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Analytical Methods

Identification and kinetics of oxidized compounds from phosphatidylcholine molecular species

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ABSTRACT

Heat-induced oxidative modifications of two phosphatidylcholine molecular species as potential functional food components were evaluated. 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) were chosen as models. The optimal temperature for hydroperoxide formation was determined by MS for each standard: 125 °C for SLPC and 150 °C for SOPC. Oxidation was performed at these temperatures and degradation products were identified using LC–ESI-MS combined to an acid treatment. Kinetics of formation of oxidation products from SOPC and SLPC were monitored over 120 min and curves were drawn for each identified structure. Results showed that native phospholipids rapidly decreased with heat and that oxidation products showed functions, such as hydroxyl, oxo or epoxy groups. Kinetics pointed out that some of these quite stable oxidation products are likely to be found in sizable amounts in processed foods containing phospholipids.

1. Introduction

Soy lecithin, that consists primarily of phospholipids (PL), is widely used in the food industry. The amphiphilic properties of PL make lecithin a good emulsifying and smoothing agent in chocolate, margarines, mayonnaise and instant products (Lin & Blank, 2003; Ulkowski, Musialik, & Litwinienko, 2005). Three main classes of PL were identified in soy lecithin: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). In addition to their technological properties, PL, especially PC, are of great nutritional interest: soy PL are rich in essential fatty acids (FA) such as linoleic and alpha-linolenic acids (Wang, Hammond, Cornette, & Fehr, 1999; Wang, Hammond, & Fehr, 1997) and in choline, a constitutive part of one of the polar heads, which is known to be essential for the synthesis of the neurotransmitter acetylcholine or for protecting liver (Shahidi, 2006). As far as assimilation of nutrients is concerned, several studies (Amate, Gil, & Ramirez, 2001; Lemaitre-Delaunay et al., 1999; Wijendran et al., 2002) showed that PC is a better carrier of polyunsaturated fatty acids (PUFA) than triacylglycerols (TAG). Cellular permeability to PUFA and their intracellular level were indeed much higher when linked to PL than to TAG, which suggests that food supplementation with PUFA-rich PL could enhance essential FA assimilation and could help the consumers achieve the daily recommended intake of lecithin (Chanussot, 2008; Shahidi, 2006).

Food supplementation with lecithin or PL-PUFA rises however the question of their stability during processing, storage and cooking. Minor quantities of PL added to oils are known to improve stability against oxidation (Koprivnjak et al., 2008; Nwosu, Boyd, & Sheldon, 1997; Sugino et al., 1997), but very few information is available on changes in PL structures when added as antioxidants. It is however known that the most common change in lipid structure is oxidation, which is often caused by heat treatment, the most widely used unit operation during food processing and preparation. Lipid oxidation starts with the formation of hydroperoxides, defined as primary oxidation products, followed by further oxidative changes of the alkyl chains of fatty acids (Frankel, 2005). Other downstream changes result in the formation of dimeric compounds or in degradation products (volatiles). Few studies were however conducted on thermal oxidation of PL. Most of these studies used drastic oxidative treatments (Fenton reaction: $FeCl_2 + H_2O_2$ or $FeSO_4$ + *tert*-butylhydroperoxide) that did not reflect processing or cooking conditions that a food matrix is usually subjected to (Reis, Domingues, Amado, Ferrer-Correia, & Domingues, 2005, 2007; Reis, Domingues, Ferrer-Correia, & Domingues, 2004; Spickett, Pitt, & Brown, 1998). In addition, while volatiles were investigated (Jewell & Nawar, 1980; Lin & Blank, 2003; Meynier, Genot, & Gandemer, 1998; Stephan & Steinhart, 1999), no data is available on heat-generated oxidation products.

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The present study set out to investigate the oxidation conditions and products of PC during heat treatment as a step towards understanding the behaviour of PL with regard to thermal oxidation. Two molecular species of PC were selected as models: 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC). SLPC was previously identified in soy PC (Wang et al., 1997, 1999), while SOPC was chosen because of the limited number of oxidation products it would generate due to its single double bond. In this study, only the formation of hydroperoxides and oxidative changes that do not include dimerization and degradation were investigated.

