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Liposomes as a Tool to Study Lipid Peroxidation in Retina

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1. Introduction

In living organisms, the oxidative stress is associated with several physio- pathological affections (e.g. atherosclerosis, cancer, aging, neurodegenerative diseases). The oxidative stress is generally initiated by generation of reactive oxygen (ROS) and nitrogen species (RNS) (Halliwell & Gutteridge, 1990). ROS are continuously formed during cellular metabolism and are removed by antioxidants defences. ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components and alters many cellular functions. The most vulnerable molecules to oxidative damage are proteins, lipids and DNA (Kohen & Nyska, 2002; Catalá, 2009, 2011a, 2011b).

In mammalian retina, free radicals and lipoperoxides seem to play important roles in the evolution of different retinopathies including glaucoma, cataractogenesis, diabetic retinopathy, ocular inflammation and retinal degeneration (Ueda et al., 1996; De La Paz & Anderson, 1992). Because of free radicals production induces the lipid peroxyl radical formation, known as secondary free radicals products; this chain reaction of lipid peroxidation can damage the retina, especially the membranes that play important roles in visual function (Catalá, 2006). The retina is the neurosensorial tissue of the eye. It is very rich in membranes and therefore in polyunsaturated fatty acids (PUFAs) such as docosahexenoic acid (22:6 n-3), that are quite vulnerable to lipid peroxidation. Also, the human retina is a well oxygenated tissue. High-energy short-wavelength visible light promotes the formation of ROS which can initiate lipid peroxidation in the macula and elsewhere. The macular carotenoids are thought to combat light-induced damage mediated by ROS by absorbing the most damaging incoming wavelengths of light prior to the formation of ROS and by chemically quenching ROS once they are formed.

Although peroxidation in model membranes may be very different from peroxidation in biological membranes, the results obtained in model membranes may be used to progress

our understanding of subjects that cannot be studied in biological membranes. Nevertheless, in spite of the relative simplicity of peroxidation of liposomal lipids model, these reactions are still relatively complex because they depend in a complex fashion on liposome type, reaction initiator and reaction medium (Fagali & Catalá, 2009). This complexity is the most likely cause of the apparent contradictions of literature results.

Biological membranes are complex systems. In view of this complexity and in order to avoid collateral effects that may arise during lipid peroxidation process of whole retinal membranes, we have attempted to gain understanding of the mechanisms responsible for peroxidation in a simple model system, made by dispersing retinal lipids in the form of liposomes.

This chapter describes a very useful method to prepare liposomes with natural phospholipids and the necessary methodology to follow the lipid peroxidation of these liposomes.

2. Materials and methods

2.1. Materials

Chloroform, methanol, trizma base, butylated hydroxytoluene (BHT), NaCl, $FeSO_4$ heptahydrate and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. Suitable plastic lab ware was used throughout this study to avoid effects of adventitious metals. Other reagents were of the highest quality commercially available. All solutions were prepared using distilled water treated with a Millipore Q system.

2.2. Isolation of bovine retina

Eyes were enucleated at slaughter (Frigorífico Gorina), transported in ice to laboratory where retinas were taken out within 1–2 h. Under red light and with all tubes and solutions in ice buckets, corneas were excised; lenses and vitreous were subsequently removed. Eye cups were inverted and retinas were carefully peeled from the eyes. Retinas were briefly homogenized in 0.15 M NaCl (1 ml/retina) 120 s (20on-20off) at 4 ºC in an Ultraturrax X25 homogenizer at 7000 rpm.

2.3. Lipid extraction

Total lipids were extracted from retinal homogenates with chloroform/methanol (2:1 v/v) (Folch et al, 1957) at 4 ºC (sample:Folch = 1:5). A volume of water corresponding to 20 % of total volume was added. This mixture was shaken and kept in rest in cold to allow phases separation. Chloroformic phase was kept at -22 °C.

2.4. Preparation of liposomes made of retinal lipids

Total lipids obtained from retinal homogenates dissolved in chloroform were evaporated under nitrogen until constant weight and submitted to vacuum to remove traces of

chloroform. Resultant films were dispersed at room temperature in a saline solution (0.15 M NaCl). Dispersed lipids were mixed to homogeneity using a vortex-mixer to obtain non-sonicated liposomes (NSL). Sonicated liposomes (SL) were prepared by sonication of NSL under nitrogen and ice cooling (Huang, 1969), using a Sonics vibra cell, probe-sonicator Model VCX 750 (750 W, 20kHz) at 75% of maximal output. Preparation of liposomes required about 2.5 min of sonication to reach apparently minimal optical density values.

