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Influence of fat and phytosterols concentration in margarines on their degradation at high temperature. A study by ^{1}H Nuclear Magnetic Resonance

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ABSTRACT

The objective of this work was to study the influence of several factors, especially fat and phytosterols concentration, on the behavior of margarine under thermo-oxidative conditions. For this purpose, margarines with similar compositions in acyl groups, but differing in the concentration of both fat and phytosterols, were heated at 180 °C. The changes in the main components of margarine lipids and the formation of new compounds throughout the thermal treatment were monitored by ^{1}H Nuclear Magnetic Resonance. The results show that the presence of high concentrations of phytosterols seems to have an antioxidant effect, since it slows down the thermo-oxidation rate of margarine and, consequently, the generation rate and concentrations of secondary oxidation products such as some aldehydes, epoxides and alcohols. The oil–water ratio also seems to have an important effect on margarine behavior, in such a way that the lower the fat concentration is, the higher its thermo-oxidation rate.

1. Introduction

Margarine is a foodstuff whose consumption is growing. The great versatility in its manufacturing process makes it possible for this product to be turned into a vehicle for food components that are considered beneficial for human health, such as omega-3 acyl groups, or vegetable stanols and/or sterols. These latter types of components are well known for their cholesterol-lowering ability in humans (Cusack, Fernandez, & Volek, 2013). Moreover, various studies have shown that some phytosterols can exhibit antioxidant properties (Aladedunye & Przybylski, 2012; Gordon & Magos, 1983; Singh, 2013); these could be displayed not only in foods but also in humans after their intake. However, the antioxidant ability of phytosterols has received little attention, scientific interest being mainly focused on their antihipocholesterolemic effect.

Chang and Mone (1960) were the first to suggest that 4-methyl-sterols improved the polymerization resistance of cooking fats heated to high temperatures. Later, other researchers (Boskou & Morton, 1976; Gordon & Magos, 1983; Sims, Fioriti, & Kanuk, 1972) attributed this ability not only to 4-methyl-sterols but also to 4-desmethyl-sterols with an ethylidene group in the side chain of their structure, such as Δ5-avenasterol and fucosterol. Conversely, other sterols lacking this feature like β-sitosterol or stigmasterol were considered ineffective as oil antipolymerization agents.

However, additional studies carried out over the years, have revealed that phytosterols without an ethylidene group can also exhibit antipolymerization activity at high temperatures. In particular, Singh (2013) has proved this antipolymerization effect for different levels of β-sitosterol (1%, 2% and 5%) in triolein, and in several vegetable oils with different unsaturation degrees heated at 180 °C.

Notwithstanding, it should be noticed that divergent results can be found concerning the antipolymerization and/or antioxidant ability of β-sitosterol and other vegetable sterols at high temperatures (Gordon & Magos, 1983; Lampi, Dimberg, & Kamal-Eldin, 1999; Singh, 2013; White & Armstrong, 1986; Winkler & Warner, 2008a; Yanishlieva & Schiller, 1984). This could be due to either the concentrations of phytosterols used, or to the sterol or oil nature, or to the experimental conditions used in the different studies. In fact, the great influence that the experimental conditions can have on the results obtained when studying the effect of phytosterols on the thermo-oxidation of lipidic matrices has been previously discussed by Lampi et al. (1999).

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Most of the studies concerning the antioxidant activity of phytosterols concentrate on the antipolymerization effect that this type of compounds can exhibit in oils heated at frying temperatures. In this context, both phytosterol mixtures (Aladedunye & Przybylski, 2012; Blekas & Boskou, 1986; Boskou & Morton, 1976; Cercaci, Passalacqua, Poerio, Rodriguez-Estrada, & Lercker, 2007; Gertz, Klostermann, & Kochhar, 2000; Kochhar & Gertz, 2004; Wang, Hicks, & Moreau, 2002; White & Armstrong, 1986) and individual phytosterols (Boskou & Morton, 1976; Singh, 2013; White & Armstrong, 1986; Winkler & Warner, 2008a) have been added either to vegetable oils (Gordon & Magos, 1983; Sims et al., 1972; Wang et al., 2002; White & Armstrong, 1986) or to model systems consisting in specific triglyceride mixtures (Gordon & Magos, 1983; Singh, 2013). In these previous studies the antipolymerization and antioxidant effect of sterols were evaluated by means of the amount of triacylglycerol dimers and polymers formed (Kochhar & Gertz, 2004; Singh, 2013), and by determining several classical parameters such as refractive index, conductivity, iodine value, concentration of unsaturated fatty acids and sterols, peroxide value and conjugated dienes and trienes.

Although several studies of the effect of phytosterols on the oxidation of edible oils at frying temperatures have been carried out, to the best of our knowledge their effect on margarine subjected to high temperature has not been studied. The margarine matrix, which is an emulsion, is much more complex than that of oils. In addition to factors such as lipid nature, concentration of oxygen in contact with lipids or presence of antioxidants or of prooxidants, all well known oxidation determining factors, others related to the characteristics of each emulsion could affect the oxidation of margarine (Mosca, Cuomo, Lopez, & Ceglie, 2013; Mosca, Diantom, Lopez, Ambrosone, & Ceglie, 2013). It should be noticed that nowadays margarines are considered the classical water-in-oil emulsions with a fat content between 80% and 90%, but also the so-called light margarines, which can present a fat content even lower than 39%. It is evident that the concentration of oil in both kinds of margarines is very different, as is the ratio between the interfacial surface of the emulsion drops and the oil volume; this may influence the resistance of margarine to degradation at high temperature. In fact, in previous studies on other types of emulsions, it has been found that oxidation is higher in those emulsions whose concentration of oil is lower (Osborn & Akoh, 2004; Sun & Gunasekaran, 2009).

