KINETIC OF PHOSPHOLIPID OXIDATION IN LIPOSOMAL SUSPENSIONS

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Abstract

In this paper, the effects of temperature, the addition of a free radical generator and the lipid content on the oxidation rate of phospholipids were investigated. Soy lecithin liposomes obtained by reverse-phase evaporation method were employed to prepare liposomal suspensions containing 1 wt%, 5 wt% and 10 wt% of lipidic fractions. Different temperatures (37 °C, 60 °C and 80 °C) or 10 mmol/l of a water soluble free radical generator were used to promote lipid oxidation of suspensions. Peroxide value was measured as the lipid oxidation indicator and used to determine the kinetic parameters of oxidation. The results showed that the investigated factor exerted a significant effect ($p<0.05$) on the lipid oxidation rate, being these effects attributed to the modification of packing degree and radius of curvature by the studied factors. Besides, the temperature dependence of the reaction rate exhibited a normal Arrhenius pattern.

Keywords: *Lipid oxidation, liposomes, lipid content, soy lecithin.*

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1. INTRODUCTION

Lipid oxidation is one of the most important deterioration processes occurring in foods. Presently, there is an increasing number of investigations addressing the issue of the effects of consuming oxidized foods on human health and their relationship with several diseases [1–3].

Besides, it is well known that lipid oxidation is the main cause of consumer's non-acceptance of foods due to unpleasant flavors and aromas originated when oxygen reacts with lipids.

Lipid oxidation has been widely studied in bulk fats and oils and there is a good understanding of factors affecting oxidation rate in such systems [4]. However, in most foods, lipids are not freely arranged as in bulk systems but are forming heterogeneous structures where the interactions between lipids and their neighboring molecules modify their chemical behavior and play an important role on lipid oxidation. Therefore, it is important to examine lipid oxidation in these systems. Nevertheless, because of the chemical complexity of foods, studies related to lipid oxidation are usually carried out in simplified model systems such as oil-in-water emulsions and, to a lesser extent, liposomes.

Liposomes, an artificially prepared vesicle composed of one or more phospholipid bilayers, have been frequently used in experimental studies to garner a deeper understanding into the mechanisms governing lipid peroxidation [5, 6]. These studies revealed the existence of specific factors that might affect the rate of oxidation in liposomal systems.

Some factors have already been studied. For example, when the lipid content is constant, liposome size has a great influence on lipid oxidation rate, being smaller liposomes oxidized more quickly than larger ones [7]. Also, liposome type is important since unilamellar liposomes are more susceptible to oxidation than multilamellar liposomes, in view of the different accessibility of the phospholipid layers to aqueous oxidants. The influence of some factors such as surface charge, membrane fluidity, heat, light, radiation, etc. still remind unclear and it is the origin of new research works.

Elucidation of the effect of each of these factors requires comparison of the oxidation in systems that differ only in the specific studied factor. A useful tool to analyze and to compare the influence of specific factors over the lipid oxidation process is kinetic modeling.

In this paper the effects of temperature, the addition of a free radical generator and the lipid content on the oxidation rate of phospholipid liposomal suspensions were studied by comparing the kinetic constants of hydroperoxides formation.

2. MATERIALS AND METHODS

2.1 Materials

Soy lecithin phosphatidylcholine-enriched fraction, composed of phosphatidylcholine (min. 92%), lysophosphatidylcholine (max. 3%), other phospholipids (max. 2%), and fatty acid (approximately 1%), was purchased from Merck (KGaA, Germany). The hydrophilic

free radical generator 2,2-azobis(2-amidinopropane) hydrochloride (AAPH) was purchased from Wako Chemicals, USA. Any other reagents used were of analytical grade of purity.

2.2 Lipid Characterization

The fatty acid profile of soy lecithin was determined by gas chromatography-flame ionization detection, according to the Fatty Acid Methyl Esters (FAME) analysis prepared by AOAC Nº 969.33 method [8]. Analyses of the FAME were carried out on an Agilent® 6850 chromatograph equipped with a Supelco 2340 capillary column using Fame Mix C4-C25 (Supelco, USA) and C19 (Sigma-Aldrich, Germany) as external and internal standard, respectively.

