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Study of the oxidative stability of salted and unsalted salmon fillets by ^{1}H nuclear magnetic resonance

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Abstract

Farmed salmon fillets were dry-salted for 24 h. Both salted and unsalted samples were submitted to oxidative conditions at 50 °C in an oven with circulating air. Lipids of both samples were extracted daily from aliquots and studied by ^{1}H nuclear magnetic resonance. The ^{1}H Nuclear Magnetic Resonance spectra of the salmon lipids, as well as their usefulness for determining the proportions of different acyl groups are commented on. Furthermore, not only the rate of degradation of different acyl groups but also the rate of generation of oxidation compounds can be evaluated simultaneously throughout the oxidation process, showing the use of this technique to study the oxidative stability of fish lipids samples and their oxidation degree. The reduction of the oxidative stability of the lipids, caused by the dry-salting process, is proved.

Keywords: Farmed salmon; Lipids; ^{1}H NMR spectroscopy; Salting; Oxidative stability; Oxidation

1. Introduction

The measure of lipid oxidative stability is an important task because this property determines the shelf-life of many foods. Foods having low oxidative stability degrade quickly, producing undesirable flavours and unhealthy compounds. This property is especially significant in foods containing high proportions of unsaturated acyl groups, such as in fish. However, it has been commented that studies on the oxidative stability of fats containing high proportions of polyunsaturated acyl groups have produced very different results, which are, in some cases, difficult to understand (Frankel, Satué-Gracia, Meyer, & German, 2002). This may be due to various reasons one being the absence of a generally accepted approach to the measurement of oxidative stability; there is no agreement about the oxidative conditions to which the samples should be submitted, nor to which parameter or parameters should be used, to achieve the highest accuracy to define the oxidation degree reached, nor which methodology is the most convenient. The inaccuracy and/or disadvantages of some methods used to measure lipid oxidation such as peroxide value, thiobarbituric acid reactive substances, weight-gain, oxygen absorption, or fatty acid analyses by gas chromatography have been widely criticised (Cho, Miyashita, Miyazawa, Fujimoto, & Kaneda, 1987; De Koning & Silk, 1963; Endo, Hoshizaki, & Fujimoto, 1997; Frankel et al., 2002; Kaitaranta, 1992; Miyashita, Nara, & Ota, 1993; Song & Miyazawa, 1997; Yu & Sinhuber, 1967).

Some authors only use one parameter to determine oxidation degree of fish lipids; this can be based on the concentration of primary or of secondary oxidation products. Others determinate two parameters, one related to the concentration of primary oxidation products, and a second one related to the concentration of secondary oxidation products. However, in some cases, there is discrepancy between the oxidative stability of a system defined by primary and that defined by secondary oxidation compounds; this may be due to the different rate of formation of primary and secondary oxidation products in different samples. Furthermore, it should be taken into account that primary oxidation compounds do not confer odour to the food sample and only secondary oxidation products are responsible for

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rancidity. For these reasons the development of new methods to evaluate oxidation degree and oxidative stability has been encouraged (Connell, 1990; Hernandez-Herrero, Roig-Sagués, Lopez-Sabater, Rodríguez-Jerez, & Mora-Ventura, 1999; Smith, 1995).

Some spectroscopic techniques provide information about the sample as a whole, giving information on the various functional groups contained. This is true for Fourier transform infrared spectroscopy and also for ^{1}H nuclear magnetic resonance (^{1}H NMR). Both have been considered very useful for the study of edible oils (Guillén & Cabo, 1997, 1998; Guillén & Ruiz, 2001), as well as for determining the proportions of the different acyl groups (Ketshajwang, Holmback, & Yeboah, 1998; Miyake, Yokomizo, & Matsuzaki, 1998; Sacchi, Addeo, & Paolillo, 1997) and for studying of the oxidative stability of fats and oils (Abou-Gharbia, Shahidi, Shehata, & Youssef, 1997; Guillén & Cabo, 1999, 2000; Saito, 1997; Shahidi, Wanasundara, & Brunet, 1994). Concretely, the usefulness of the ^{1}H NMR spectroscopy in the study of edible lipids has been recently reviewed (Guillén & Ruiz, 2001). New contributions in this field have been made in relation with the usefulness of shape and chemical shift of the signals to discriminate between edible oils (Guillén & Ruiz, 2003a) and with the ability of ^{1}H NMR spectroscopy in quantification of oils and fats (Guillén & Ruiz, 2003b, 2003c, 2003d). All these findings can be applied in the study of fish lipids.