Taking into account all the above mentioned, in this study the influence of the presence of phytosterols in high concentrations and of the fat concentration are considered in relation to the thermo-oxidative degradation of three different margarines submitted to high temperature. The evolution of their composition is followed by means of ^{1}H Nuclear Magnetic Resonance (^{1}H NMR). This technique has proved to be very useful in following lipid degradation processes (Martínez-Yusta, Goicoechea, & Guillén, 2014) and is also able to give a comprehensive view of the thermo-oxidation process of the lipidic matrix of margarine both in the presence and absence of vegetable sterols and/or stanols (Ibargoitia, Sopelana, & Guillén, 2014). Therefore, ^{1}H NMR could provide, in a fast and simple way, more complete information than other techniques previously used to assess the effect of phytosterols on oil thermo-oxidative processes. It allows one to monitor not only the evolution of margarine components but also the formation of diverse oxidation products. The components which are the subjects of study are the different kinds of acyl groups, both mono- and di-glycerides, as well as primary, if any, and secondary oxidation compounds such as aldehydes, epoxides and alcohols. As far as we know, there are no studies dealing either with the influence of fat concentration or with the effect of the presence of high concentrations of phytosterols in margarines on the formation at high temperature of secondary oxidation products such as aldehydes or epoxides. This issue could be considered of interest since phytosterols are sometimes added to food commodities rich in polyunsaturated acyl groups, and the oxidation of these latter can result in the generation of toxic compounds (Guillén & Goicoechea, 2008).

2. Materials and methods

2.1. Samples

The samples subject of study were three commercial margarines of similar composition in acyl groups, all of them rich in polyunsaturated groups, mainly linoleic ones. These were two light margarines, named VSLM and LM, the first enriched with 7.5% vegetable sterols, and a third margarine with a higher fat content, called M. At this point, it is worth remembering that, strictly speaking, margarine is a water-in-oil emulsion derived from vegetable and/or animal fats, with a fat content of between 80% and 90% and a milk fat content of no more than 3%. Other water-in-oil emulsions with different fat proportions receive different designations (Guillén, Ibargoitia, & Sopelana, 2016). In spite of this, and in order to simplify matters, in this work, we will use the term "margarine" in a general way, regardless of the fat concentration of each sample. The percentage in weight of fat and the molar percentages of the different kinds of acyl groups, in each margarine, are given in Table 1. The data regarding fat content were taken from the information provided by the manufacturer, whereas the molar percentages of acyl groups were determined by ^{1}H NMR as in previous studies (Ibargoitia et al., 2014; Sopelana, Arizabaleta, Ibargoitia, & Guillén, 2013). Table 1 also shows the content of vitamin E, the main antioxidant present in the three margarines above mentioned, as stated by the producers (9 mg vitamin E/100 g margarine in all cases), as well as its concentration expressed as mg of vitamin E/g of tryglyceride (TG), which is different in each margarine.

The vegetable sterols present in margarine VSLM, also determined by ^{1}H NMR as in a previous work (Sopelana et al., 2013), were predominantly β-sitosterol + campesterol. As well as these main sterols, some phytostanols such as β-sitostanol + campestanol, together with smaller quantities of Δ7-avenasterol, Δ7-sitosterol + Δ7-campesterol and probably brassicasterol, were also detected. Δ7-Phytosterols were identified according to data from Zhang et al. (2006). The concentrations of all these vegetable sterols and stanols are also shown in Table 1.

In addition, Table 1 also shows all data before mentioned for a margarine named IM, previously studied (Ibargoitia et al., 2014; Sopelana et al., 2013) under the same conditions as here. The data of IM margarine will be used in the discussion of the results found in this study.

2.2. Thermal treatment

20 g of margarine were weighed into pyrex glass receptacles of approximately 13.5 × 10.3 × 3 cm, and the receptacles were introduced together with the samples into a conventional oven with aeration at a temperature of 180 °C for 240 min. Aliquots were taken before and throughout the thermal treatment every 40 min and these were kept frozen until their study. The thermal treatment was performed at least in duplicate with each of the margarines.

The weight of the aliquots taken was approximately 0.16 g in all cases. The presence of detectable levels of water in the margarines could distort the signals of the ^{1}H NMR spectra to a certain extent; for this reason, the aliquots taken before thermal treatment were subjected to a liquid–liquid partition between deuterated chloroform (1 ml) and water (1 ml).

2.3. Monitoring of the thermo-oxidation of margarine lipids by ^1H NMR

The changes in the composition of the lipidic fraction of the margarines studied throughout heating were monitored by ^1H NMR, in the same way as in a previous work (Ibargoitia et al., 2014). For this purpose, the ^1H NMR spectra of the margarine aliquots taken periodically during the thermal treatment were registered on a Bruker Avance 400 spectrometer operating at 400 MHz. Each aliquot was mixed in a 5 mm diameter tube with 400 μl of deuterated chloroform that contained 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane (TMS) as internal references. In the case of the aliquots subjected to the liquid–liquid partition described above, 600 μl of the corresponding chloroform extracts were introduced into the RMN tube.

In order to select the most appropriate values to obtain accurate quantitative results in the shortest period of time, a very broad range of recycling times and relaxation delays were tested in the acquisition of the ^1H NMR spectra of unoxidized and oxidized samples. As a result of these tests, the acquisition parameters used were: spectral width 5000 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.744 s, and pulse width 90°, with a total acquisition time of 12 min 54 s. The experiments were carried out at 25 °C, as in previous works (Ibargoitia et al., 2014; Sopelana et al., 2013).

