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The antioxidant behaviour of melatonin and structural analogues during lipid peroxidation depends not only on their functional groups but also on the assay system

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ABSTRACT

There is no general agreement yet on the antioxidant effect of pineal indoles against lipid peroxidation. Accordingly, the main goal of the present work was to study the antioxidant activity of melatonin (MLT), *N*-acetylserotonin (NAS), 5-HO-tryptophan (5HO-TRP) and 5-methoxytryptamine (5MTP) in two different lipid systems with high content of polyunsaturated fatty acids (PUFAs): triglycerides (rich in 20:5 n-3, 22:6 n-3) dissolved in chloroform and sonicated liposomes made of retinal lipids (rich in 22:6 n-3). In the triglyceride–chloroform-system the peroxidation reaction was initiated by cumene hydroperoxide (CHP) whereas liposomes were peroxidized with Fe^{2+}. The techniques employed at the present work were: (1) TBARS production, (2) DPPH assay, (3) determination of conjugated dienes production and (4) analysis of fatty acid profile by GC–MS. Butylated hydroxytoluene (BHT) was employed as a reference because of its well known antioxidant capacity. Our results showed that MLT and 5MTP were unable to protect PUFAs against lipid peroxidation in both systems, whereas NAS and 5HO-TRP were better antioxidants that BHT in the triglyceride-system but ineffective in the liposome-system. We conclude that the antioxidant behaviour of pineal indoles depends not only on their functional groups but also on the assay system and could be explained by the polar paradox theory.

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1. Introduction

Lipids are important components of food and biological systems and are susceptible to oxidation that may occur at any step of food processing and storage, as well as under physiological and/or pathological conditions in living organisms [1]. Lipid peroxidation is a complex process mediated by free-radicals, whose detailed mechanism of action is not fully understood. However, it proceeds through three stages of initiation, propagation, and termination and involves initiators or promoters, such as heat, light, oxygen, enzymes, transition metals, metalloproteins, and/or microorganisms [2]. Among the methods employed for preventing lipid peroxidation, the addition of antioxidants is the most effective, suitable, and economical approach for stabilizing food and non-food supplies [3]. Antioxidants can avoid or delay oxidation by scavenging free radicals, quenching singlet oxygen, inactivating peroxides and other reactive oxygen species (ROS), chelating pro-oxidant metal ions, quenching secondary oxidation products, and inhibiting pro-oxidative enzymes, among others [4]. The efficiency of antioxidants is determined by their chemical structures and may fluctuate depending upon the concentration, temperature, type of oxidation substrate, and physical state of the system media, as well as the presence of antagonists and synergists [5]. Therefore, all significant factors must be taken into account when selecting or designing antioxidants for a particular application. For example, antioxidants are found to behave differently when used in various media; their activity in bulk oil is different from that in oil-in-water emulsion systems.

With respect to antioxidant efficiency in different lipid media, the "polar paradox theory" was proposed. This theory states that polar antioxidants are more effective in less polar media, such as bulk oils, whereas non-polar antioxidants are more efficient in

Abbreviations: 18:2 n-6, linoleic acid; 20:4 n-6, arachidonic acid; 22:6 n-3, docosahexaenoic acid; 22:5 n-3 (EPA), eicosapentaenoic acid; DPPH, 2,2-diphenyl-2-picrylhydrazyl; BHT, butylated hydroxytoluene; GC–MS, gas chromatography–mass spectrometry; MLT, melatonin; 5MTP, 5-methoxytryptamine; 5HO-TRP, 5-HO-tryptophan; NAS, *N*-acetylserotonin; PUFAs, polyunsaturated fatty acids; TBARS, thiobarbituric reactive substances; ROS, reactive oxygen species; SL, sonicated liposomes.