Therefore, in this paper ^{1}H nuclear magnetic resonance is used to study the oxidative degradation of the lipids of salted and unsalted farmed salmon fillets, with a new perspective. This paper has as aims to test not only the usefulness of the most significant signals of the ^{1}H NMR spectra in determining the oxidation degree but also to evaluate the ability of this technique in the simultaneous detection of primary and secondary oxidation products and, lastly, to study the effect of the salting process on the oxidation and oxidative stability of farmed salmon, because contradictory results have been reported (Bal & Khvalova, 1972; Basby, Jeppesen, & Huss, 1998; Hultin, 1994; Toshiyuki, 1997; Yankah, Ohshima, & Koizumi, 1993).

2. Materials and methods

2.1. Samples, salting and oxidation conditions

Farmed salmon were acquired at a local supermarket. After gutting and cleaning, some of the fillets were salted by covering them completely with culinary salt for 24 h at 3 °C, afterwards the surface salt was removed. The salted and unsalted fish fillets were placed in a *Selecta* convection oven, at 50 °C with a stability of ±0.5%, with circulating air to provoke their oxidation.

2.2. Extraction of lipids

Lipids of the samples were extracted from aliquots of meat fillets daily, using carbon di-sulphide as solvent, and an ultrasonic bath for one hour in order to ensure an exhaustive extraction. This solvent was selected because of its ability to extract lipids, its high volatility and because it does not give signals in ^{1}H NMR. The solvent was eliminated by means of a rotative evaporator at 25 °C and under reduced pressure.

2.3. Spectral acquisition conditions and study of the ^{1}H NMR spectra

The ^{1}H NMR spectra were recorded on a Varian 300 Plus spectrometer operating at 299,862 MHz. Each lipid sample, weighing 0.2 g, was mixed with 400 μl of deuterated chloroform and a small proportion of TMS as 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 32, acquisition time 3.744 s, pulse width 90°, with a total acquisition time of 3.37 min. The experiment was carried out at 25 °C. The assignment of the signals was made taking into account previous studies (Guillén & Ruiz, 2001). The area of the signals was determined by using the software of the equipment and the integrations were made three times to obtain average values.

The whole experiment was done in duplicate in order to ensure consistency of the results.

3. Results and discussion

Salmon lipids are constituted basically by triacylglycerols, having saturated, monounsaturated, di-unsaturated and polyunsaturated (eicosapentanoic and docosahexaenoic) acyl groups, and small proportions of phospholipids (Aursand, Rainuzzo, & Grasladen, 1993). The ^{1}H NMR spectrum of the lipids of salted and unsalted salmon fillets before to be submitted to oxidative conditions is made up of eleven signals of significant intensity, due to protons of the main components. Fig. 1 (days 1–17) shows the ^{1}H NMR spectrum of the lipids of unsalted salmon fillets before to be submitted to oxidative conditions; the assignment of its signals is given in Table 1. Fig. 2 gives de enlargement of some of the most important signals of the ^{1}H NMR spectrum of farmed salmon lipids. Signal **1**, between 0.83 and 0.93 ppm (Figs. 1 and 2), has four peaks, at 0.899, 0.889, 0.879 and 0.856 ppm, due to the overlapping of the triplets of methylic protons of the saturated, ω-7 and/or ω-9 monounsaturated acyl groups and that of the ω-6 di-unsaturated acyl groups; taking into account the height of these peaks it can be said that, in this sample, saturated and monounsaturated acyl groups are in