Compounds such as 1,2-dioleoylglycerol, dioleoylglycerol, dipalmitin, 1-oleoyl-*rac*-glycerol, 1-linoleoyl-*rac*-glycerol, 2-oleoylglycerol, 2-monopalmitin, propanal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, 4,5-epoxy-(*E*)-2-decenal, 12,13-epoxy-Z, 9-octadecenoic acid methyl ester (isoleukotoxin methyl ester), (*E*)-2-penten-1-ol, 1-hexanol and β-sitosterol, acquired from Sigma-Aldrich (St. Louis, MO, USA), 4-hydroxy-(*E*)-2-nonenal, acquired from Cayman Chemical (Ann Arbor, MI, USA), and campesterol and brassicasterol from Larodan (Malmö, Sweden) were used as standard compounds for identification purposes. Likewise, trilinolein and trilinolenin were acquired from Sigma-Aldrich for quantitative purposes.

2.4. Data derived from ^1H NMR spectra

The fact that the signal intensity in ^1H NMR spectra is proportional to the number of protons that generates it makes it possible to determine from them the relative concentrations of certain compounds without using standard compounds. This determination can be made throughout the thermal degradation process. Thus, as commented above, the molar percentage of the different types of acyl groups was determined using the same approach as that employed in previous works (Ibargoitia et al., 2014; Sopelana et al., 2013). In addition to the molar proportions of acyl groups, the concentrations of mono- and di-glycerides, as well as of phytosterols, were also determined, in this case referred to that of triglycerides (TG) and expressed as mmol/mol TG. Likewise, the concentrations of compounds newly formed as a consequence of margarine lipid degradation, such as aldehydes, epoxides and alcohols were also determined in relation to the total acyl groups (AG), and given in mmol/AG. The method used for all these determinations is thoroughly explained in a previous paper (Ibargoitia et al., 2014). All these experimental data, represented in Figs. 1, 2 and 4, are average values coming from at least two different thermal treatments of each margarine.

2.5. Statistic and kinetic studies

The graphic tools of Microsoft Office Excel 2007 were used to find equations that fit the molar percentage of the different types of acyl groups in the three margarines studied throughout the degradation process and heating time. Given that the proportions of the different types of acyl groups hardly exhibited changes after the first 40 min of heating, the equations were obtained with the data from min 40 onwards. Likewise, equations that fit heating time and the molar concentrations of aldehydes, epoxides and alcohols were also found using the same program.

The IBM SPSS Statistics 22 software package was also used to study relationships between the thermo-oxidation rate of the different margarines and their fat and phytosterols concentration.

3. Results and discussion

In the oxidation of lipids, their unsaturation degree, the presence of oxygen, the system temperature, the presence or absence of antioxidants or of prooxidants are important factors that influence both the beginning and the advance of the lipid oxidation process. In this study, the temperature and the amount of oxygen in contact with the sample surface are the same for all the experiments. However, the three margarines of this study show small differences in the molar percentages of the different kinds of acyl groups and much greater ones in the concentration of vegetable sterols, this latter being far higher in VSLM than in the other two margarines; in addition, the concentration of fat is much higher in sample M than in the other two.

3.1. Evolution throughout the thermal treatment of some original lipidic components present in the margarines

The components considered in this group are triglycerides, whose original composition in acyl groups is indicated in Table 1, and also di- and mono-glycerides.

3.1.1. Evolution of the different kinds of acyl groups

The acyl groups of the triglycerides of any kind of lipids submitted to thermal treatment undergo important changes. In the study of the thermal degradation of edible oils at high temperature, it has been proved that the highest rate of decrease of the molar percentage is observed in the unsaturated acyl group with the highest percentage in the oil, regardless of its unsaturation degree; the molar percentage of the types of acyl groups having a higher unsaturation degree than the main one also decreases, and that of those with lower unsaturation degree than the main one remains almost unchanged or suffers very small changes (Guillén & Uriarte, 2009; Guillén & Uriarte, 2012a, 2012b). However, since margarines are emulsions, their behavior under similar degradative conditions could be different from that of oils.

Fig. 1 shows the evolution, throughout the thermal process, of the molar percentages of the different kinds of acyl groups in the three margarines studied. It can be observed in this figure that, in agreement with the results obtained for oils, the molar percentage of the main acyl group in margarines VSLM and M, linoleic group (L), is the one showing the highest decreasing rate. In the case of margarine LM, despite the slightly higher proportion of monounsaturated (MU) than of linoleic groups (see Table 1), the higher unsaturation degree of linoleic determines that this type of group exhibits the highest decreasing rate.

The molar percentage of the acyl group more unsaturated than the main one, that is of linolenic group (Ln), also decreases in each margarine, although at a slower rate than linoleic groups. The molar percentage of the unsaturated acyl group less unsaturated than the main one, which is oleic or monounsaturated groups (MU) in samples VSLM and M, varies very little or increases slightly. Finally, the sum of saturated plus modified acyl groups (S + M) increases in all cases.

