decadienal, both with higher boiling points (244 °C) than the frying temperature (190 °C), and for that reason their ability to escape from the oil matrix into the atmosphere is greatly diminished, so they remain in the oil liquid matrix to a large extent. Nevertheless, it has to be noted that the formation of aldehydes in thermo-oxidation processes at 190 °C is affected by the air–oil contact surface in relation to the oil volume (Guillén and Ruiz 2006, 2008; Guillén and Uriarte 2009, 2012c).

In short, under frying conditions, once again, EVO oil generates lower concentrations of the most reactive aldehydes than oils rich in polyunsaturated groups, such as SF and VL oils.

In addition to aldehydes, other secondary compounds are also formed under these conditions and they too are different depending on the oil nature. So, the $^1$H NMR spectra of SF oil submitted to deep-frying conditions do not have signals of epoxides or of the diol derivatives; the only signals that are visible here are the side bands of the bis-allylic protons (see Figure 11a). However, when it comes to EVO oil, the formation of 2 epoxides derived from oleic acyl groups, such as (E)-9,10-epoxystearate (signal at 2.63 ppm) and (Z)-9,10-epoxystearate (signal at 2.88 ppm), is observed (Du and others 2004; Marmesat and others 2008; Guillén and Uriarte 2012a; Martínez-Yusta and Guillén 2014a). And, finally, no signals of epoxidic protons were observed in the spectra of VL oil submitted to the same conditions as above.

Concerning alcohols, only signals attributable to primary alcohols were detected in SF oil spectra, whereas in that of EVO oil signals attributable to primary and to secondary ones were observed, the formation of this functional group in the degradation of VL oil under deep-frying conditions being unclear. The formation of hydroxyl groups in edible oils submitted to frying temperature has been reported previously (Schwartz and others 1994).

In short, when oils are submitted to deep-frying conditions at 190 °C, in the absence of food, their thermo-oxidation evolves without the occurrence, in measurable concentrations by this technique, of primary oxidation compounds (hydroperoxy groups and conjugated dienic systems). In comparison with thermo-oxidation processes at lower temperatures with aeration, lower concentrations of oxygenated secondary oxidation compounds are generated. Differences between the performance of oils as a function of their compositions can be observed.

Conclusions

$^1$H NMR gives very valuable information about the different types of protons present in food lipids, which can be supported by
main and minor compounds. This spectroscopic technique is fast and simple, requires no chemical modification of the sample, and provides a great deal of information on the lipid sample as a whole, this latter fact being very important. It is very useful not only for the study of the nondegraded food lipids, but also for the study of the degraded ones. For this reason it is very useful to follow, in a qualitative and quantitative way, the evolution of food lipids when they are submitted to degradative conditions. It allows not only the identification of many of the main functional groups formed, but also to quantify them providing, in this way, information of the thermodegradative processes.

From the results described here, it can be concluded that the use of classical methods to determine the concentration of functional groups in a global way (hydroperoxides, conjugated dienes, aldehydes, and so on) in food lipids of different compositions when submitted to different degradation conditions, is a very crude way of analyzing their thermo-oxidation processes. It should be taken into account that each functional group includes compounds of very different reactivities and toxicities.

The studies mentioned here have demonstrated that both food lipid composition and the conditions under which the thermo-oxidation process takes place govern the mechanisms by which the process evolves, as well as affecting the nature and concentrations of intermediate and finally formed compounds.

From the above-mentioned studies it can also be concluded that polysaturated food lipids submitted to oxidative conditions at low temperatures are able to generate a great number of different kinds of very reactive oxygenated compounds, some of which are known to be toxic, such as 4-hydroperoxy-, and 4-hydroxy-(E)-2-alkenals, or 9,10-epoxy-12-octadecenoate (leukotxin) and 12,13-epoxy-9-octadecenoate (isoleukotxin) derivatives. As the temperature increases, the number of different kinds of compounds which may be formed from these lipids decreases. At low temperatures the process is slow; however, at high temperatures it takes place very quickly. Although the number and concentration of the most reactive compounds which may form from polysaturated lipids at high temperatures is smaller than at low temperatures, they can accumulate with heating time.

Food lipids with small concentrations of polysaturated groups and significant concentrations of compounds that exhibit antioxidant ability, when submitted to thermo-oxidative conditions at low temperature, not only generate much lower concentrations of the most reactive and toxic lipid-derived compounds, but this formation occurs much more slowly than in the case of the food lipids, which are rich in polysaturated groups. When these lipids are submitted to high temperatures the rate of their degradation increases, as does that of those rich in polysaturated groups, but the range of compounds formed is different from those formed in the latter.

In addition to the influence of both food lipid nature and the temperature during which the thermo-oxidation evolves, another important factor is the food lipid surface exposed to the oxygen. When this surface is high in relation to the oil volume involved, then higher concentrations of oxygenated derivatives are formed than when the exposed surface is small. This fact is very important because some of the oxygenated compounds are very reactive and toxic.

The results summarized here are worthy of consideration because some of the above-mentioned toxic compounds detected by this technique during food lipid thermo-oxidation processes have also been found in human cells and tissues. Finally, it only remains to be added that the results commented on and discussed here may be helpful in the interpretation of results obtained in metabolomic and lipidomic studies based on $^1$H NMR techniques.

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