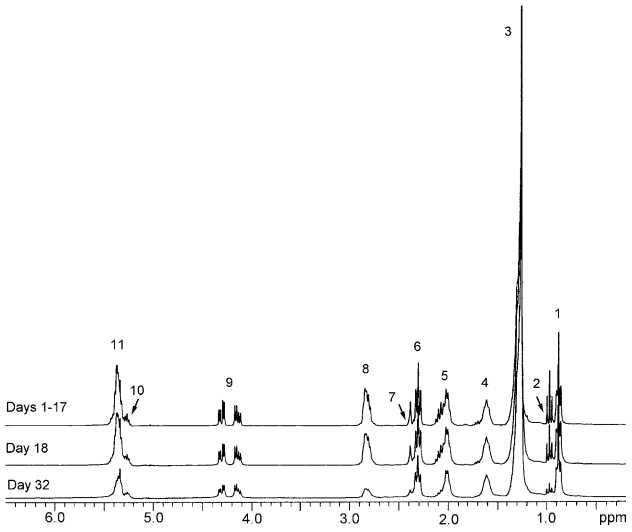

Fig. 1. ^{1}H NMR spectra of the unsalted salmon fillets lipids in different days of the oxidation process.

higher proportions than diunsaturated acyl groups, in agreement with previous studies (Guillén & Ruiz, 2003a). Signal **2**, between 0.93 and 1.03 ppm (Figs. 1 and 2), has three peaks, at 0.944, 0.969 and 0.994 ppm, due to the triplet of methylic protons of the ω-3 polyunsaturated acyl groups. These two signals have been used to determine the proportions and concentrations of ω-3 polyunsaturated acyl groups in fish lipids (Aursand et al., 1993; Igarashi et al., 2000; Sacchi, Medina, Aubourg, Addeo, & Paolillo, 1993). Signal **3**, between 1.22 and 1.42 ppm (Figs. 1 and 2), has one peak at 1.259 ppm and three shoulders, at 1.28, 1.30 and 1.31 ppm, due to methylenic protons either in position β or further, in relation to double bonds, or in position γ or further, in relation to the carbonyl group in the different acyl groups; obviously docosahexaenoic (DHA) and eicosapentaenoic (EPA) acyl groups make no contribution to this signal. The height of the peak at 1.259 ppm, in relation to the shoulders in this signal, indicates that the

Table 1
Assignment of the signals of the ^{1}H NMR spectra of salmon lipids

Signal	Chemical shift (ppm)	Functional group	Intensity[a]
1	0.83–0.93	–**C**H$_3$ (saturated, oleic and linoleic acyl group)	m
2	0.93–1.03	–**C**H$_3$ (linolenic acyl group)	v
3	1.22–1.42	–(**C**H$_2$)$_n$– (acyl group)	l
4	1.52–1.70	–OCO–CH$_2$–**C**H$_2$– (acyl group)	m
5	1.94–2.14	–**C**H$_2$–CH=CH– (acyl group)	m
6	2.23–2.36	–OCO–**C**H$_2$– (acyl group)	m
7	2.36–2.41	–OCO–**C**H$_2$– (docosahexanoic acyl group)	m
8	2.70–2.84	=HC–**C**H$_2$–CH= (acyl group)	v
9	4.10–4.32	–**C**H$_2$OCOR (glyceryl group)	m
10	5.20–5.26	>**C**HOCOR (glyceryl group)	s
11	5.26–5.40	–**C**H=**C**H– (acyl group)	m

The signal number agrees with those in Fig. 1.
[a] l, large; m, medium: s, small; v, variable.