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comparatively more polar media, such as oil-in-water emulsions or liposomes [6]. The polar paradox hypothesis has been tested and confirmed by studies using antioxidants of different polarity and rationalised by the interfacial phenomenon. In addition, synthetic lipophilic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were found to be more active in emulsions than in dry lard or vegetable oil; α-, β-, γ-, and δ-tocopherols showed opposite trends in efficiency in liposomes and bulk oil [7].

Previous estimations of the antioxidant activity of MLT have used a diversity of approaches which suggests indirect effects such as activation of antioxidant enzymes and up regulation by modulating gene expression [8]. The antioxidant activity of MLT in lipid model systems has been extensively investigated; however results of these studies are doubtful. Conversely, antioxidant activity of structural analogues of MLT was not studied enough.

With these concepts in mind, the aim of the present study was to evaluate in vitro the antioxidant activity of MLT and structural analogues in two different lipid systems with high content of polyunsaturated fatty acids (PUFAs): triglycerides (rich in 20:5 n-3 and 22:6 n-3) dissolved in chloroform and sonicated liposomes (rich in 22:6 n-3) made of retinal lipids in an aqueous media.

As far as we know, this is the first time that the antioxidant properties of MLT and structural analogues are compared in two different lipid systems with high content of PUFAs. In our view, one of the most important interpretations is that results of experiments conducted in bulk solution cannot extrapolate to reactions that occur at the oil/water interface. In the light of the above considerations, the results of this experimental approach are discussed under concepts emerging from the polar paradox theory.

2. Materials and methods

2.1. Materials

Commercial fish oil (triglycerides enriched in n-3 long chain fatty acids, Tg n-3 PUFAs) that was stabilized, deodorized, refined, and bleached (EPA: 18.43%; DHA: 13.11%) was donated by *Winterization Europe Fécamp Cedex* (France). Bovine eyes were donated by *Frigorífico Gorina* (La Plata, Buenos Aires, Argentina). Cumene hydroperoxide (CHP), 1,2-diphenyl-2-picrylhydrazyl (DPPH·) as free radical form (90% purity), chloroform, methanol, butylated hydroxytoluene (BHT), melatonin (MLT), 5-methoxytryptamine (5MTP), 5-OH-tryptophan (5HO-TRP), *N*-acetylserotonin (NAS) were from Sigma Chemical Co. (St. Louis, MO, USA). These compounds were dissolved in methanol (5HO-TRP) or ethanol (others). Sodium chloride, iron (II) sulfate heptahydrate, 2-thiobarbituric acid (TBA), boron-trifluoride–methanol complex were from Fluka. 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. Methods

2.2.1. Preparation of liposomes made of retinal lipids

Isolation of bovine retina and preparation of homogenates, extraction of total lipids, preparation of liposomes and measurement of vesicle size, was done as previously described [9].

2.2.2. Radical scavenging capacity assay of melatonin and structural analogues

The free radical scavenging activities of MLT and structural analogues was tested by their ability to bleach the stable radical DPPH [11]. This assay has often been used to estimate the anti-radical activity of antioxidants. DPPH· presents a maximum of absorbance at 515 nm; when DPPH· reacts with an antioxidant compound, which can donate hydrogen, this absorbance diminishes and can be measured on a visible spectrophotometer. The DPPH assay was run by the following procedure: DPPH solution (3.9 ml, 60 μM) in methanol was mixed with 100 μl of different concentrations of sample solution (BHT; MLT; 5MTP, 5HO-TRP or NAS) or methanol (blank sample). Each experiment was performed in triplicate and BHT was used as a reference compound. The percentage of the DPPH remaining at the steady state is inversely proportional to the antioxidant efficiency, and the concentration that causes a decrease in the initial DPPH concentration by 50% was defined as EC_{50}. The radical scavenging activity (%), at a fixed concentration (10 μM), was obtained from the equation:

$$\text{Radical scavenging activity} = \frac{(\text{Abs blank} - \text{Abs sample})}{\text{Abs blank}} \times 100$$

where Abs blank denotes absorption of the blank sample at steady state and Abs sample denotes absorption of tested compound at steady state.