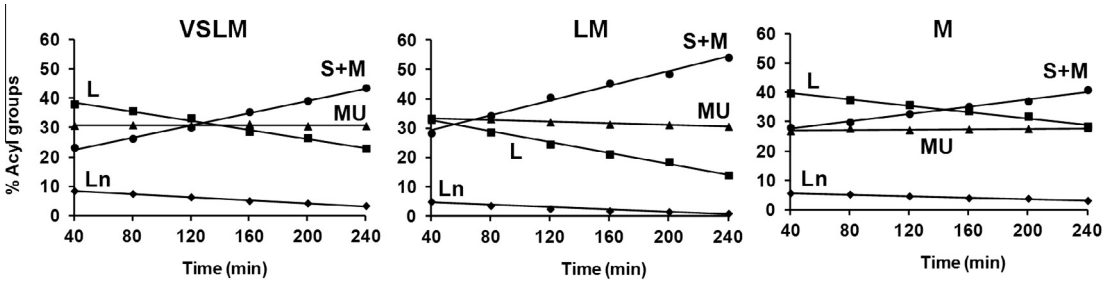

Fig. 1. Evolution with heating time of the molar percentages of the different types of acyl groups in the three margarines. Ln: linolenic; L: linoleic; MU: monounsaturated; S + M: saturated plus modified.

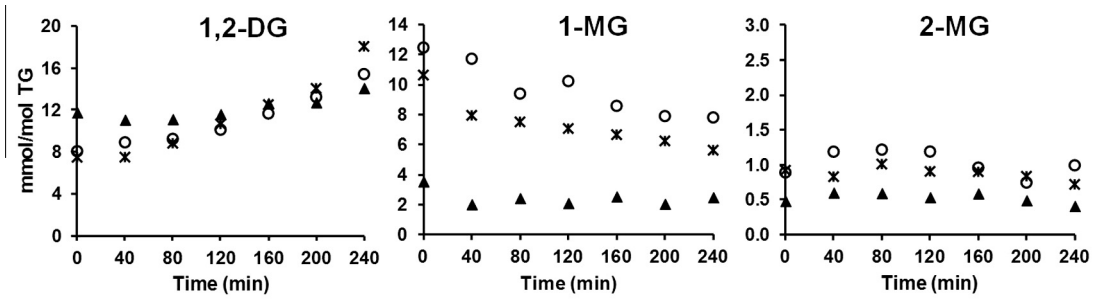

Fig. 2. Evolution with heating time of the concentrations of 1,2-diglycerides (1,2-DG), 1-monoglycerides (1-MG) and 2-monoglycerides (2-MG) in the three margarines subject of study, in mmol/mol of triglyceride (TG).

In spite of this common behavior, clear differences are observed in the evolution of the molar percentages of the different types of acyl groups in these margarines. Experimental data represented in Fig. 1 were fitted to linear equations with high correlation coefficients; the slope of these lines, which are given in Table 2, represents the rate of positive or negative variation of the molar percentage of the above mentioned acyl groups in each margarine throughout the thermal process. Table 2 also gives these same data for IM margarine; they were obtained in a previous study (Ibargoitia et al., 2014) which was carried out under the same conditions as this one and will be used in the discussion of the results.

Of the various factors influencing the thermo-oxidation of margarine, their *unsaturation degree* could be considered as important. However, the differences in the unsaturation degree of the margarines involved in this study are not large, as Table 1 shows. For this reason, this factor would not be expected to have a great influence on the difference in the thermo-oxidation rate of these margarines. This is corroborated by comparison of data in Tables 1 and 2. It can be observed that the most saturated margarine, LM, exhibits the greatest degradation rate, whereas one of the most unsaturated, IM, has an intermediate rate of degradation. This indicates that although this factor can play a certain role in relation to the thermo-oxidation rate, in margarines with similar unsaturation degree, like those here considered, other factors can have more influence.

Fat and vitamin E, as well as phytosterol content, could also influence margarine thermo-oxidation rate. In relation to *fat content*, the four margarines considered in this study covered a

Table 1
Percentage in weight of fat, vitamin E concentration, molar percentages of the different kinds of acyl groups, and concentrations of 1,2-diglycerides (1,2-DG), 1- and 2-monoglycerides (MG), and vegetable sterols and/or stanols, in mmol/mol of triglyceride (TG) in the several margarines.

Parameter	VSLM[a]	LM[a]	M[a]	IM[b]
% Fat in weight	35[c]	40.0	70.0	60
Vitamin E (mg/g margarine)	9	9	9	20
Vitamin E (mg/g TG)	0.26	0.23	0.13	0.33
Linoleic (molar %)	38.4 ± 0.7	33.0 ± 0.7	39.7 ± 2.7	43.4 ± 1.0
Linolenic (molar %)	8.4 ± 0.0	5.0 ± 0.7	5.7 ± 0.2	6.3 ± 0.2
Monounsaturated (molar %)	31.2 ± 0.4	35.1 ± 1.4	27.7 ± 1.5	28.8 ± 0.2
Saturated (molar %)	22.0 ± 1.1	26.9 ± 0.7	26.9 ± 1.2	21.5 ± 1.4
1,2-DG (mmol/mol TG)	8.1 ± 1.0	7.5 ± 0.9	12.4 ± 0.4	8.3 ± 0.4
1-MG (mmol/mol TG)	12.5 ± 1.4	10.7 ± 0.4	3.6 ± 0.4	7.4 ± 0.9
2-MG (mmol/mol TG)	0.9 ± 0.1	0.9 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
β-Sit + camp (mmol/mol TG)	382.4 ± 19.1	4.4 ± 0.3	3.5 ± 0.1	4.3 ± 0.1
Sitn + campn (mmol/mol TG)	39.4 ± 3.8	nd	nd	nd
Brassicasterol (mmol/mol TG)	9.7 ± 1.5	nd	nd	nd
Δ7-Avenasterol (mmol/mol TG)	4.5 ± 0.2	1.3 ± 0.1	1.6 ± 0.1	1.8 ± 0.1
Δ7-Sit + Δ7-camp (mmol/mol TG)	1.4 ± 0.2	nd	nd	nd

Sit: sitosterol; Camp: campesterol; Sitn: sitostanol; Campn: campestanol; nd: not detected.
[a] Margarines subject of study.
[b] Margarine previously studied (Ibargoitia et al., 2014).
[c] Concentration without considering the added phytosterols.