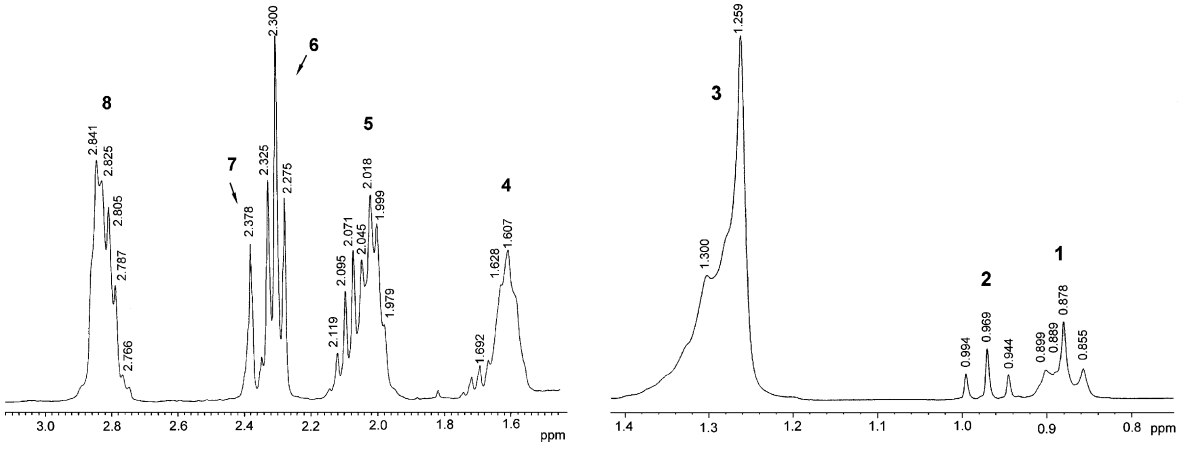

Fig. 2. Enlargement of some signals of the ^{1}H NMR spectrum of the farmed salmon lipids.

proportion of saturated acyl groups in farmed salmon lipids is much greater than in vegetable oils. Signal **4**, between 1.52 and 1.70 ppm (Figs. 1 and 2), is due to methylenic protons in β position in relation to the carbonyl group; DHA acyl group does not contribute to this signal. Signal **5**, between 1.94 and 2.14 ppm (Figs. 1 and 2), having peaks at 2.00, 2.02, 2.05, 2.07, 2.10 and 2.12 ppm, and a shoulder at 1.98 ppm, is due to the overlapping of the various signals of allylic protons, that is to say of α methylenic protons in relation to a single double bond in the different functional groups; this signal appears to be basically the result of the overlapping of the peaks of monounsaturated acyl groups causing a doublet at 2.00 and 2.02 ppm, together with a shoulder at 1.98 ppm and those of polyunsaturated acyl groups which cause multiplets with peaks of different heights at 2.05, 2.07, 2.10 and 2.12 ppm. It must be noted that each DHA acyl group contributes only two protons to this signal. Signal **6** due to methylenic protons in α position in relation to the carbonyl group appears between 2.23 and 2.36 ppm (Figs. 1 and 2), DHA acyl group does not contribute to this signal (Pouchert & Behnke, 1993). Signal **7**, between 2.36 and 2.41 ppm (Figs. 1 and 2), is a singlet (2.38 ppm) due to methylenic protons in α and β positions in relation to the carbonyl group of DHA acyl group; this signal is typical of fish lipids and is absent in the ^{1}H NMR spectra of vegetable oils. Signal **8**, between 2.70 and 2.84 ppm (Figs. 1 and 2), has four peaks at 2.79, 2.81, 2.83 and 2.84 ppm due to the overlapping of several triplets of the methylenic protons in α position in relation to two double bonds of the different acyl groups, also named bis-allylic protons; this signal appears at higher chemical shifts than that of di-unsaturated (2.75, 2.77 and 2.79 ppm) and triunsaturated (2.78, 2.80 and 2.82 ppm) acyl groups (Guillén & Ruiz, 2003a). Signal **9** at 4.10–4.32 ppm (Fig. 1), is due to the protons on 1 and 3 carbon atoms of the glyceryl group and signal **10**, at 5.20–5.26 ppm (Fig. 1), is due to protons on the carbon atom 2 of the same glyceryl group. This latter signal overlaps slightly with signal **11**, at 5.26–5.40 ppm (Fig. 1), due to olefinic protons of the different acyl groups.

The most significant differences between the ^{1}H NMR spectra of farmed salmon lipids and those of vegetable edible oils are to be seen not only in the shape, the number of peaks, the chemical shifts and the intensity of some common signals but also in the presence of a new signal at 2.38 ppm corresponding to methylenic protons in α and β positions in relation to the carbonyl group of DHA acyl group.

In addition to the above mentioned signals due to the main functional groups, there are others which are only observable by means of the enlargement of the spectrum. Among these there is a weak broad signal centred near 5.62 ppm, a very weak multiplet between 5.72 and 5.88 ppm and a weak triplet between 5.90 and 6.10 ppm, centred at 6.00 ppm, all of them assignable tentatively to protons of different conjugated diene systems. Furthermore, very weak signals assignable tentatively to phospholipids (Aursand et al., 1993), at 3.30, 3.72, 3.80 and 4.00 ppm have also been observed.