First, in order to form the maximum of degradation products of interest, the temperature that allowed the production of the highest yield of hydroperoxides was determined. The second part of this work was aimed at elucidating the structures of the oxidation products and studying the kinetics of their formation, as well as the kinetics of degradation of the starting material (SOPC and SLPC).

2. Materials and methods

2.1. Chemicals and stock solutions

2.1.1. Chemicals

SOPC and SLPC were purchased from Avanti Polar Lipids (Alabaster, USA). HPLC grade methanol and analytical grade formic acid (99–100%) were purchased from VWR (Strasbourg, France). Ultrapure water was produced by a Synergy UV purification system (Millipore, Molsheim, France). Chloroform, ammonium hydroxide and sodium hydroxide were of analytical grade and were purchased from Riedel de Haën (Sigma–Aldrich, Seelze, Germany).

2.1.2. Stock solutions

Stock solutions of 10 mg/mL were prepared by weighing SOPC or SLPC standards into amber vials and dissolving them in chloroform. Aliquots were prepared by introducing 100 μL of SOPC or SLPC stock solution into a 4 mL amber vial (VWR, vials 4 mL, height: 44 mm, internal diameter: 12 mm). Each vial was dried under nitrogen, closed and kept at −20 °C until oxidative treatment.

2.2. Oxidative treatments

2.2.1. Determination of optimal oxidation temperature

Several temperatures were tested on SOPC and SLPC samples in order to determine the best conditions for hydroperoxide formation. All samples were analyzed in triplicate. Both PC were oxidized in an oven for 30 min at temperatures ranging from 50 to 175 °C. Accuracy of each indicated temperature was ±1 °C. Vials were left open to air during heating. After oxidation, samples were cooled down at room temperature. One millilitre methanol was added in vial, then a 1/50 dilution was performed to obtain 0.02 mg/mL. Solutions were filtered through 0.45 μm PTFE filters (Macherey–Nagel, Hoerdt, France) as a precaution to eliminate potential particles, as no guard column was used. Filtered and non-filtered samples were not significantly different as far as responses (peak areas) were concerned (data not shown).

2.2.2. Kinetics

Aliquots from stock solutions of SOPC and SLPC were oxidized in open vials at the appropriate temperature determined for each PC molecular species for durations between 0 and 120 min. Samples were cooled down at room temperature and 1 mL methanol was added to obtain 1 mg/mL PC. Each sample was filtered through 0.45 μm PTFE filters as described above and 10 μL were injected in the HPLC system.

2.3. Liquid chromatography and mass spectrometry

2.3.1. Determination of optimal temperature for hydroperoxide formation by MS

Mass analysis was carried on 5 μL of each sample in a 1200 L triple quadrupole mass spectrometer (Varian, Les Ulis, France) fitted with an ESI source using methanol as mobile phase. High purity nitrogen, produced by a nitrogen generator (Domnik Hunter, Villefranche-sur-Saône, France) was used as nebulizing gas, set at 46 psi, and as a drying gas, set at 300 °C. Spectral data was acquired in positive mode (single quadrupole analysis) and the *m/z* scan range was from 500 to 1000. The best temperature of oxidation was defined at the temperature where the percentage of hydroperoxide was the highest for each starting material. The percentage was calculated as follows:

$$\frac{(A_{[M+H+32]})_T}{A_{[M+H]}} \times 100$$

where $(A_{[M+H+32]})_T$ is the area of the peak corresponding to the extracted ion *m/z* of the hydroperoxide [M+H+32] formed at a given temperature (T) and where $A_{[M+H]}$ is the area of the peak corresponding to the extracted ion *m/z* of the starting material [M+H] without oxidative treatment.

2.3.2. Identification of oxidation products with LC–ESI-MS

The HPLC system was made of two Prostar 210 solvent delivery modules (Varian), a Prostar 410 autosampler (Varian) and an ESI mass spectrometer as described above. SOPC and SLPC oxidation products were separated on a reverse phase C8 Lichrospher RP Select B (Interchim, Montluçon, France) using a linear gradient ranging from 15% ultrapure water in methanol containing 0.1% NH_4OH to 100% methanol containing 0.1% NH_4OH. The flow rate was set to 1 mL/min through the column and split to derive 0.2 mL/min to the mass spectrometer. Mass spectra were acquired in a *m/z* range from 500 to 1000. To confirm oxidation products structures, 10 μL formic acid (1 M) were added to samples and immediately neutralized with 10 μL sodium hydroxide (1 M). Samples were then analyzed by LC–ESI-MS as described above. Indeed, by adding formic acid, an epoxy group will be opened and converted into a diol group (Mori, Porzio, & Schaleger, 1972), which will result in an *Rt* shift.