2.5. Determination of liposomes size by Dynamic Light-Scattering (DLS)

The time correlation $G(q,t)$ of the light-scattering intensity was measured at 90° with a goniometer, ALV/CGS-5022F, with a multiple-τ digital correlator, ALV-5000/EPP, covering a 10^{-6}-10^3 s time range. The light source was a helium/neon laser with a wavelength of 633 nm operating at 22 mW. Each correlation function was analyzed by the well known cumulant fit yielding the apparent mean diffusion coefficient and the distribution δD of this value (Koppel, 1972). The measurements were carried out with 80 µl of SL and NSL (lipid concentration= 2 mg/ml) in water, 0.15 M NaCl and Tris-HCl buffer 20 mM (final volume= 2 mL).

2.6. Measurements of lipid peroxidation by detection of conjugated dienes and trienes production

In order to determine conjugated dienes and trienes production, absorption spectra were recorded by means of a Shimadzu UV-1800 spectrophotometer, in the range 200 to 300 nm, at 22 °C, with 1 cm path length quartz cell. Liposomes made of retinal lipids (80 µl, 2 g/l of lipids) were diluted to 2 ml with water, 0.15 M NaCl or 20 mM Tris-HCl pH 7.4, and oxidation was initiated by the addition of $FeSO_4$ (final concentration = 25 µM). Lipid peroxidation was assessed continuously by measuring the increase in absorbance at 234 nm (formation of conjugated dienes) and 270 nm (formation of conjugated trienes) taken at 1 min intervals. Oxidation rates were determined as the slope of a regression line drawn through linear range of absorbance versus time curve. Lag times were determined as time corresponding to intersection of oxidation rate regression line with a regression line drawn through initial phase of oxidation (Sargis & Subbaiah, 2003).

2.7. Measurements of lipid peroxidation by detection of Thiobarbituric Reactive Substances (TBARS)

During Fe^{2+}- initiated reactions, extent of liposomal lipid peroxidation was assessed using a TBA assay. In this procedure, 850 µL of TBA (0.375% w/v TBA, 0.25 N HCl) were added to aliquots of 150 µl of reaction mixture containing BHT (0.1 % w/v in ethanol) to prevent possible peroxidation of liposomes during incubation. The aliquots were taken at different intervals of time. Samples were heated for 30 min at 75 °C. Absorbance was measured at 532 nm for determination of aldehydic breakdown products of lipid peroxidation.

2.8. Preparation of Fatty Acids Methyl Esters (FAME)

Lipids from retina, liposomes or liposomes exposed to peroxidation initiated by Fe^{2+}, in absence or presence of BHT, were extracted according to the method of Folch et al (1957). A similar reaction mixture to that used in the analysis of conjugated dienes but scaled up 7.5 times was used to analyze the fatty acid composition of the samples

After one hour of incubation of liposomes with or without Fe^{2+} in the presence or absence of BHT, the samples were mixed with 15 ml of chloroform:methanol (2:1 v/v) containing 0.01 % BHT to stop the reaction. The mixture was stirred, gassed with nitrogen and kept in refrigerator overnight to achieve separation of phases. The lower chloroform phase was filtered through paper filter containing anhydrous sodium sulphate. The solvent was evaporated to dryness under nitrogen. Dry lipids of retina and/or liposomes were transmethylated with 300 μl of 1.3 M BF_3 in methanol at 65°C during 180 min. After incubation 1 ml of 0.15 M NaCl was added and the fatty acid methyl esters were extracted with 1 ml of hexane. This phase was injected onto the chromatograph.