2.3 Liposome Preparation

Liposomes were prepared by reverse-phase evaporation method [9] slightly modified. Soy lecithin (7.5 g) was dissolved in diethyl ether (100 ml) and mixed with Sorensen's phosphate buffer (10% of final volume, pH 7.17). The mixture was sonicated for 30 minutes at 400 W output power (40 kHz frequency) in a laboratory ultrasonic device (Teslab; model TB010TA, Argentine). Then, it was placed on a rotary evaporator (Figmay, model SV1, Argentine) and the organic solvent was removed under reduced pressure at 25 °C and 200 rpm. Finally, buffer phosphate (90% of final volume) was added and the system was gently mixed to obtain 1 wt%, 5 wt% and 10 wt% liposomal suspensions.

2.4 Lipid Oxidation Experiments

Lipid oxidation was initiated by both heat and AAPH addition. Liposomal suspensions were disposed into open recipients, maintaining a constant air exposed surface/weight rate of 1.56 cm^2/g, and incubated in the dark at three different temperatures (37 °C, 60 °C y 80 °C) in heat catalyzed experiments, or added with 10 mmol/l of AAPH and incubated in the dark at 37 °C in free radical catalyzed experiments. In all cases, liposomal suspension aliquots were taken at regular intervals of time to determine peroxide value, a primary indicator of oxidation.

2.5 Peroxide Value Measurements

Peroxide value (PV) was determined by the ferric thiocyanate method, adapted from the FIL-IDF 74A method [10]. Samples (0.02 g) were dissolved in a 9.8 ml of methanol:chloroform (70:30 v/v) solution and mixed with 0.1 ml of 30% ammonium thiocyanate. Subsequently, 0.1 ml of ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture, and after 5 minutes, the absorbance was measured at 501 nm against the solvent solution as blank. PV was expressed in terms of miliequivalent of active oxygen per kilogram of oil (meq O_2/kg of oil). Each analysis was performed at least in duplicate.

2.6 Kinetic of Lipid Autoxidation

According to the classical free radical model for lipid oxidation, oxidation is a complicated process which includes typical initiation, propagation, and termination stages. Although lipid oxidation involves a large number of possible reactions, the formation and decomposition of hydroperoxides, the primary oxidation product of lipidic substrates [11, 12], may be described by the following elementary steps as proposed by Labuza et al. [13].

Initiation

$$ROOH \xrightarrow{k_i} RO\bullet + \bullet OH \quad (1a)$$

$$ROOH \xrightarrow{k_i} ROO\bullet + \bullet H \quad (1b)$$

$$ROOH \xrightarrow{k_i} R\bullet + \bullet OOH \quad (1c)$$

Propagation

$$R\bullet + O_2 \xrightarrow{k_0} ROO\bullet \quad (2a)$$

$$ROO\bullet + RH \xrightarrow{k_p} R\bullet + ROOH \quad (2b)$$

Termination

$$RO_2\bullet + RO_2\bullet \xrightarrow{k_t} \text{Non Radical Products} \quad (3)$$

Where ROOH is the lipid hydroperoxide, ROO• the lipid peroxyl radical, RO• the lipid alkoxyl radical, R• the lipid radical, RH the lipid molecule, k_i the initiation rate constant, k_p the propagation rate constant and k_t the termination rate constant. The following equation for the rate of hydroperoxide formation, which is a measure of the rate of oxidation, was proposed to describe the entire autoxidation process [14, 15].

$$[ROOH]^{1/2} \cong k_G.t \quad (Eq.\ 1)$$

Where [ROOH] is the hydroperoxide concentration, k_G is the global rate constant of oxidation, defined as

$$k_G \cong k_p.\left(\frac{k_i}{2.k_t}\right)^{1/2}.[RH] \quad (Eq.\ 2)$$

and t is the heating time.
The effect of temperature on the kinetic constants of lipid oxidation was evaluated by means of Arrhenius equation:

$$k_G = A\ .\ e^{-\frac{E_a}{R.T}} \quad (Eq.\ 3)$$

where T is the absolute temperature, R is the ideal gas constant (8.314 $J.mol^{-1}.K^{-1}$), A is the pre-exponential factor and E_a is the apparent activation energy ($J.mol^{-1}$).