2.3.3. Kinetic curves

Spectral data acquisition was performed using SIM (single ion monitoring) mode. Molecular ions corresponding to each identified product were selected. Based on areas of the chromatographic peaks, kinetic curves were drawn for each identified degradation product and for the starting material.

3. Results and discussion

3.1. Determination of optimal oxidative temperatures

To determine the optimal oxidative conditions of SOPC and SLPC, the percentage of hydroperoxides [M+H+32] generated at several temperatures was determined. Fig. 1 shows that the temperature allowing the highest yield of hydroperoxides was different for thermally oxidized SOPC and SLPC. For the same oxidation time (30 min), production of hydroperoxides was the highest at 125 ± 1 °C for SLPC and at 150 ± 1 °C for SOPC. This is in accordance with reports showing that free linoleic acid is more sensitive to oxidation than free oleic acid (Shahidi, 2006). It is also noteworthy that the percentage of hydroperoxides produced by SOPC is higher than in the case of SLPC. This is also in accordance with previous studies showing that the amount of hydroperoxides

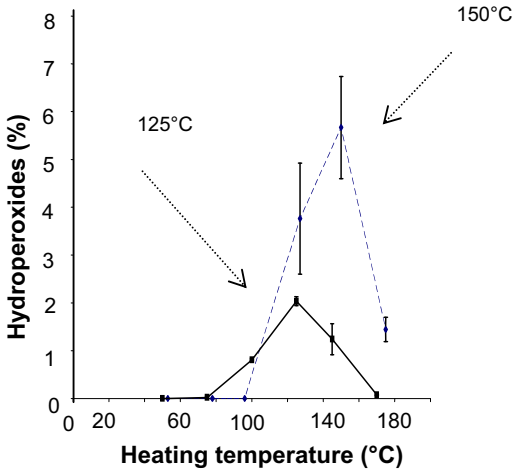

Fig. 1. Evolution of SOPC (–♦–) and SLPC (–■–) hydroperoxides production during thermal oxidation at different temperatures for 30 min.

generated through oxidation decreased with the increase of the number of double bonds, which is ascribed to a higher rate of hydroperoxide decomposition (Frankel, 2005; Shahidi, 2006). For temperatures below 80 °C for SLPC and below 100 °C for SOPC, the oxidative process seems to be slow, which explains the small amounts of hydroperoxides produced, either from SOPC or from SLPC (Fig. 1). Hydroperoxides were not formed or at a very small yield, so they could not have been further decomposed into other oxidation products in a significant way. This implies a relative stability of the system until the activation energy is sufficient to initiate the oxidation process. At heating temperatures over 80 °C for SLPC and 100 °C for SOPC, activation energy is apparently high enough to form large amounts of hydroperoxides and to destabilize the system by breaking down the produced hydroperoxides at the same time. In fact, while it is known that the oxidation process is accelerated with the increase of temperature, it was also reported that heat promotes the decomposition of hydroperoxides on free fatty acids (Frankel, 2005; Shahidi, 2006). The overall process is in a way a competition between formation and decomposition of hydroperoxides. To study the kinetics of formation of oxidized products from hydroperoxides, temperatures that yielded the largest amounts of hydroperoxides were chosen.

Table 1
Possible structures of SOPC and SLPC oxidation products with the corresponding *m/z* and retention times.