Statistically significant differences between means were determined by ANOVA and multiple range test based on Tukey's HSD (Honestly Significant Difference) test, with a 95% confidence interval.

2.2.3. Measurements of lipid peroxidation by detection of thiobarbituric reactive substances (TBARS)

During lipid peroxidation the breakdown of fatty acids occurs and small molecular weight products, such as aldehydes emerge. Among these the primary product is malondialdehyde, which was proposed as a diagnostic marker of in vivo lipid peroxidation [12]. The samples (Tg PUFAs and sonicated liposomes) were analyzed for assessment of TBARS by the method of Buege and Aust [13]. The absorbance was measured at 532 nm in a spectrophotometer.

Significant differences between means were determined by ANOVA.

2.2.4. Measurements of lipid peroxidation of liposomes by detection of conjugated dienes

Conjugated dienes production was measured as we previously described [9], lipid peroxidation was assessed by plotting the increase in absorbance at 234 nm vs time, every 9 min along 9 h. The area under this curve was calculated to estimate the inhibition produced by BHT or indoleamines.

Statistically significant differences between means were determined by ANOVA and multiple range test based on Tukey's HSD test, with a 95% confidence interval.

2.2.5. Analysis of PUFAs loss by GC–MS

Preparation of fatty acids methyl esters and gas chromatography–mass spectrometry analyses were performed as we previously described [10].

3. Results

3.1. Radical scavenging activity of melatonin and structural analogues

The radical scavenging activity of the indole derivatives at different concentration was analyzed by the DPPH method and compared to BHT. In Fig. 1 we can see the comparative radical scavenging activity of BHT and indoleamines at a fixed concentration (10 μM). These results indicate that 5HO-TRP and NAS exhibited 85% and 74% radical scavenging activity respectively, compared to 51% activity of BHT. MLT and 5MTP showed only 2% and 5% activity, respectively. Given that BHT, NAS and 5HO-TRP

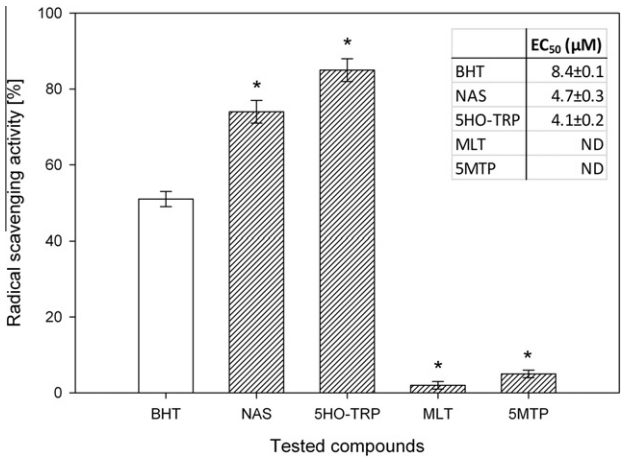

Fig. 1. Radical scavenging activity of BHT and indoleamines (10 μM) determined by DPPH assay. Each bar represents $\bar{x} \pm SD$ from three experiments. An asterisk (*) indicates significant differences between samples and BHT. Inset: EC_{50} indexes calculated from fitting curves of absorbance of DPPH vs tested compounds concentrations (ND: no determined).

exhibited a dose-dependent free-radical scavenging ability at all of the tested concentrations, the EC_{50} indexes were calculated (inset Fig. 1). Thus, higher radical scavenging activity corresponds to lower EC_{50} value. According to these data 5HO-TRP (EC_{50} = 4.1 ± 0.2 μM) and NAS (EC_{50} = 4.7 ± 0.3 μM) were more efficient radical scavengers that BHT (EC_{50} = 8.4 ± 0.1 μM). The EC_{50} index could not be calculated to MLT and 5MTP because these compounds did not present relevant radical scavenging activity at analyzed concentrations.