From a quantitative point of view the proportion of certain acyl groups can be determined from the area of some signals. As is known, the area of the ^{1}H NMR spectra signals is proportional to the number of protons of each type in the sample. Bearing in mind that each acyl group has in turn a methyl group, using the area of the methylic protons both the proportion of ω-3 acyl groups and of saturated, ω-9, ω-7 monosaturated and ω-6 di-unsaturated acyl groups can be calculated. Table 2 gives the proportions thus obtained. Furthermore, the area of the signals of the methylene groups in α and β position in relation to the carbonyl group in DHA acyl groups (signal between 2.36 and 2.41 ppm) and that of methylene groups in α position in relation to the carbonyl group of the other acyl groups (signal between 2.23 and 2.36 ppm) can give the proportion of DHA acyl groups in the sample; this is close to 10% (see Table 2). Taking into account that the proportion of mono- and di-glyceride as well as of phospholipid molecules in these farmed salmon lipids samples is not significant, the area of signal **9**, corresponding to the four protons on 1 and 3 carbon atoms of the glyceryl group, was fitted to four units; thus the values of the other signal areas are referred to a triglyceride molecule in an approximate way. This being so, the area of the signal of allylic protons, taking into account that each DHA acyl group contributes only two protons and that any other unsaturated acyl groups contribute four protons to the signal, gives the proportion of saturated and so of unsaturated acyl groups. The proportion of monounsaturated, together with di-unsaturated, acyl groups can be determined by difference, starting from the values obtained above. Obtained data are indicated in Table 2; these proportions are in satisfactory agreement for Atlantic salmon with those obtained by other authors using different techniques (Aursand et al., 1993; Espe, Nortvedt, Lie, & Hafsteinsson, 2002).

As commented above, salted and unsalted salmon fillets were submitted to oxidative conditions and daily lipids of aliquots were extracted and studied by ^{1}H NMR. The spectra of the lipids of unsalted salmon fillets

Table 2
Proportions of some acyl groups in lipids of the farmed salmon fillets

Acyl group	Percentage	Acyl group	Percentage
ω-3	25.3	SMDU	74.7
DHA	10.2	MDU	47.2
OHU	15.1	U	72.5

ω-3, omega-3 acyl groups; DHA, docosahexaenoic acyl group; OHU, other highly unsaturated different from DHA acyl groups; SMDU, saturated, mono- and di-unsaturated acyl groups; MDU, mono- and di-unsaturated acyl groups; U, unsaturated acyl groups.

under oxidative conditions do not show variations from the beginning of the experiment to day seventeen. However from day seventeen onwards some variations are observed in the spectra, not only with regard to changes in the intensity of the signals of the main components, but also to the appearance of new signals due to components generated in the oxidation process.

Fig. 1 shows changes in the intensity of some signals in the ^{1}H NMR spectra of the lipids of unsalted salmon fillets throughout the oxidation process. It can be observed that the intensity of those signals due exclusively to polyunsaturated acyl groups, such as signals **2**, **7** and **8,** are apparently much more affected by oxidation than those due to unsaturated acyl groups in general. From the integration of different signal areas and bearing in mind the above mentioned considerations, the changes throughout the oxidation process, in the proportions of the different acyl groups in the lipids of unsalted salmon fillets, can be determined. Fig. 3 shows the variation of the percentage of different acyl groups in the lipids of unsalted salmon fillets throughout the process, which can be taken as the kinetic of their degradation. It can be observed that between days 1 and 17 no changes are observed in the proportions of the different acyl groups; however, from day 18 the proportion of polyunsaturated acyl groups, such as ω-3 polyunsaturated, DHA, and the, so denominated, ω-3 OHU (other highly unsaturated acyl groups different from DHA), as well as of mono- and di-unsaturated acyl groups decreases. The highest rate in degradation is shown by the ω-3 polyunsaturated acyl groups and within this, the ω-3 OHU acyl groups; DHA also shows a high rate of degradation. It is also noteworthy that mono- and di-unsaturated acyl groups are degraded in very important proportions though at a lower rate than the above mentioned acyl groups. Apparently, only the saturated acyl groups are not affected by the oxidative degradation. From the results obtained it is evident that ^{1}H NMR is a powerful technique not only for determining the oxidative stability or resistance to oxidation of salmon lipids, but also to know the oxidation level reached by any salmon lipid sample; at the same time this technique provides information on the rate of degradation of the different acyl groups throughout the oxidation process.