Ion *m/z* [M+H]	Possible structure(s)[a]	*Rt* (min)	Reaction with formic acid[b]
SOPC oxidation products			
788.6	***18 :0–18 :1-PC***	38.7	–
802.6	18 :0–18 :1 (Ep)-PC	29.9	–
	18 :0–18 :1 (Ke)-PC		
804.6	18 :0–18 :0 (Ep)-PC	29.1	–
	18 :0–18 :0 (Ke)-PC		
	18 :0–18 :1 (OH)-PC		
804.6	***18 :0–18 :0 (Ep)-PC***	32.7	+
	18 :0–18 :0 (Ke)-PC		
	18 :0–18 :1 (OH)-PC		
820.6	***18 :0–18 :1 (OOH)-PC***	29.5	+
SLPC oxidation products			
786.6	***18 :0–18 :2-PC***	37.7	–
800.6	***18 :0–18 :2 (Ep)-PC***	28.9	±
	18 :0–18 :2 (Ke)-PC		
802.6	18 :0–18 :1 (Ep)-PC	28.3	–
	18 :0–18 :1 (Ke)-PC		
	18 :0–18 :2 (OH)-PC		
802.6	***18 :0–18 :1 (Ep)-PC***	32.4	+
	18 :0–18 :1 (Ke)-PC		
	18 :0–18 :2 (OH)-PC		
816.6	***18:0–18:1 (Ep, Ke)-PC***	24.5	+
	18:0–18:2 (Ep, OH)-PC		
818.6	***18 :0–18 :2 (OOH)-PC***	27.7	+
	18 :0–18 :2 (OH, OH)-PC		
	18 :0–18 :1 (OH, Ep)-PC		
	18 :0–18 :1 (Ke, OH)-PC		
	18 :0–18 :0 (Ke, Ke)-PC		
834.6	***18 :0–18 :1 (Ep, OOH)-PC***	22.5	+
	18 :0–18 :2 (OH, OH, OH)-PC		

Bold italic characters refer to the identified structures.
[a] 18:0 refers to stearic acid, 18:1 refers to oleic acid, 18:2 refers to linoleic acid. Ep refers to an epoxy group, Ke to an oxo group, OH to a hydroxyl group and OOH to a hydroperoxide group.
[b] –, no change in *Rt*; +, change in *Rt*.

3.2. Oxidation products from SOPC and oxidation kinetics

For the identification of thermally induced degradation products, samples of SOPC were heated at 150 ± 1 °C as determined above for 45 min. Acquisition in RIC (Reconstructed Ion Monitoring) mode allowed the identification of several *m/z*. Table 1 shows the different ions, retention times (*Rt*) and possible structures of the products detected in chromatograms (Fig. 2a). *m/z* 788.6, detected at 38.7 min, refers to the molecular ion $[M+H]^+$ of SOPC. Three other *m/z* appeared in chromatograms after thermal treatment: *m/z* 802.6, *m/z* 804.6 and *m/z* 820.6. Each *m/z* corresponds to several possible structures. *m/z* 802.6 may carry an epoxy group (Ep): 18:0–18:1 (Ep)-PC or an oxo group (Ke): 18:0–18:1 (Ke)-PC on oleic acid. By adding formic acid, no change in *Rt* for the peak

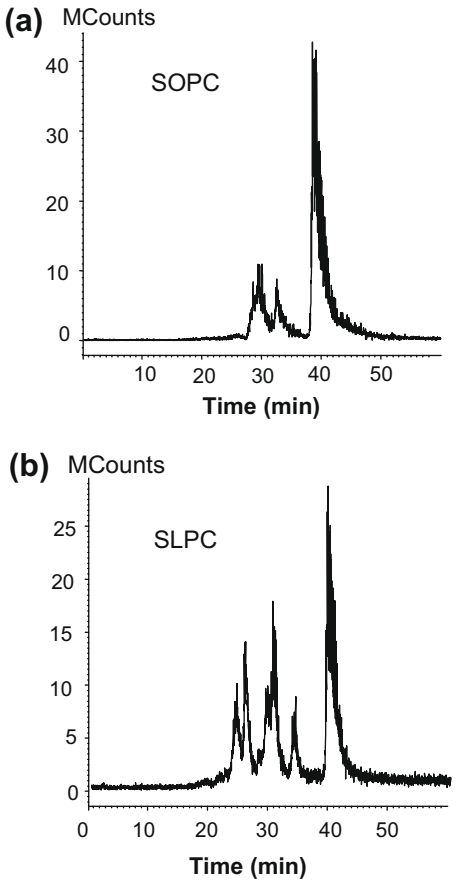

Fig. 2. Chromatograms of oxidized (a) SOPC and (b) SLPC.