3.2. TBARS assay

3.2.1. Triglycerides system

The lipid peroxidation process of Tg PUFAs n-3 initiated by CHP originates increasing amounts of TBARS along time. The TBARS concentration at steady state was compared with a blank sample and with samples treated with BHT and indoleamines. Fig. 2 shows the inhibition of TBARS production caused by different concentrations of BHT, NAS and 5HO-TRP. IC_{50} index was calculated since inhibition is concentration-dependent (inset Fig. 2). BHT (IC_{50} = 1.4 ± 0.1 mM) and 5HO-TRP (IC_{50} = 1.3 ± 0.1 mM) inhibited the TBARS production in a more efficient way that NAS (IC_{50} = 9.3 ± 0.5 mM). IC_{50} indexes could not be calculated to MLT and 5MTP given that none of them could inhibit TBARS production, moreover it was enhanced greatly.

3.2.2. Liposomes system

As in the triglycerides system, the lipid peroxidation of sonicated liposomes initiated by Fe^{2+} originates increasing amounts of TBARS along time. Fig. 3 shows the inhibition of TBARS production caused by different concentrations of BHT and indoleamines. IC_{50} index was calculated only for BHT (IC_{50} = 0.6 ± 0.1 μM) (inset Fig. 3) because the indoleamines tested were not able to inhibit TBARS production at any analyzed concentrations. Moreover, 5HO-TRP presented pro-oxidant behaviour given that it increased the TBARS production.

3.3. Measurements of lipid peroxidation of liposomes by detection of conjugated dienes

Prompt increased production of conjugated dienes (absorbance at 234 nm) occurs after addition of Fe^{2+} as initiator of peroxidation of liposomes. With addition of increasing concentrations of the antioxidant BHT the start of the reaction is delayed and initial reaction rates are lower. Fig. 4 shows the inhibition on global conjugated dienes production generated by different concentrations of BHT and indoleamines. With addition of increasing concentrations of NAS, MLT and 5MTP (1, 2 and 5 μM), the reaction was not significantly modified. However, when 5HO-TRP was tested, it showed a significant pro-oxidant effect at all assayed concentrations.

3.4. Lipid peroxidation of liposomes analyzed by gas chromatography–mass spectrometry

Table 1 shows the fatty acid composition (area%) of retinal lipids and of liposomes made of these retinal lipids (control). This table also compares fatty acid profiles of control samples with liposomes incubated with Fe^{2+} for 1 h with and without BHT, MLT and related indoleamines (5 μM). Retinal lipids show a high percent (25.8 ± 0.4%) of docosahexaenoic (22:6 n-3) acid, characteristic of this tissue. Sonicated liposomes prepared with these lipids show a decrease of 22:6 n-3. PUFAs diminished significantly after incubation with Fe^{2+} during 1 h. This produced a relative increase of saturated and monounsaturated fatty acids. 5 μM BHT protected PUFAs avoiding lipid peroxidation effects. The fatty acid profile of samples treated with BHT did not have significant differences with control. MLT and relative indoleamines did not avoid

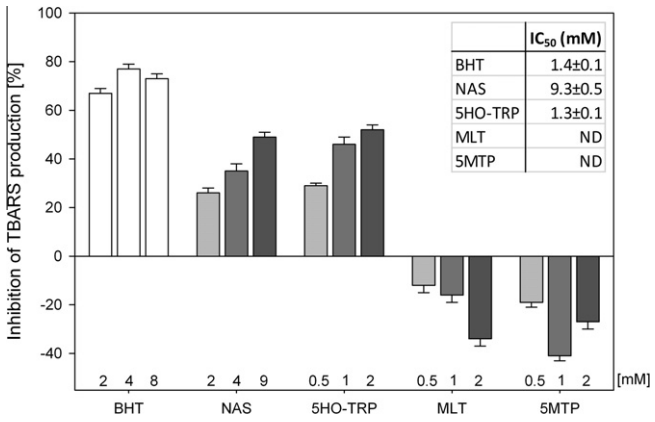

Fig. 2. Inhibition of TBARS production by BHT and indoleamines on lipid peroxidation initiated by CHP of Tg PUFAs. Each bar represents $\bar{x} \pm SD$ from three experiments. Inset: IC_{50} indexes calculated from fitting curves of inhibition% vs tested compounds concentrations (ND: no determined).