2.7 Statistical Analysis

Statistical analysis were conducted using Microsoft® Excel, Statgraphics® for Windows and SigmaPlot© for Windows software. Normal distribution and homoscedasticity of data were evaluated using Shapiro-Wilk test and Levene test, respectively. The analysis of variance (ANOVA) was performed to evaluate the differences in the kinetic constants of peroxide formation. When ANOVA indicated the presence of a significant difference, post-hoc comparisons using the Holm-Sidak method were applied to determine multiple pairwise differences. All data were given as mean ± standard deviation. The level of significance was set at α=0.05

3. RESULTS AND DISCUSSION

The fatty acid composition of soy lecithin, used as phospholipid source in oxidation experiments was determined by gas chromatography. Fatty acid detected were palmitic acid (10.7%), stearic acid (3.3%), oleic acid (8.8%), linoleic acid (70.3%) and linolenic acid (6.9%).

From results, it can be mentioned that soy lecithin employed in oxidation tests had a high content of unsaturated fatty acids, being very susceptible to oxidation.

Phospholipids were used to obtain liposomal suspension by reverse-phase evaporation method. To confirm the liposome formation, freshly prepared suspensions were observed through an optical microscopic.

3.1 Oxidation of Liposomal Suspensions Catalyzed by Heat

Freshly prepared liposomal suspensions were exposed to heat to initiate lipid oxidation. Figure 1 shows the changes in PV in liposomal suspensions exposed at different temperatures.

It can be seen that PV increased in time for all temperatures and all lipid contents tested. Besides, the lower the lipid content of liposomal suspension, the higher the development of PV.

The global rate constants of liposome oxidation were obtained by fitting the kinetic model proposed (Eq. 1) to the measured PV. The values of kinetic constants and correlation coefficients (R^2) are shown in Table 1.

Analysis of calculated constants indicated that both temperature and lipid content exerted a significant effect on lipid oxidation. As expected, the relationship between the oxidation rate constants and temperature was directly proportional. This behavior was previously observed in oxidation of liposomes prepared from cuttlefish phospholipids [16].

The temperature dependence of kinetic constants was determined through application of Arrhenius law. Apparent activation energies, determined from the slopes of the lines generated by regression of ln k_G versus 1/T, are presented in Table 2.