at 29.9 min could be observed (Table 1), which indicated that the structure of *m/z* 802.6 is 18:0–18:1 (Ke)-PC. *m/z* 804.6 was detected with two different *Rt*: 29.1 and 32.7 min (Fig. 3a). Three structures could be envisioned for this *m/z*: an epoxy group: 18:0–18:0 (Ep)-PC, an oxo group: 18:0–18:0 (Ke)-PC or an hydroxyl group: 18:0–18:1 (OH)-PC. The addition of formic acid made the latter peak disappear while the former remained unchanged. This result showed that the peak eluted at 32.7 min presents a 18:0–18:0 (Ep)-PC structure. For the *m/z* 804.6 at 29.1 min, it can be suggested that its structure is most probably 18:0–18:1 (OH)-PC, an 18:0–18:0 (Ke)-PC structure being less likely since it has never been reported so far. The other structures (epoxy-octadecanoate, oxo-octadecenoate and hydroxy-octadecenoate) have in fact been identified in previous investigations of thermal degradation of oleic acid and methyl oleate (Frankel, 2005; Lercker, Bortolomeazzi, & Pizzale, 1998; Velasco, Berdeaux, Marquez-Ruiz, & Dobarganes, 2002). Finally, *m/z* 820.6, which was detected at 29.5 min, may carry only one possible structure: a hydroperoxy group: 18:0–18:1 (OOH)-PC (Table 1). Adding formic acid made the peak disappear, which confirms the high reactivity of the molecule and therefore its hydroperoxide nature.

Kinetics of degradation of SOPC and of production of its oxidation products were monitored over time at 150 ± 1 °C (Fig. 4). The behaviour of the degradation products provided information on their heat stability, which was an additional clue that helped confirm their structures. SOPC peak area gradually decreased between 30 and 90 min of oxidation then seemed to have stabilized (Fig. 4a). With regard to the oxidation products, their formation could only be detected after a minimal heating period of 30 min. It is noteworthy that *m/z* 820.6 was the first to reach a maximum before decreasing until it totally disappeared after 90 min. Its behaviour with regard to oxidation (Fig. 4b) confirmed its hydroperoxide structure.

As far as the other oxidation products are concerned, *m/z* 802.6 (18:0–18:1 (Ke)-PC) reached a maximum after 45 min of heating before decreasing and stabilizing by 90 min. *m/z* 804.6 (18:0–18:1 (OH)-PC and 18:0–18:0 (Ep)-PC) reached their maxima after 60 min of heating and stabilized (Fig. 4b). These kinetics showed that the oxidation products monitored here are stable. However, as carbonyl compounds (18:0–18:1 (Ke)-PC for example) are very reactive, they would certainly be involved in Maillard reactions in complex food matrices. This way, they would probably be converted into other degradation products such as imines (Frankel, 2005) and would not be present in sufficient concentrations to be detected. Nevertheless, as hydroxyl groups are less reactive than the carbonyl ones, they would probably be more readily detected in food matrices containing PL after oxidative treatment.

3.3. Oxidation products from SLPC and oxidation kinetics

Samples of SLPC were heated at 125 ± 1 °C as determined above for 30 min to determine structures of SLPC degradation products. The obtained *m/z*, identified in RIC mode, are listed in Table 1. *m/z* 786.6 with a *Rt* of 37.7 min refers to the molecular ion $[M+H]^+$ of SLPC. Five other *m/z* were found in chromatograms (Fig. 2b). *m/z* 800.6 gave an asymmetrical peak at an *Rt* of 28.9 min. Table 1 shows that two structures are likely for this products, an epoxy group 18:0–18:2 (Ep)-PC and an oxo group 18:0–18:2 (Ke)-PC on linoleic acid. By adding formic acid, the peak did not entirely disappear, which revealed that the peak with *m/z* 800.6 is in fact made of two overlapped peaks, each corresponding to one of the possible structures. Another product, *m/z* 802.6, showed a profile quite sim-