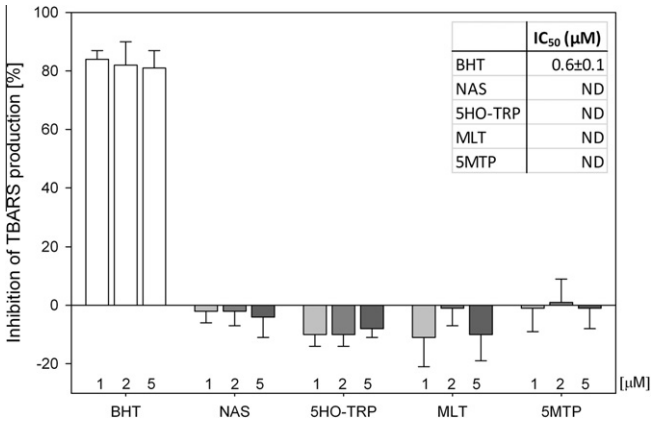

Fig. 3. Inhibition of TBARS production by BHT and indoleamines during lipid peroxidation initiated by Fe^{2+} of sonicated liposomes. Each bar represents $\bar{x} \pm SD$ from three experiments. Inset: IC_{50} indexes calculated from fitting curves of inhibition% vs tested compounds concentrations (ND: no determined).

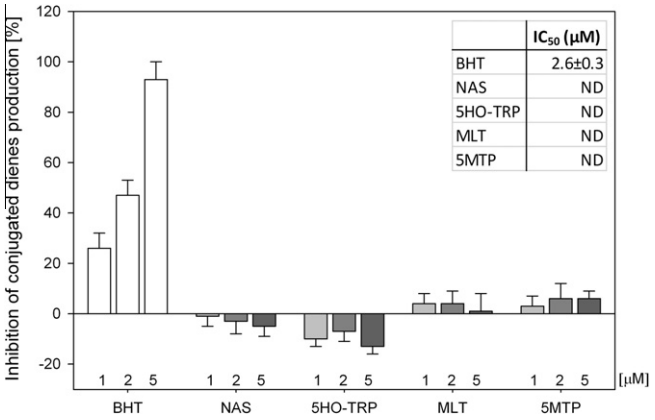

Fig. 4. Inhibition of conjugated dienes production by BHT and indoleamines during lipid peroxidation initiated by Fe^{2+} of sonicated liposomes. Each bar represents $\bar{x} \pm SD$ from three experiments. Inset: IC_{50} indexes calculated from fitting curves of inhibition% vs tested compounds concentrations (ND: no determined).

the peroxidation effect on PUFAs; these fatty acids diminished significantly and the fatty acid composition was similar to peroxidized liposomes.

4. Discussion

Some studies have questioned MLT's ability to inhibit the autoxidation of lipids in homogeneous solution and in model heterogeneous systems [14,15]. In the light of the above considerations, we may attempt to interpret the different effects of MLT and the indole derivatives assayed in this study.