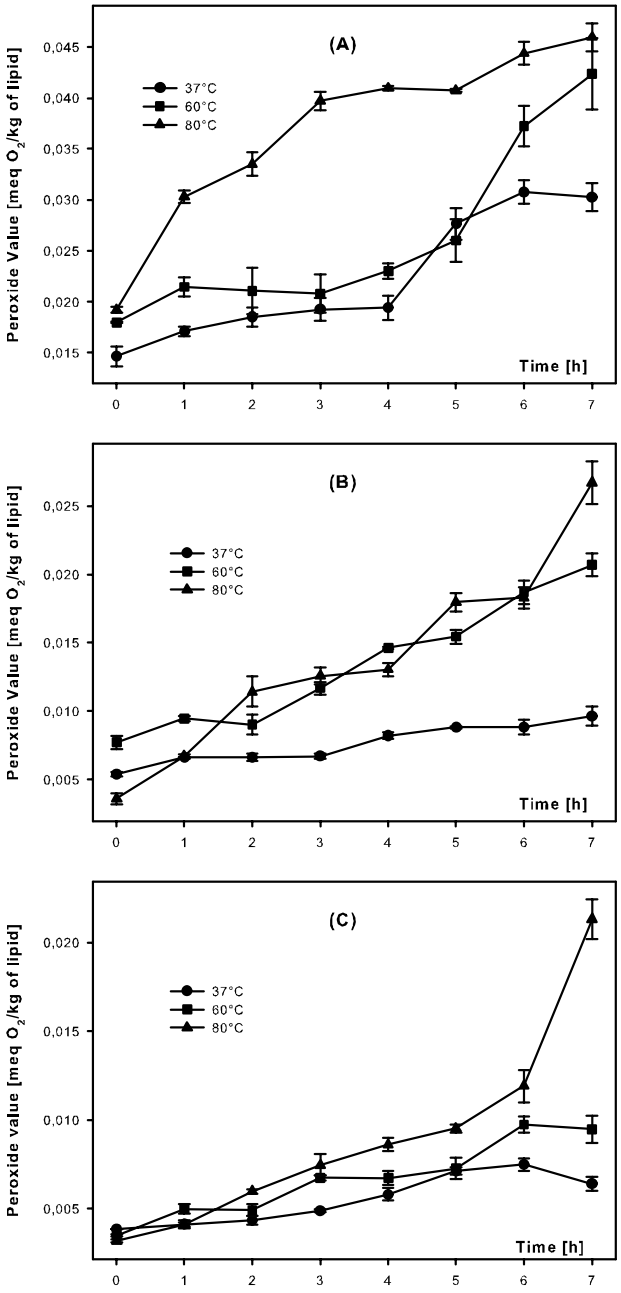

Fig-1 Peroxide value obtained in 1 wt% (A), 5 wt% (B) and 10 wt% (C) soy lecithin liposomal suspensions heated at different temperatures. Data markers represent the mean ± standard deviation of two determinations.

The results indicated that the lower the lipid content of liposomal suspensions, the lower the calculated apparent activation energies. This would suggest that oxidation of liposomal suspensions are less sensitive to temperature changes at lower levels of lipid content.

In contrast to temperature-rate constant relationship, in heated liposomal suspensions the relationship between

oxidation rate constants and lipid content was observed as inversely proportional. This was also perceived in a previous work [17] although the authors did not expose any explanation for this behavior. This behavior was unexpected since, in unilamellar liposomes, a higher content of the lipid phase leads to the formation of larger liposomes, which results in an increased interfacial area [18], and this should favor the oxidation in higher lipid content liposomal suspensions.

Therefore, the results would indicate that the interfacial area is not a determining factor in the rate of oxidation in unilamellar liposomes.

Table-1. Calculated rate constants for peroxide formation (k_G), obtained for soy lecithin liposomal suspensions, heated at different temperatures

	1 wt% Lipidic phase		5 wt% Lipidic phase		10 wt% Lipidic phase	
Temperature [°C]	$k_{G1}.10^3$	R^2	$k_{G5}.10^3$	R^2	$k_{G10}.10^3$	R^2
37	8.15 ± 0.07^{aC}	0.90	6.45 ± 0.64^{bC}	0.92	3.56 ± 0.16^{cC}	0.85
60	9.40 ± 0.14^{aB}	0.81	8.25 ± 0.07^{bB}	0.97	5.39 ± 0.75^{cB}	0.94
80	13.02 ± 0.02^{aA}	0.77	12.55 ± 0.07^{bA}	0.94	10.93 ± 0.18^{cA}	0.91

Values, expressed in [(meq O_2/kg of lipid)$^{1/2}$ h^{-1}], represent the mean ± standard deviation of two determinations. ^{abc} Means with different letters in each row are significantly different ($p<0.05$). ^{ABC} Means with different letters in each column are significantly different ($p<0.05$).

In compartmentalized systems such as liposomes, it is reasonable to assume that free radical inducers present in the external medium must first gain access to the unsaturated fatty acyl chains buried inside the membrane bilayer to start the chain reaction of lipid oxidation. If this is the case, either the transition metal ions and oxygen or the hydroxyl radicals should penetrate into the membrane bilayer and a higher water permeability of the latter would definitely facilitate this process. Numerous physical studies of the acyl chain structure of liposome suggest that acyl chain packing partly depends on the radius of curvature of the liposome [18–20]. With a higher surface curvature, the unsaturated fatty acyl chains of small liposomes should have a greater exposure to water, resulting in a higher accessibility of these potential targets of oxidation reactions to the water soluble oxidants (Figure 2).