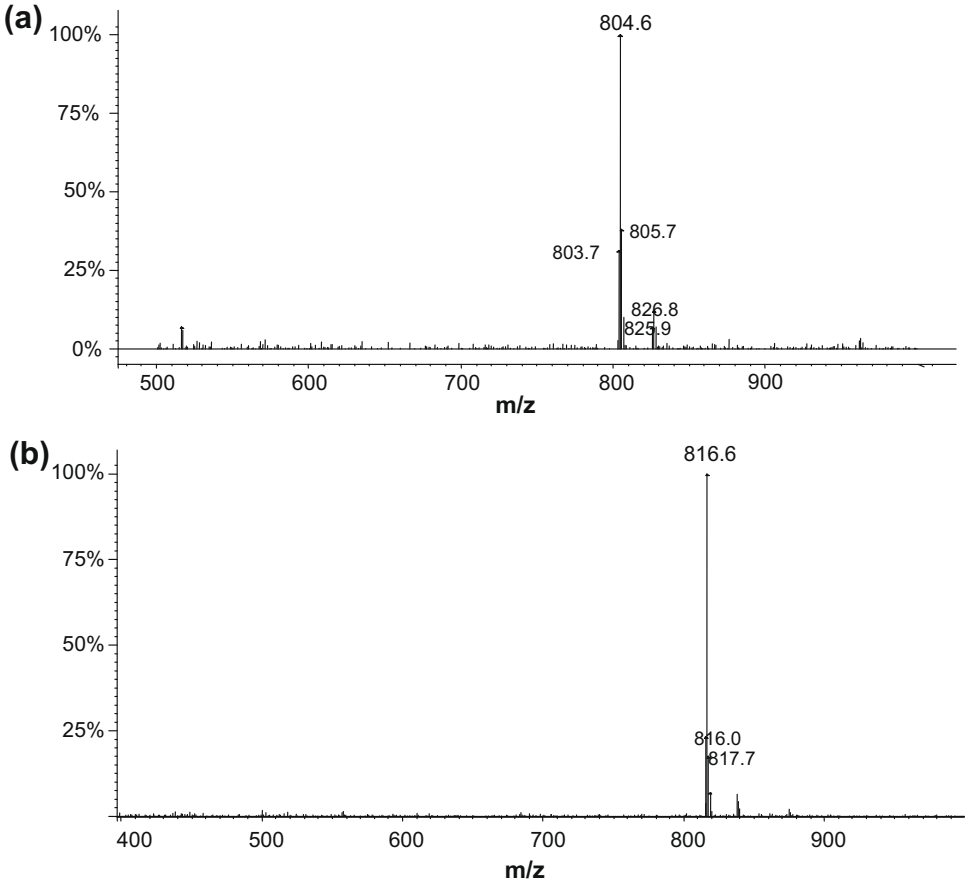

Fig. 3. Mass spectra of oxidation products at two different retention times: (a) SOPC (*Rt* = 32.7 min) and (b) SLPC (*Rt* = 24.5 min).

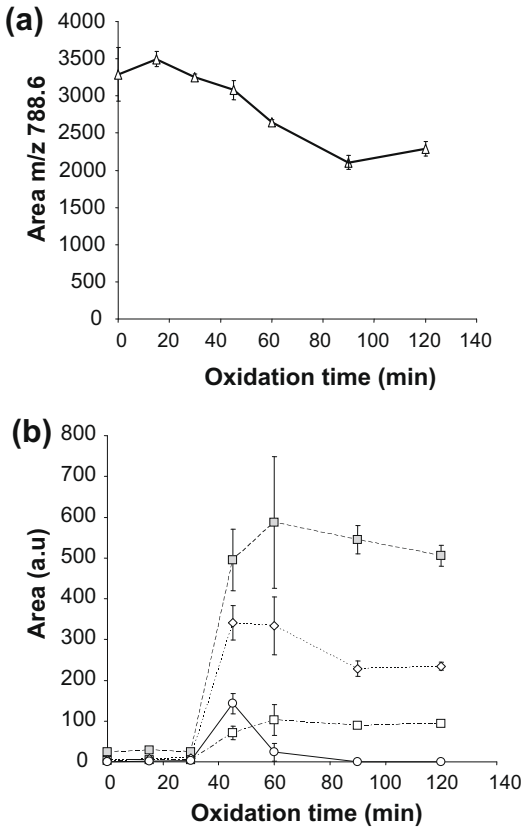

Fig. 4. Kinetics of SOPC oxidation during heat treatment at 150 °C: (a) evolution of SOPC area; (b) formation of oxidized products: –○– *m/z* 820.6, - -◇- - *m/z* 802.6, – –□– – *m/z* 804.6 (2), *m/z* 804.6 (1).