Table 2
Rate of change of the molar percentage of the different kinds of acyl groups of the several margarines, throughout the thermal treatment, expressed in % per minute of heating (slopes of lines in Fig. 1).

Acyl group	VSLM[a]	LM[a]	M[a]	IM[b]
Linoleic	−0.0771	−0.0933	−0.0541	−0.0848
Linolenic	−0.0260	−0.0193	−0.0126	−0.0188
Monounsaturated	−0.0008	−0.0132	0.0037	0.0136
Saturated + modified	0.1038	0.1258	0.0629	0.0900

[a] Margarines subject of study.
[b] Margarine previously studied (Ibargoitia et al., 2014).

broad range, varying from around 35% (without considering phytosterol content) in VSLM to 70% in M margarine. This factor seems to be determinant in the thermo-oxidation rate of margarines which are not enriched in phytosterols. In fact, an inverse relationship has been found, with a high correlation coefficient, between thermo-oxidation rate, expressed by the rate of increase of the molar percentage of saturated plus modified acyl groups during thermo-oxidation of margarines (R_{S+M}) (data given in Table 2), and the fat content (%*F*) (data given in Table 1). The corresponding equation for the three mentioned margarines LM, M and IM is $R_{S+M} = 0.2092 - 0.0021$ (%*F*) ($n = 3$, $R = 0.9937$), where R_{S+M} is expressed in molar percentage per minute of heating and %*F* as percentage in weight. This result indicates that, for these margarines, the greater the fat content, the lower the thermo-oxidation rate. This fact is in agreement with previous results on other kinds of emulsions analyzed by using other parameters and methodologies (Osborn & Akoh, 2004; Sun & Gunasekaran, 2009) and suggests that fat content has a great influence on the thermo-oxidation rate of margarines.

In addition, the above results concerning the influence of the fat content on the margarine thermo-oxidation rate also suggest that the effect of the *vitamin E concentration* in the margarines subject of study is not as important as might be expected due to its well known antioxidant ability. The concentration of vitamin E in margarines LM and M is 9 mg/g of margarine, and 20 mg/g of margarine in IM. Bearing in mind the different fat content in these margarines, a more appropriate way to express the concentration of vitamin E is referring to the compounds which are able to be oxidized: to triglyceride weight instead of to total margarine weight. Taking as reference the weight of triglycerides, the concentration of vitamin E expressed as mg/g of triglyceride is 0.23, 0.13 and 0.33 in margarines LM, M and IM respectively. Comparing these data in Table 1 and the thermo-oxidation rate expressed by the rate of increase of the molar percentage of saturated plus modified acyl groups given in Table 2, it is evident that, unexpectedly, the margarine M having the smallest concentration of vitamin E (0.13 mg vitamin E/g triglyceride) shows the smallest rate of thermo-oxidation, whereas margarine IM, which has the greatest concentration of vitamin E (0.33 mg vitamin E/g triglyceride), shows an intermediate rate of thermo-oxidation. These results indicate that there is not a determinant effect of the concentration of vitamin E on the thermo-oxidation rate of these margarines.

Margarine VSLM has not been included in the above discussion on the influence of the fat and vitamin E content due to its high *content in phytosterols*. In fact, the phytosterols concentration in this margarine is near a hundred times higher than in LM, M or IM. In order to analyze the possible effect of the occurrence of these compounds at high concentration in the thermo-oxidation rate of margarines, VSLM and LM margarines are considered. Data in Table 1 indicate that both margarines have a very similar concentration of vitamin E, a slightly smaller fat content in VSLM (considering only triglycerides) than in LM, and a higher unsaturation degree in VSLM than in LM. For these reasons, it would be expected a somewhat higher thermo-oxidation rate in VSLM than in LM under the same degradative conditions. However, as Table 2 shows, the results indicate the contrary. In the light of the data available on these margarines, this fact only can be attributed to the high concentration of phytosterols in VSLM.

These results suggest that the differences existing in the unsaturation degree of these margarines do not seem to have a great influence on their thermo-oxidation rate. The same can be said of their vitamin E content. However, fat concentration could have a major influence on the thermo-oxidation rate of margarine lipids, since the lower the fat concentration the higher is the global degradation rate of acyl groups. Likewise it seems that the presence of phytosterols at high concentrations slows down the margarine thermo-oxidation rate to a certain extent.

All the above commented can be summarized in the existence of a very close relationship between the thermo-oxidation rate (R_{S+M}) of the four margarines involved in this discussion and their concentrations of fat (%*F*) and phytosterols (Phy). This relationship is described by a biparametric equation [$R_{S+M} = 0.2097 - 0.0021$ (%*F*) $- 0.0778$ (Phy), $n = 4$, $R = 0.9941$], with a very high correlation coefficient. The thermo-oxidation rate is expressed in terms of rate of increase of saturated + modified acyl groups (%/min), fat in percentage in weight and phytosterols in mol/mol TG.

3.1.2. Evolution of mono- and di-glycerides

Mono- and di-glycerides are common components of margarines, both kinds of compounds being used as emulsifiers. Their concentrations in the original margarines, in mmol/mol of triglyceride, are given in Table 1. It can be observed that in all cases the concentration of 1-monoglycerides (1-MG) is much greater than that of 2-monoglycerides (2-MG), being the concentration of these latter very small in the four samples, although slightly higher in VSLM and LM than in M and IM. Furthermore, the ratio between the concentration of monoglycerides (1-MG + 2-MG) and that of 1,2-diglycerides (1,2-DG) is greater than 1 in margarines having low fat content; this ratio decreases as the fat content increases to become smaller than 1 in the margarine with the highest fat content (M).