Thus, it would be the effect of the lower degree of membrane packing of small size liposomes the reason why lipid oxidation proceeds faster in liposomal systems with lower lipid content.

The explanation above might also be helpful to explain the effect of temperature on kinetic constants observed in Table 2.

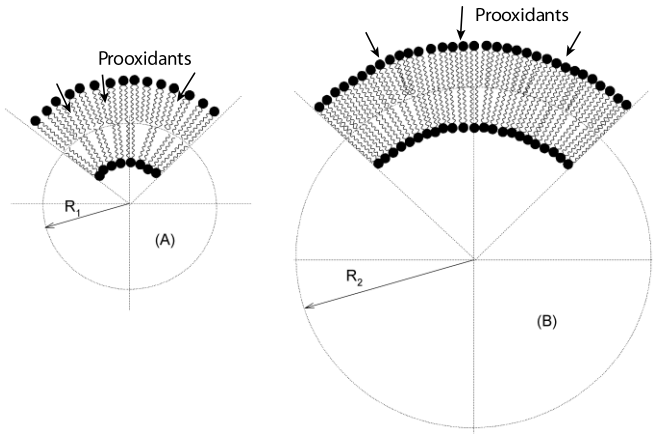

Fig-2 Influence of radius of curvature of liposome on molecular packing of lipid bilayer of small (A) and large (B) liposomes Radius $R_2 >> R_1$

Table-2. Calculated apparent activation energies of liposomal suspensions

Soy lecithin liposomal suspension	Ea	R^2
1 wt%	9.70 ± 0.21^{B}	0.91
5 wt%	28.75 ± 3.72^{A}	0.99
10 wt%	23.35 ± 0.41^{A}	0.95

Values, expressed in [$kJ.mol^{-1}$], represent the mean ± standard deviation of two determinations. ^{AB} Means with different letters are significantly different ($p<0.05$).

It is known that temperature reduces molecular ordering and increases molecular dynamics in lipid bilayers [19]. Due to the fact that larger liposomes with larger radius of curvature have a more ordered molecular structure there is a chance that they were more affected by temperature than smaller liposomes, modifying their degree of packing and, consequently, their oxidation rate.

3.2 Oxidation of Liposomal Suspensions Catalyzed by AAPH Addition

In these experiments, the liposomal suspensions were oxidized at 37 °C due to the constant flux of initiators radicals generated in the aqueous phase by thermal scission of AAPH. The PV, measured through time for all assayed liposomal systems is shown in Figure 3. It can be noticed that an increase in lipid peroxides content was observed in all tests performed.

The obtained PV was used to determine the global rate constants of liposome oxidation by fitting the kinetic model proposed (eq. 1) to the measured data. The values of kinetic constants and correlation coefficients are shown in Table 3.

A subsequent statistical analysis of estimated constants showed that AAPH addition exerted a significant effect on lipid oxidation in all systems, being its effect relatively higher in less concentrated liposomal suspensions. Once again, this effect could be attributed to the lesser packing of smaller liposomes presents in 1 wt% liposomal suspensions, since this model system was the most significantly affected when there was a constant flux of radical initiators in the aqueous medium.

As a consequence, the obtained results in free radical catalyzed experiments reinforced the explanation that the radius of curvature plays an important role in liposome oxidation.