ilar to that of *m/z* 804.6 from SOPC, with two peaks were detected. Three structures were possible for *m/z* 802.6 as shown in Table 1. By treating samples with formic acid, only the second of the two peaks disappeared (Table 1). As for SOPC, the first peak was identified as SLPC with a hydroxyl group: 18:0–18:2 (OH)-PC and the second one as SLPC with an epoxy group: 18:0–18:1 (Ep)-PC. *m/z* 816.6, identified at an *Rt* of 24.5 (Fig. 3b), could be associated with two possible structures as shown in Table 1: 18:0–18:1 (Ep, Ke)-PC and 18:0–18:2 (Ep, OH)-PC. In this case, acidic treatment confirms that this oxidation product carries an epoxy group, since the peak disappeared. Moreover, since the starting material (SLPC) carries two double bonds which are sensitive to oxidation, an 18:0–18:1 (Ep, Ke)-PC structure was considered the most likely, which is in agreement with a previous report (Toschi, Costa, & Lercker, 1997). An 18:0–18:2 (Ep, OH)-PC structure would on the other hand be probably found in degradation products of more unsaturated fatty acids, such as linolenic acid (Frankel, 2005). *m/z* 818.6, with an *Rt* of 27.7, which could be related to five possible structures (Table 1), was in fact quite instable and its peak disappeared with acid treatment. This helped narrow down the possibilities to the two acid-sensitive structures: 18:0–18:2 (OOH)-PC and 18:0–18:1 (OH, Ep)-PC. The last identified *m/z*, 834.6, had an *Rt* of 22.5 and could be related to two possible structures as shown in Table 1: 18:0–18:1 (Ep, OOH)-PC and 18:0–18:2 (OH, OH, OH)-PC. The corresponding oxidation product was sensitive to acidic treatment, which indicated that the most likely structure for *m/z* 834.6 was 18:0–18:1 (Ep, OOH)-PC. The suggested structures of oxidation products from SLPC were further confirmed by *Rt* values that were in accordance with their polarity (Table 1). In addition, structures, such as keto-linoleate, epoxy-linoleate, hydroxyl-linoleate, ketoepoxy-oleate and epoxyoleate, have previously been reported by studies carried out on free linoleic acid and linoleic acid methyl ester (Frankel, 2005; Toschi et al., 1997; Velasco et al., 2002). As far as hydroperoxides are concerned, epoxy-hydroperoxide could be obtained from hydroperoxides of linoleate (Frankel, 2005).

Kinetics of degradation of SLPC and of production of its oxidation products were monitored over time at 125 ± 1 °C (Fig. 5). Fig. 5a shows changes in the amounts SLPC and its oxidation products during heating at 125 ± 1 °C. SLPC peak area decreased in a sharp and rapid way, and did not stabilize before 90 min of treatment. The decrease of SLPC was therefore more important than that of SOPC for the same heating durations (Figs. 4 and 5a). As previously mentioned, this result is in accordance with the fact that SLPC is more sensitive to oxidation than SOPC.

As far as the formation of thermally induced oxidation products is concerned, two groups could be identified: the first included products that rapidly reached a maximum before completely disappearing (Fig. 5b); the second included more stable products that