2.9. Gas chromatography – Mass spectrometry analyses

GC–MS analyses were done using a Perkin Elmer Clarus 560D MS - gas chromatograph equipped with a mass selective detector with quadrupole analyzer and photomultiplier detector and a split/splitless injector. In the gas chromatographic system, a Elite 5MS (Perkin Elmer) capillary column (30 m, 0.25 mm ID, 0.25 μm df) was used. Column temperature was programmed from 130 to 250 °C at a rate of 5 °C/min and 250 °C for 6 min. Injector temperature was set to 260 ºC and inlet temperature was kept at 250 °C. Split injections were performed with a 10:1 split ratio. Helium carrier gas was used at a constant flow rate of 1 ml/min. In the mass spectrometer, electron ionization (EI+) mass spectra was recorded at 70 eV ionization energy, in full scan mode (50-400) unit mass range. The ionization source temperature was set at 180 °C. The fatty acid composition of the lipid extracts was determined by comparing their methyl derivatives mass fragmentation patterns with those of mass spectra from the NIST databases.

3. Results

3.1. Size of sonicated and non-sonicated liposomes made of retinal lipids in different aqueous media

Average hydrodynamic radii of liposomes determined by DLS studies are presented in Table 1. We noted that NSL display a multimodal size distribution when analyzed by inverse Laplace transform (CONTIN), a result that is compatible with the high polydispersity index (PI > 0.4) from cumulants fit. Thus, hydrodynamic radii for NSL, at room temperature in different aqueous media, cover a broad range with intensity weighted maxima centered between 190 and 320 nm. On the other hand, results for liposomes formed by sonication gave, through cumulant method, hydrodynamic radii in the order of 76.4-83.3

nm, showing as expected significant influence of sonication on size and distribution. It is clear that NSL possessed higher hydrodynamic radii than SL. Either NSL or SL in water were slightly smaller than that in 0.15 M NaCl and Tris-buffer.

Type of liposome	Aqueous media	Hydrodynamic radii (nm)	Polydispersity index
Sonicated	Water	76.4	0.31
	0.15 M NaCl	83.3	0.27
	20 mM Tris–HCl	83.3	0.27
Non-sonicated	Water	190–225	0.44
	0.15 M NaCl	260–320	0.45
	20 mM Tris–HCl	200–240	0.43

Table 1. Summary of values obtained by dynamic light scattering of SL and NSL made of retinal lipids in different aqueous media. Hydrodynamic radii values are the average of at least three representative determinations in each media.

3.2. Evolution of UV spectra as a function of time for Fe^{2+} initiated lipid peroxidation of SL and NSL in different aqueous media

Figure 1 shows evolution of UV spectra as a function of time, for Fe^{2+} initiated lipid peroxidation of SL and NSL, in different aqueous media. This figure showed increases in UV absorption with a maximum at 234 nm and at 270 nm, due to conjugated dienes and trienes respectively, and a decrease of absorbance at 200-215 nm, due to loss of methylene interrupted double bonds (unoxidized lipids). When lipid peroxidation was carried out in water or 0.15 M NaCl decreases at 200-215 nm were more notorious than in reactions carried out in Tris-buffer.

3.3. Conjugated dienes, trienes and TBARS are excellent markers of lipid peroxidation of liposomes made of retinal lipids

Figure 2 shows changes in TBARS production and variation of absorbance at 234 nm and 270 nm as a function of time.

When SL were peroxidized in water (**Figure 2A**) a lag phase of 30 min, followed by a fast rate, was observed in TBARS production. Absorbance final value at 532 nm reached was 0.24. Increase of absorbance at 234 nm showed a small lag phase followed by a fast initial phase until 40 min, since then speed of reaction became slighter. This behaviour was also observed in measured absorbance at 270 nm, but all absorbance values were lower than that at 234 nm in the range of time studied.