The precursors of MLT, 5HO-TRP and NAS, were the most efficient radical scavengers in our experiments carried out in *triglycerides dissolved in chloroform*. In this regard in a recent paper it has been demonstrated that 5-HO-TRP is a more potent in vitro hydroxyl radical scavenger than MLT or vitamin C [16]. As our results indicate, 5HO-TRP showed the highest capacity to inhibit de TBARS production and this situation agrees with its high activity against DPPH radical. These results lead to the conclusion that this compound has a strong trend to give a hydrogen atom ($H^{\cdot}$) to free radicals generated during lipid peroxidation process. In consequence, the chain of lipid peroxidation is cut and the PUFAs are protected. Other 5-HO- derivative, NAS, also was able to inhibit TBARS production and was an efficient DPPH radical scavenger. Wherever, its effectiveness was lesser than that of 5HO-TRP. MLT and 5MTP (5-methoxy derivatives) were not efficient $H^{\cdot}$ donors as they were analyzed by DPPH assay. These results agree with the lack of inhibition of both compounds on TBARS production (indeed, they presented pro-oxidant effect). Therefore, MLT and 5MTP were not good antioxidants in the system of triglycerides in chloroform.

The comparison between structural analogues revealed a critical importance of functional groups. The existence of a methoxy residue in position 5, as in 5MTP and MLT, strongly reduced the capacity of radical scavenging. On the other hand, the presence of the hydroxyl group in position 5 of the indole ring, as in 5HO-TRP and NAS, established high free-radical scavenging activity and efficient antioxidant activity during the lipid peroxidation of Tg PUFAs n-3. However, the mechanism by which the indole ring interacts with free radicals is still only partially understood. Several evidences are on line with our results (i) Teixeira et al. have demonstrated that MLT shows low reactivity with DPPH [17], (ii) other study showed that MLT exerts only limited direct antioxidant activities [18], (iii) the antioxidant activity of MLT in soybean PC liposomes is much lower than that of alpha-tocopherol, under comparable assay conditions [15], and (iv) the ability of MLT to scavenge the lipid peroxyl radical ($LOO^{\cdot}$) is debated [19].

Peroxidation of liposomes made of retinal lipids using Fe^{2+} as initiator was assayed by detection of conjugated dienes, TBARS and analysis of fatty acid profiles. NAS, MLT, 5HO-TRP and 5MTP did no present antioxidant effect when it was determined by TBARS and conjugated dienes techniques; moreover, 5HO-TRP presented significant pro-oxidant effect. Analysing fatty acid profile, we can see that PUFAs present in liposomes were affected by peroxidation to almost disappear. BHT (5 μM) was able to protect them while MLT and related indoleamines, at the same concentrations, were not. We think the PUFAs loss is a conclusive result, since the lack of protective effect becomes apparent and this technique is a direct way to evaluate antioxidant power of indoleamines.

We have performed studies using a simple liposomal model that facilitate evaluation of the kinetic of the lipid peroxidation process without the interference produced by retinal proteins. Liposomes, whose phospholipid composition, structure and dynamics can be controlled, are frequently accepted as an appropriate model for in vitro studies of membrane structures and properties. They are composed by lipid bilayers, structurally similar to cell membrane lipidic environment. Conjugated dienes formed from oxidised PUFAs, and TBARS products derived from the breakdown of these fatty acids located in phospholipids could be analyzed during peroxidation of liposomes made of retinal lipids using Fe^{2+} as initiator. Through both techniques it was noticeable the powerful antioxidant effect of BHT, while MLT, 5MTP and NAS did not show antioxidant effect when they were added to the reaction mixture, even when we assayed higher concentrations of MLT (10 and 20 μM). 5HO-TRP not only did not show antioxidant activity but it is possible that it have prooxidant effect as we observed with TBARS and conjugated dienes assays. Our results do not allow us

Table 1
Fatty acid composition (%) of retina and liposomes made with retinal lipids. Liposomes were incubated during 1 h with and without Fe^{2+} (SL + Fe and SL − Fe) and with Fe^{2+} and BHT or studied indoleamines. Each value represents $\bar{x} \pm SD$ to *n* experiments. Significant differences with control (SL − Fe) were marked with an asterisk(*).