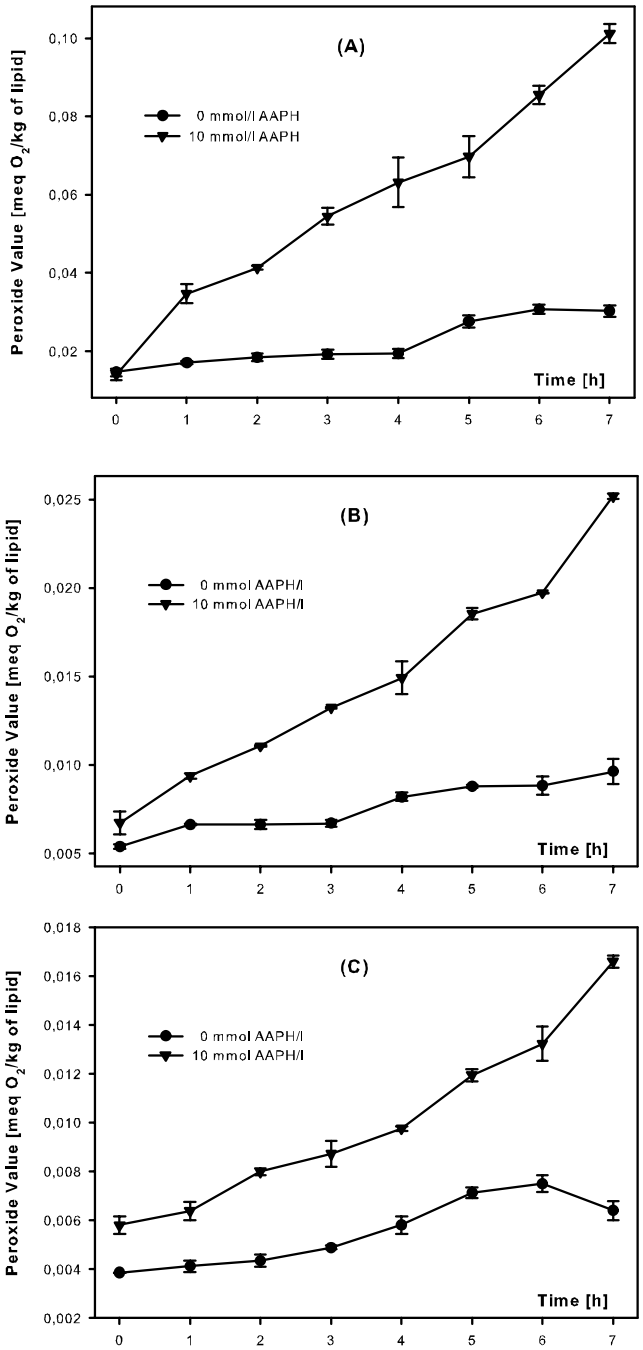

Fig-3 Peroxide value obtained in 1 wt% (A), 5 wt% (B) and 10 wt% (C) soy lecithin liposomal suspensions heated a 37 °C and with or without 10 mmol/l of AAPH added. Data markers represent the mean ± standard deviation of two determinations.

Table-3. Calculated rate constants for peroxide formation (k_G), obtained for soy lecithin liposomal suspensions, heated at 37 °C and with or without 10 mmol/l of AAPH added.

	1 wt% Lipidic phase		5 wt% Lipidic phase		10 wt% Lipidic phase	
AAPH [mmoles/l]	$k_{G1}.10^3$	R^2	$k_{G5}.10^3$	R^2	$k_{G10}.10^3$	R^2
0	8.2 ± 0.1^{aB}	0.90	6.4 ± 0.6^{bB}	0.92	3.6 ± 0.2^{cB}	0.85
10	25.3 ± 0.6^{aA}	0.95	10.2 ± 0.3^{bA}	0.99	7.2 ± 0.6^{cA}	0.98

Values, expressed in [(meq O_2/kg of lipid)$^{1/2}$ h^{-1}], represent the mean ± standard deviation of two determinations. ^{abc} Means with different letters in each row are significantly different ($p<0.05$). ^{ABC} Means with different letters in each column are significantly different ($p<0.05$).

4. CONCLUSION

In liposomal suspensions, the lipid oxidation rate is affected by several factors. In this work the effects of temperature, the addition of a free radical generator and the lipid content over the kinetic of lipid oxidation developed in liposomal suspensions are reported. All factors analyzed exerted a significant effect on lipid oxidation rate of liposomal suspensions, being the influence of temperature and AAPH addition directly proportional and the effect of lipid content inversely proportional. These behaviors were attributed to the modification of packing degree and radius of curvature by the studied factors.

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