Lipid peroxidation of SL in 0.15 M NaCl (**Figure 2B**). showed an immediate and fast production of TBARS without lag phase, reaching a final value (Abs≈ 0.23) similar to that obtained in water. The absorbance at 234 nm increased with an initial speed greater than
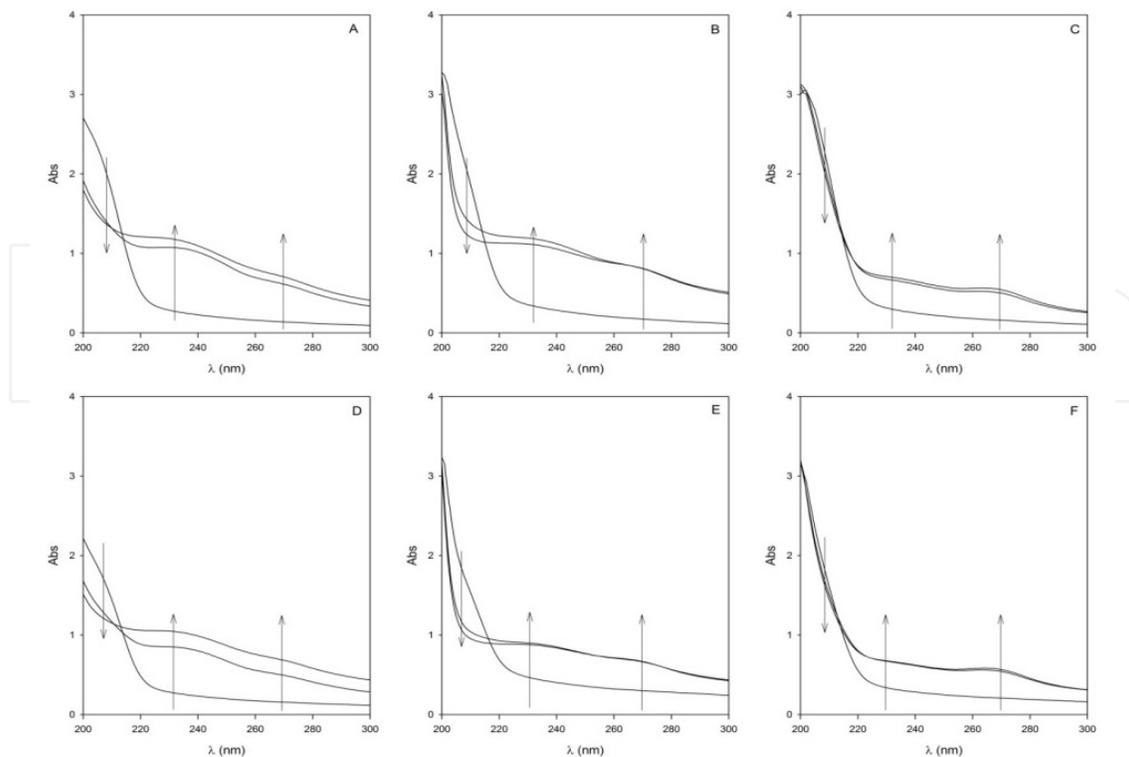
Figure 1. Time evolution (0, 90 and 180 min) of UV spectra of liposomes peroxidized with Fe^{2+} as an initiator of the reaction. SL in A) water, B) 0.15 M NaCl, C) buffer Tris. NSL in D) water and) 0.15 M NaCl, F) buffer Tris
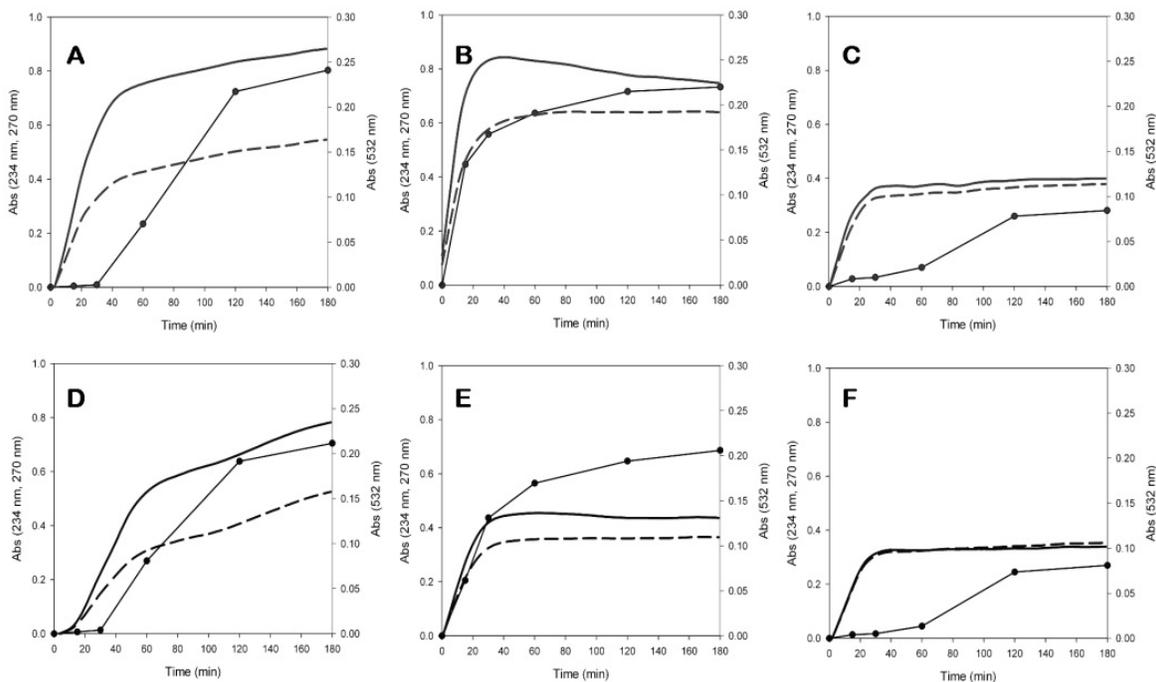
Figure 2. TBARS production (—•—) and variation of absorbance at 234 nm (—) and 270 nm (--) as a function of time, during Fe^{2+}-catalyzed lipid peroxidation of SL (top) and NSL (bottom). TBARS were determined at 0, 15, 30, 60, 120 and 180 min after addition of Fe^{2+}. Aqueous media where reactions were carried out: A, D: water; B, E: 0.15 M NaCl; C, F: 20 mM Tris-HCl pH 7.4.