Variations in the concentration of these compounds during thermal treatment are probably due to hydrolysis, the changes in 1-MG and 2-MG being due to hydrolysis of either DG or TG and also to its own hydrolysis to give fatty acids. Likewise, the changes in the concentration of 1,2-DG are due to its own hydrolysis and to that of TG. As Fig. 2 shows, the changes in the concentration of 1,2-DG and 1-MG are more noticeable in the margarines with higher water proportion (VSLM and LM) than in M. The balance, throughout the thermal process, between formation and hydrolysis in 1,2-DG is positive whereas in 1-MG is negative. Concerning 2-MG, the changes in their concentrations are very small throughout the thermal treatment. It is evident that the occurrence of phytosterols in high concentration, and the unsaturation degree of margarine fat does not seem to affect the hydrolytic changes; however, as might be expected, water concentration influences the evolution of the concentration of mono- and di-glycerides. Even so, neither 1- nor 2-MG disappear from the system.

3.2. Formation of new compounds derived from acyl groups degradation throughout the thermal process

It is well known that the thermal degradation of the acyl groups of triglycerides in presence of oxygen has as a consequence the formation of new compounds. In processes at low or intermediate temperature primary oxidation compounds have been detected, identified and quantified by ^{1}H NMR. However, in accordance with that observed in vegetable oils (Martínez-Yusta et al., 2014), in the degradation at high temperature of these margarines, the first

compounds detected as newly formed are the so called secondary oxidation compounds. This could be due to the instability of primary oxidation products at high temperature, which would lead to their rapid degradation before they reach high enough concentrations to be detected, and to the generation of secondary oxidation compounds; some of these latter, such as aldehydes, epoxides and alcohols, are detectable by ^{1}H NMR.

3.2.1. Formation of aldehydes and evolution of their concentrations with the heating time

The aldehydes formed in oxidation processes deserve special attention due to the potential toxic effect of some of them (Guillén & Goicoechea, 2008). Fig. 3 shows the enlargement of the region between 9.45 and 9.82 ppm, of the ^{1}H NMR spectra of the lipids of each margarine, at the different stages of the thermal treatment. All the spectra have been plotted at a fixed value of absolute intensity for comparative purposes. This figure enables the observation of the appearance of signals corresponding to aldehydic protons and their evolution throughout heating. In addition, Fig. 4A–H show the evolution of the concentrations of these aldehydes, as well as of total aldehydes, throughout the heating time. From Figs. 3 and 4, it can be observed that, in all cases, (*E*)-2-alkenals (a), (*E,E*)-2,4-alkadienals (b) and n-alkanals (c) are the most abundant aldehydes during the entire heating period, and that (*Z,E*)-2,4-alkadienals (f) and oxygenated α,β-unsaturated aldehydes such as 4,5-epoxy-alkenals (d) and 4-hydroxy-(*E*)-2-alkenals (e) are in lower concentrations; these latter are well known for their toxicity (Guillén & Goicoechea, 2008). Moreover, a small signal tentatively attributed to 4-oxo-alkanals (g) can also be observed from 120 min onwards in samples VSLM and LM, and at the end of the heating period in M.

In general, aldehydes are detected earlier in sample M (after 40 min of heating) than in VSLM and in LM (after 80 min). However, it should be mentioned that intermediate times between 40 and 80 min were not tested, so the exact heating time at which aldehydes are formed in VSLM and LM margarines is not known. Although the formation of aldehydes is detected in M before it is in VSLM and LM samples, Figs. 3 and 4 show that the generation rate of aldehydes in these latter margarines, once the process has begun, is higher than in M for all kinds of aldehydes at any heating time. This finding, which is in agreement with the lower decreasing rate of linoleic and linolenic acyl groups observed in M *versus* VSLM and LM margarines, and also with the lower rate of increase of saturated plus modified acyl groups, demonstrates the influence of fat concentration in margarines on their degradation rate. Likewise, the slower degradation of VSLM margarine *versus* LM, attributed to the presence of high concentrations of vegetable sterols in the former, is also evidenced by the lower concentrations of most aldehydes, as well as of total aldehydes, in VSLM margarine at each one of the heating times studied throughout the process (see Fig. 4). Only at very advanced thermo-oxidation stages are the concentrations of some aldehydes coming from polyunsaturated acyl groups, such as (*E,E*)-2,4-alkadienals (Fig. 4D) (after 160 min of heating), 4,5-epoxy-alkenals (Fig. 4E), 4-hydroxy-(*E*)-2-alkenals (Fig. 4F) and (*Z,E*)-2,4-alkadienals (Fig. 4G) (after 240 min of heating), higher in VSLM than in LM sample. This could be due to the higher degradation rate of linolenic groups in VSLM margarine than in LM.

The fact that the formation of secondary oxidation products begins earlier in margarine M than in the other two ones, could be interpreted as its oxidative stability was the least of the three margarines. This would be so if oxidative stability was considered to be resistance to oxidation and consequently measured by the time at which the first oxidation markers are detected. However, the results of this study suggest that the concept of oxidative stability should be defined in a more complete way, including not only the time when the first oxidation markers are detected but also their subsequent rate of formation or of degradation of acyl groups.