Previously, the use of ^{1}H NMR had been proposed as an alternative method to determine the rate at which lipid oxidation advances, based on the relative ratio

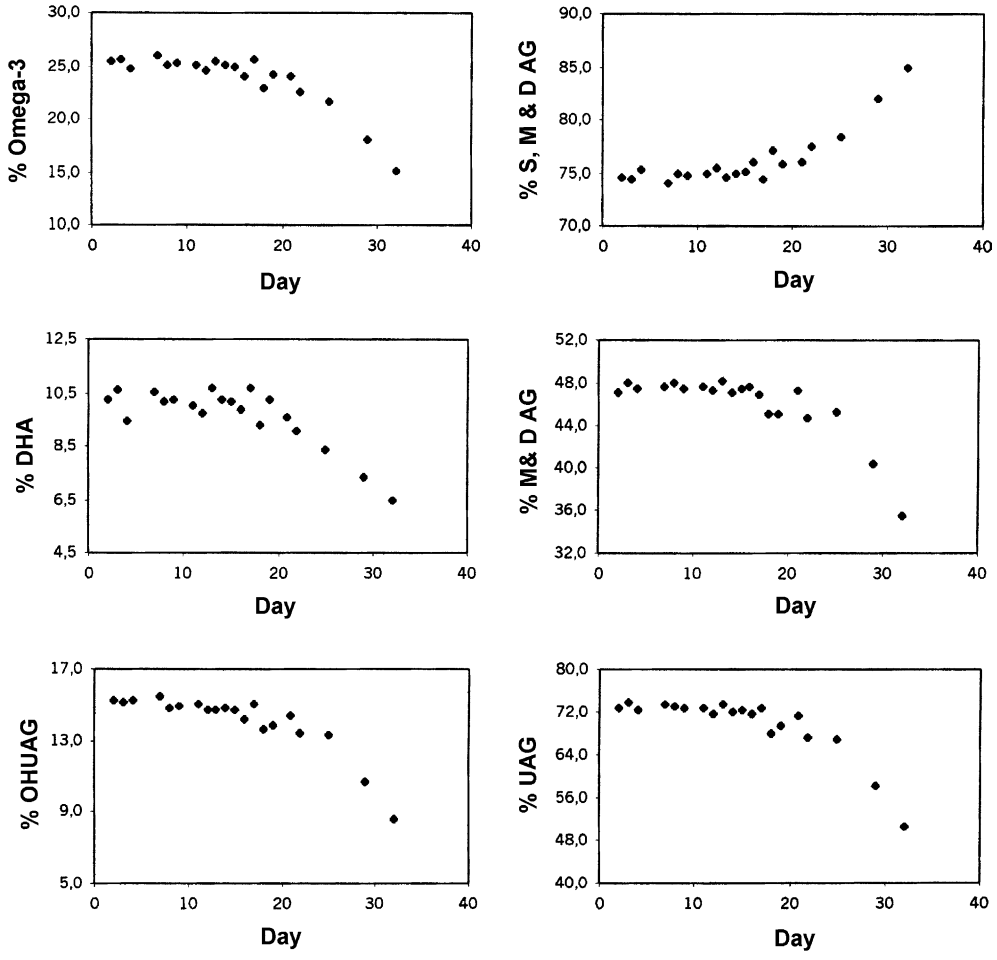

Fig. 3. Changes in the proportion of some acyl groups of the unsalted salmon fillet lipids throughout the oxidation process.

changes of aliphatic to olefinic proton (R_{ao}, that is to say the ratio between the area of signals **1**, **2**, **3**, **4**, **5**, **6**, **7**, and **8** together and the area of signal **11**) or of aliphatic to di-allylmethylene proton (R_{ad}, that is to say the ratio between the area of signals **1**, **2**, **3**, **4**, **5**, **6**, **7**, and **8** together and the area of signal **8**) or the inverse ratios R_{oa} and R_{da} (Abou-Gharbia et al., 1997; Saito, 1997; Shahidi et al., 1994). The variation of these ratios for lipids of unsalted salmon fillets throughout the oxidation process are shown in Fig. 4; it can be observed that from the start of the experiment to day 17 the values of these parameters are constant and from day 18 onwards they begin to increase, showing that oxidation has begun.