that observed in water, became the highest to 30 minutes and, then, diminished slowly. The absorbance at 270 nm increased with an initial speed greater than that observed in water, became the highest around the 30 min and then remained constant.

Lipid peroxidation of SL in Tris- buffer (**Figure 2C**) showed the largest lag phase and the lowest final value of absorbance (Abs$_f$= 0.09) for TBARS formation. The initial speed of TBARS production was also the lowest. Initial speed of reaction observed, by increase of absorbance at 234 nm, was lower than that measured on water and 0.15 M NaCl. Absorbance reached the maximum at 30 minutes and then remained constant. Conjugated trienes production was very similar to that of conjugated dienes.

Lipid peroxidation of NSL in water (**Figure 2D**) showed a lag phase of 30 min for the TBARS production and a final value of absorbance of 0.21. Changes of absorbance at 234 nm displayed a lag of 16 min, increased quickly from this time to 60 min and since then continued increasing with lower speed. Values of absorbance at 270 nm were below than those observed at 234 nm, although the behavior was similar.

Lipid peroxidation of NSL in 0.15 M NaCl (**Figure 2E**) showed an initial speed of TBARS production greater than that observed in water, but with a very similar final value (Abs$_f$= 0.21). Changes in absorbance at 270 nm and 234 nm showed greater initial speeds than the corresponding ones in water. These speeds stayed until 30 minutes and since then, absorbance values did not change. Final values of absorbance in 0.15 M NaCl were smaller than the water ones.

Lipid peroxidation of NSL in Tris-buffer (**Figure 2F**) showed the greatest lag phase (60 min) in TBARS production and the smallest initial reaction rate. The final value was 0.08, a result much smaller than those obtained in water and 0.15 M NaCl. Values of change of absorbance determined at 270 nm and 234 nm were practically the same. Initial speeds were similar to those obtained in water and slower to those observed in 0.15 M NaCl. The reached final values were below to those obtained in water and 0.15 M NaCl.

SL were more susceptible to lipid peroxidation than NSL both in water as in 0.15 M NaCl. Nevertheless, both types of liposomes were equally peroxidized in Tris-buffer.