3.2.2. Formation of epoxides and evolution of their concentrations with the heating time

Other secondary oxidation compounds formed in thermal oxidation processes are epoxides. Fig. 4I–K show the concentrations of different types of monoepoxides and also of diepoxides, all of them derived from the degradation of unsaturated acyl groups, *versus* heating time at the different stages of the heating process in the three margarines studied, in mmol/acyl group. This figure reveals clear differences among margarines concerning both epoxides formation rate and concentrations. In sample LM, (*E*)-epoxystearates, derived from monounsaturated groups, are detected after 80 min of heating, whereas in VSLM and M, these become detectable later (after 120 min). The earlier onset and the higher concentrations of (*E*)-epoxystearates in sample LM *versus* VSLM and M throughout the entire thermo-oxidative process seem to be in accordance with the higher proportion of monounsaturated groups in the former

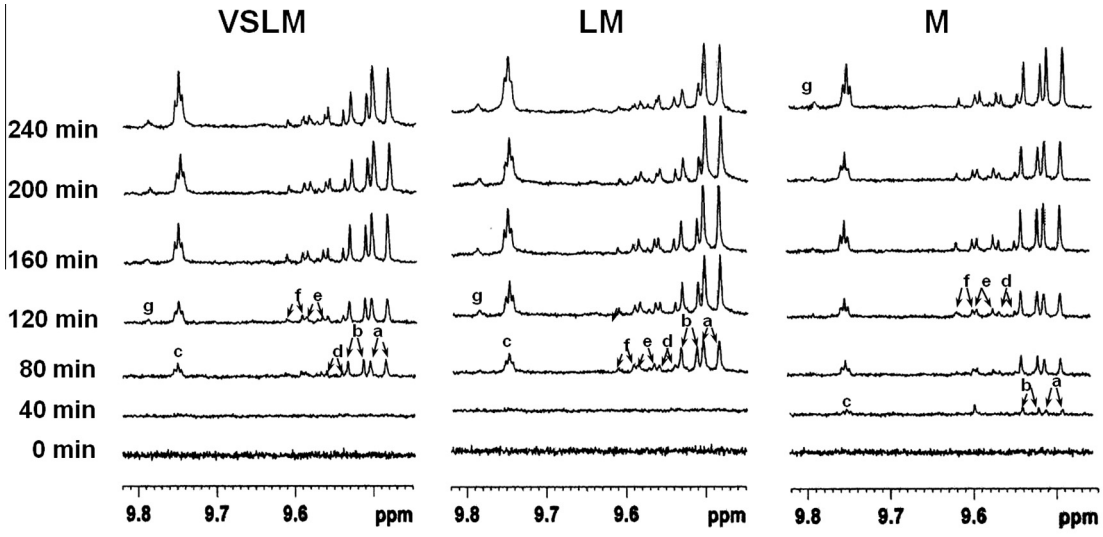

Fig. 3. Evolution with heating time of the ^{1}H NMR spectral signals of aldehydes in the three margarines subject of study. a: (*E*)-2-alkenals; b: (*E,E*)-2,4-alkadienals; c: n-alkanals; d: 4,5-epoxy-alkenals; e: 4-hydroxy-(*E*)-2-alkenals; f: (*Z,E*)-2,4-alkadienals (tentative); g: 4-oxoalkanals (tentative).