In the same way, many other ratios between area of signals (As), in which protons of saturated and unsaturated acyl groups are involved provide information about the changes suffered by the sample under oxidative conditions; for example ratios As**1**/As**2**, As**1**/As**5**, As**1**/As**7**, As**1**/As**8**, As**1**/As**11**, As**3**/As**2**, etc. The changes shown by all these parameters coincide with that of Rao and Rad and with those previously observed in the variation of the proportions of the several acyl groups above mentioned.

In addition to changes in the proportion of the different acyl groups throughout oxidation the appearance of new signals due to products generated in the process can be observed. Thus, in the oxidation of edible oils the appearance and disappearance of hydroperoxides, or primary oxidation compounds, as well as the appearance of secondary oxidation products such as aldehydes, or secondary oxidation products, has been observed by this technique (Guillén, Ibargoitia, Cabo, & Ruiz, 2000). Fig. 5 shows the enlargement of the region comprised between 5.40 and 10 ppm of the ^{1}H NMR spectra of the lipids of unsalted salmon fillets submitted to oxidative conditions on different days of the process. From the beginning of the experiment to day 17 no changes are observed, but from day 18 a modification of the signals between 5.40 and 7.20 ppm is visible; this can be probably due to the appearance of new conjugated systems. Signals of saturated aldehydic protons between 9.70 and 9.90 ppm are visible from day 18. In the following days, signals of saturated and unsaturated aldehydic protons, between 9.30 and 9.90 ppm, and of hydroperoxides, between 8.00 and 8.20 ppm, appear. Although in the study of the oxidation of safflower oil the presence of hydroperoxides was observed before the formation of aldehydes (Guillén et al., 2000), in the oxidation of lipids of unsalted salmon fillets hydroperoxides were observed almost at the same time as aldehydes, but in much lower proportions. The almost simultaneous appearance of the so called primary and secondary oxidation products could be due to the high rate of degradation of these primary oxidation compounds to yield secondary oxidation products, making their observation by ^{1}H NMR difficult. The small presence of hydroperoxides in the oxidation of salmon lipids and the coincidence in time with the presence of aldehydes has also been observed by means of Fourier transform infrared spectroscopy. The appearance of hydroperoxides and aldehydes detectable by this technique can be also taken as a measure of oxidative stability; the detection of the appearance of aldehydes is especially important because they are responsible for rancidity.

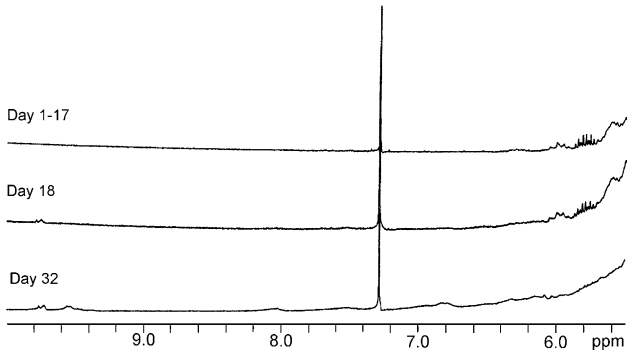

Fig. 5. Enlargement of the ^{1}H NMR spectral region comprised between 5.5 and 10.0 ppm in different days of the oxidation process.

The same study was carried out on the lipids of salted salmon fillets. Fig. 6 shows the evolution of the proportions of some acyl groups of the salted sample throughout the different days of the process. It is evident