3.4. Fatty acid composition of retinal lipids and liposomes made of these retinal lipids

Figure 3 shows the fatty acid composition (area %) of retinal lipids and of liposomes made of these retinal lipids (SL-Fe, control). This table also compares fatty acid profiles of control with liposomes incubated with Fe^{2+} for 1 h, in absence and in presence of BHT. Retinal lipids show a high percent (25.8 ± 0.6 %) of docosahexaenoic acid (22:6 n-3), characteristic of this tissue. The retina has approximately 40 percent of PUFAs and 60 percent of saturated and monounsaturated fatty acids. SL prepared with these lipids show a decrease of 22:6 n-3. The PUFAs diminished significantly after incubation with Fe^{2+}. This produce a relative increase of saturated and monounsaturated fatty acids. 5 μM BHT protected PUFAs avoiding lipid peroxidation effects and the fatty acid profile there was not significant differences with control.
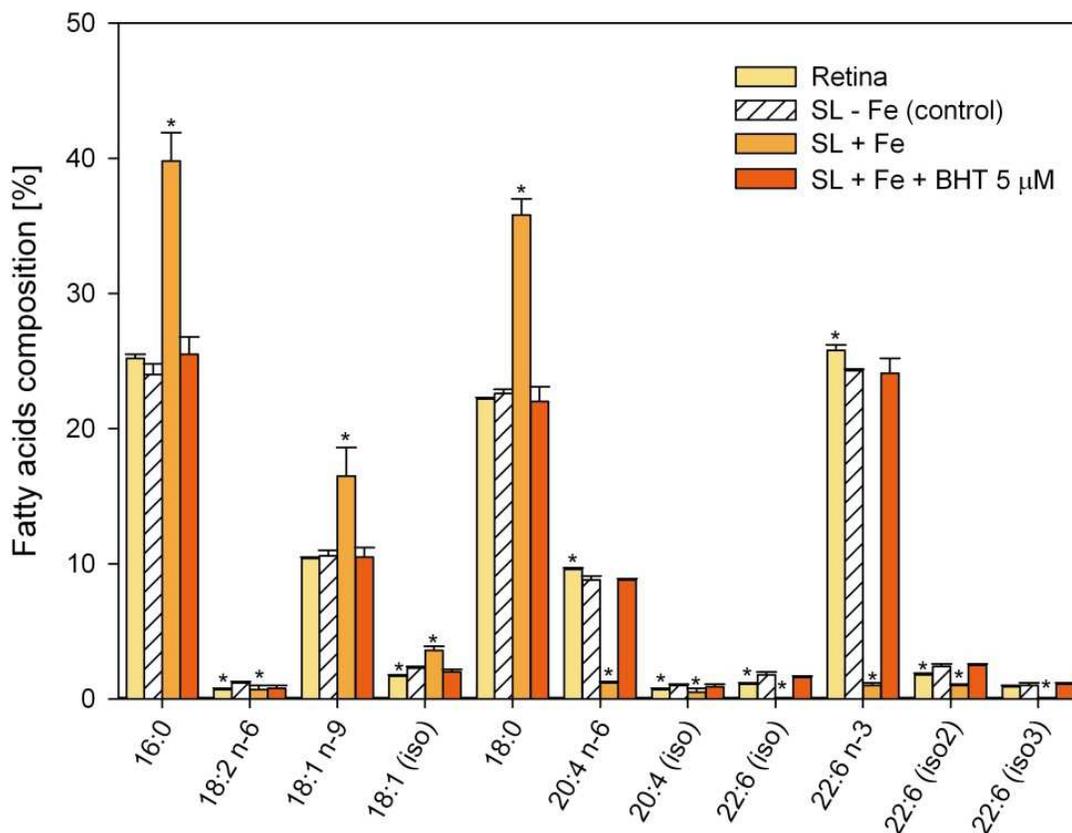
Figure 3. Fatty acid composition (area %) of retinal lipids, liposomes made of retinal lipids (SL -Fe, control), liposomes incubated with Fe^{2+} (SL + Fe) and liposomes incubated with Fe^{2+} in the presence of BHT. Results are expressed as $\tilde{x} \pm SD$. $\tilde{x}$: Average of area % of 3 assays, SD: standard deviation. Significant differences analyzed by ANOVA with control are marked with (*).

4. Conclusion

In summary, the presented results are indicative that liposomes made of retinal lipids by their structural similarities with the biomembranes constitute a very useful analytical system and can mimic the cellular membranes, providing additional information to that obtained with the whole retina. In addition, SL prepared with phospholipids obtained from selected tissues should be used in order to measure lipid peroxidation and the effect of different antioxidants. Additionally, we presented some simple techniques of many possibles that can be applied to study the lipid peroxidation process, different reaction initiators and the antioxidant effect of new compounds.

Abbreviations

16:0: palmitic acid, 18:1 n-9: oleic acid, 18:2 n-6: linoleic acid, 20:4 n-6: arachidonic acid, 22:6 n-3: docosahexaenoic acid, BHT: butylated hydroxitoluene, GC-MS: gas chromatography–mass spectrometry, PUFAs: polyunsaturated fatty acids, TBARS: thiobarbituric acid reactive substances, RNS: reactive nitrogen species, ROS: reactive oxygen species, SL: sonicated liposomes.

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