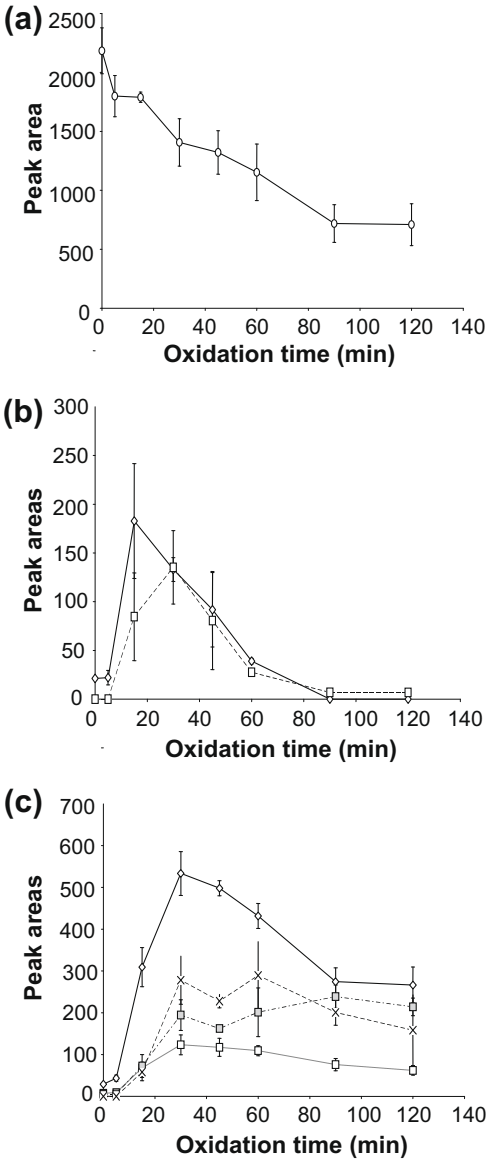

Fig. 5. Kinetics of SLPC oxidation during heat treatment at 125 °C: (a) evolution of SLPC area, (b) formation of oxidized products (group 1): –◇– *m/z* 818.6, –□– *m/z* 834.6; (c) formation of oxidized products (group 2): –◇– *m/z* 800.6, –□– *m/z* 802.6 (1), – –□– – *m/z* 802.6 (2), –×– *m/z* 816.6.

did not disappear (Fig. 5c). The first group included *m/z* 818.6 and *m/z* 834.6. The evolution of peak areas was monitored during heating. Fig. 5b shows that a maximum was reached after 15 min before the area started decreasing and totally disappeared after 90 min. As observed for SOPC and according to the identification, this behaviour is indicative of a hydroperoxide structure: 18:0–18:2 (OOH)-PC for *m/z* 818.6. *m/z* 834.6 area reached a maximum after 30 min oxidation and then it decreased until 90 min where very little product was detected (Fig. 5b). This trend, which is quite similar to that of *m/z* 818.6, suggested that *m/z* 834.6 also carries a hydroperoxide function, which is in accordance with the identified structure: 18:0–18:1 (Ep, OOH)-PC.

The second group of thermally generated products included compounds with *m/z* 816.6, *m/z* 802.6 and *m/z* 800.6. These products behaved in the same way as oxidation products from SOPC: the amount of each product reached a maximum after 30 min of oxidation, then decreased and stabilized. As for SOPC, some oxidation products of this group, which is quite stable once formed, are likely to be found in sizable amounts in processed foods containing PL. As for SOPC, oxidation products containing a carbonyl group would probably be difficult to detect in complex food matrices due to Maillard reaction (Frankel, 2005).

Finally, heat treatment of unsaturated PL (SOPC and SLPC) leads to hydroperoxides which are degraded into further oxidized products. Structures elucidated for oxidation products of these unsaturated PL could also be identified in oxidized TAG (Byrdwell & Neff, 1999, 2001; Neff & Byrdwell, 1998), which suggests that their formation in PL is not all that surprising. As heat treatment is a major and widespread food process, investigating its impact on the structure, the stability and the safety of PL is of great interest to the consumer and to the food industry. While this study does not reflect the real conditions of a food matrix during processing, it allowed however the identification of degradation products that are likely to be formed in food from two major PC species, which represents a first step towards unravelling PL thermal degradation during food processing. As kinetics is influenced by the composition of the food matrix, it would probably differ if sugars, proteins, metals or other lipids are mixed together with unsaturated PL. Since the antioxidative capacity of PL is not fully understood (Shahidi, 2006), it is still unknown whether the oxidative process would be enhanced or inhibited in presence of other food constituents. Further investigations have to be carried out that take into account the effects of complex food matrices on the oxidative process.

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