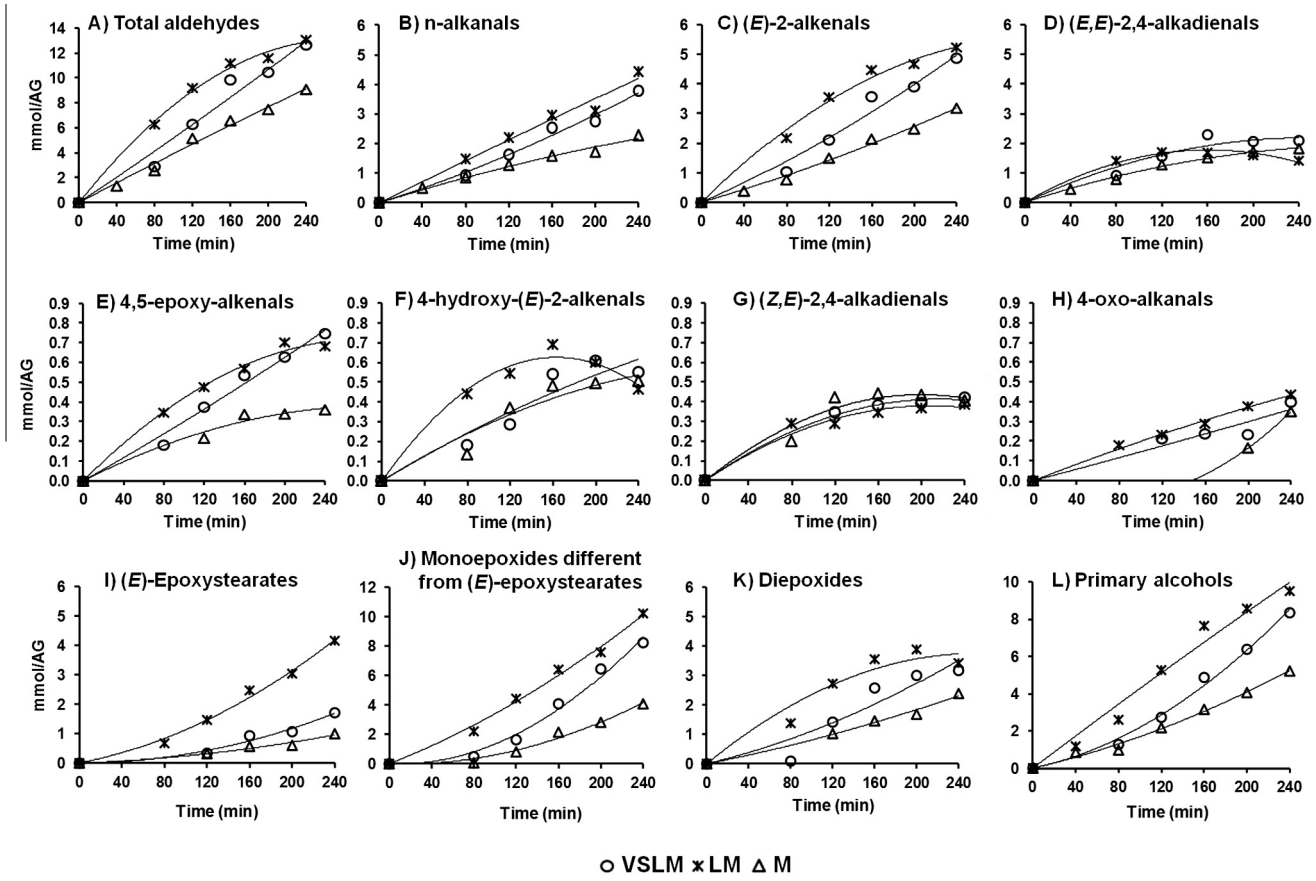

Fig. 4. Evolution with heating time of the concentrations of total aldehydes (A), each kind of aldehydes (B–H), epoxides (I–K) and primary alcohols (L) in the several margarines, all in mmol/acyl group (AG).

(see Table 1). Likewise, a greater formation rate and higher concentrations of monoepoxides different from (*E*)-epoxystearates are observed in sample LM too; these latter include (*Z*)-epoxystearates, derived from monounsaturated groups, but also different types of monoepoxides generated from polyunsaturated groups (Martínez-Yusta et al., 2014). Moreover, although according to their proportions of polyunsaturated groups, a more intense formation of diepoxides would be expected in sample VSLM and in M than in LM, the opposite tendency is observed. This could be due to the influence of the elevated concentration of vegetable sterols in VSLM and of the high concentration of fat in M; both factors slow down the thermo-oxidative process of margarine, even though the effect of fat concentration seems to be more noticeable. As for aldehydes, these results are in total agreement with the acyl groups degradation rate observed in the different margarines subject of study.

It is worth noticing that, in accordance with that observed for aldehydes, the differences between the concentrations of monoepoxides different from (*E*)-epoxystearates and of diepoxides in samples VSLM and LM tend to get smaller as the thermo-oxidative process advances.

3.2.3. Primary alcohols

Finally, among the products that may be generated during the thermo-oxidation of margarine lipids, primary alcohols should also be mentioned. The concentrations of primary alcohols in the three studied margarines throughout the whole thermal treatment, expressed in mmol/acyl group, are represented *versus* heating time in Fig. 4L. This figure shows that, in a similar way to that observed for aldehydes and epoxides, the concentrations of primary alcohols in sample LM are higher than those in VSLM, the sample with added vegetable sterols, and in this latter they are, in turn, higher than in M, the sample with the highest fat concentration.

In agreement with observations made concerning aldehydes, monoepoxides different from (*E*)-epoxystearates and diepoxides, the differences between the two light margarines (VSLM and LM), tend to get slightly smaller as the thermo-oxidative process progresses.

Regarding the effect observed for the presence of high concentration of phytosterols, it is worth noticing that the most abundant sterols in margarine VSLM are 4-desmethyl-sterols that lack structural features considered important for exhibiting an antioxidant effect, such as an ethylidene chain or more than one endocyclic double bond in the sterol nucleus (Gordon & Magos, 1983; Winkler & Warner, 2008a). Therefore, it could be thought that free radicals formed from acyl groups during heating could react with sterols, thus bringing about the oxidation of the steryl moiety (Smith, 1981) and retarding that of the triglycerides themselves. This explanation has also been suggested by other authors (Cercaci et al., 2007; Winkler & Warner, 2008b).

Finally, it is also worth pointing out that phytosterol concentration in margarine VSLM (17.65% in relation to the total fat amount) is considerably higher than those used in previous works dealing with the antioxidant and/or antipolymerization effect of vegetable sterols in oils; this could be another factor influencing the results obtained in this study, since the antioxidant effect of phytosterols seems to increase with concentration (Singh, 2013; Winkler & Warner, 2008b).

4. Conclusions

Fat concentration seems to play an important role in the thermo-oxidative process of margarines not enriched in phytosterols and with similar compositions in acyl groups. This factor affects their thermo-oxidation rate and the subsequent formation of secondary oxidation products, both being less intense in the margarine with the highest fat content. These results are in agreement with those observed previously in other kinds of oil-in-water emulsions.

The presence of significant concentrations of phytosterols seems to decrease the thermo-oxidation rate of margarine whenever margarines with similar acyl groups proportions are considered. This is evidenced by a smaller degradation rate of the phytosterol-enriched margarine; as a consequence, secondary oxidation products such as aldehydes, epoxides and primary alcohols are generated later and in lower concentrations. This effect seems to be more pronounced at the beginning of the thermo-oxidative process and slightly different depending on the oxidation products considered. Thus, whereas in the case of epoxides and primary alcohols their concentrations are lower in the sample enriched with phytosterols throughout the whole heating period, some types of aldehydes reach higher concentrations in this latter margarine at the most advanced stages of the thermo-oxidation process. The results obtained suggest that, in line with the most recent findings, vegetable sterols lacking an ethylidene chain, like β-sitosterol and campesterol, can exhibit an antioxidant effect at high temperature, at least in the conditions of this study.

A far as we know, this is the first time that the effect of fat concentration and of the presence of high concentrations of phytosterols on the thermo-oxidative degradation of margarine has been shown.

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