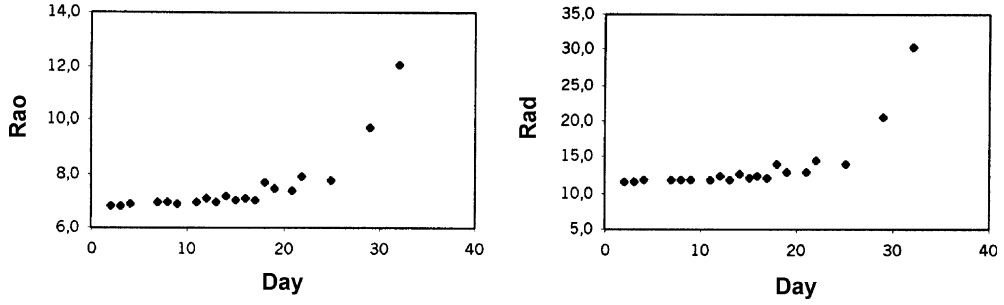

Fig. 4. Changes in the ratio of aliphatic to olefinic protons (R_{ao}) and of aliphatic to di-allylmethylene protons (R_{ad}) of unsalted salmon fillet lipids throughout the oxidation process.

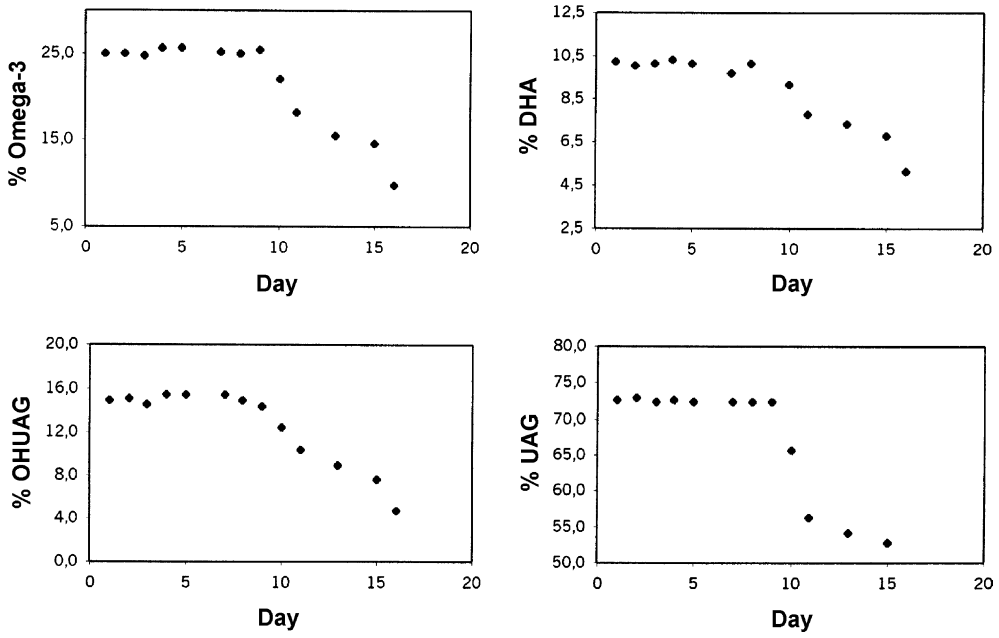

Fig. 6. Changes in the proportion of some acyl groups of the salted salmon fillet lipids throughout the oxidation process.

that in this sample the oxidation process begins the day 10, that is to say 8 days before the unsalted sample, indicating that the salting process clearly accelerates oxidation and considerably reduces the oxidative stability of salmon lipids. As in the unsalted sample, the appearance of both hydroperoxides and aldehydes is produced almost simultaneously.

In conclusion the ^{1}H NMR spectra of farmed salmon lipids contain a great deal of information which, conveniently studied, permits the determination of the proportions of several acyl groups in the sample. Furthermore, from the ^{1}H NMR spectra of the lipids of salmon fillets submitted to oxidative conditions, the changes in the proportions of several acyl groups throughout the oxidation process can be determined, providing information not only on the start of the oxidation but also on its rate, that is to say about the oxidative stability of the sample and about its kinetic. In addition the period of time before the appearance of aldehydic signals can be considered as a measure of resistance to rancidity, because these compounds are responsible for the off-flavours. The simultaneous appearance of hydroperoxides and aldehydes indicates the rapid rate of degradation from the first to the second. Finally, this technique has clearly shown that salting greatly accelerates the oxidation of lipids of farmed salmon. The advantages of this technique for evaluating oxidation degree and oxidative stability of salmon lipids in comparison with the classical methods such as peroxide and thiobarbituric values are evident, not only in relation to simplicity and rapidity but also in relation to the great deal of useful information obtained.

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