Fatty acid	Retina	SL − Fe	SL + Fe	BHT 5 μM	NAS 5 μM	5HO-TRP 5 μM	MLT 5 μM	5MTP 5 μM
16:0	25.2 ± 0.3	24.0 ± 30.8	39.8 ± 2.1*	25.5 ± 1.3	40.3 ± 0.2*	40.8 ± 1.2	40.7 ± 0.5*	37.6 ± 0.1*
18:2 n-6	0.7 ± 0.1*	1.2 ± 0.1	0.7 ± 0.3*	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.4	0.7 ± 0.1*	0.9 ± 0.1
18:1 n-9	10.4 ± 0.1	10.6 ± 0.4	16.5 ± 2.1*	10.5 ± 0.7	17.8 ± 0.5*	19.1 ± 0.8	18.2 ± 0.2*	17.5 ± 0.3*
18:1 (iso)	1.7 ± 0.1*	2.3 ± 0.1	3.6 ± 0.3*	2.0 ± 0.2	3.9 ± 0.1*	4.3 ± 0.1	3.5 ± 0.1*	3.3 ± 0.1*
18:0	22.2 ± 0.1	22.6 ± 0.3	35.8 ± 1.2*	22.2 ± 1.1	34.8 ± 0.5*	35.1 ± 1.8	33.7 ± 0.1*	33.5 ± 0.1*
20:4 n-6	9.6 ± 0.1*	8.8 ± 0.3	1.2 ± 0.1*	8.8 ± 0.1	1.6 ± 0.3*	ND*	1.6 ± 0.2*	1.9 ± 0.1*
20:4 (iso)	0.7 ± 0.1	1.0 ± 0.1	0.5 ± 0.3*	0.9 ± 0.2	0.6 ± 0.4	ND*	ND*	0.6 ± 0.2
22:6 (iso)	1.1 ± 0.1*	1.8 ± 0.2	ND*	1.6 ± 0.1	ND*	ND*	ND*	0.6 ± 0.1*
22:6 n-3	25.8 ± 0.4*	24.3 ± 0.1	1.0 ± 0.2*	24.1 ± 1.1	ND*	ND*	1.5 ± 0.1*	2.2 ± 0.1*
22:6 (iso)	1.8 ± 0.1*	2.4 ± 0.2	1.0 ± 0.1*	2.5 ± 0.1	ND*	ND*	ND*	1.4 ± 0.2*
22:6 (iso)	0.9 ± 0.1	1.0 ± 0.2	ND*	1.1 ± 0.1	ND*	ND*	ND*	0.4 ± 0.1*
	n = 3	*n* = 2	*n* = 2	*n* = 2	*n* = 2	*n* = 2	*n* = 2	*n* = 2

to conclude about the reasons why MLT and 5MTP stimulated TBARS production in the triglycerides-system and 5HO-TRP stimulated TBARS and conjugated dienes in liposomes-system, but we can speculate about the importance of theirs functional groups on this effect.

The differences found in the antioxidant behaviour of NAS and 5HO-TRP (both hydrophilic) in both sets of experiments may be explained by the known theory of the Polar Paradox [20]. This theory would also explain the fact that these compounds showed more efficient antioxidant activity than BHT (lipophilic) in the pure lipid system containing triglycerides dissolved in chloroform. This theory interprets the apparent contradiction of the fact that the hydrophilic antioxidants are more effective in purely lipid media while lipophilic antioxidants are better in aqueous media. It is known that, together with its innate power, the effectiveness of an antioxidant is also affected by its interfacial properties and in the medium partition. Early studies of pure lipid oxidation were based on the statement that peroxidation occurs in a homogeneous medium. The air–lipid was considered the start site of peroxidation propagating then into oil. Under this postulation, partially soluble antioxidants would be directed at the air–oil interface where peroxidation occurs and, hence, would protect the system from oxidative changes. However, the distribution of polar antioxidants in this interface was questioned because the air is even less polar than the oil. Thus, we supposed that micro or nanoambients affect the chemistry of lipid peroxidation and antioxidant altering the physical position of lipid substrates and antioxidants. For example, different types or micellar or lamellar structures (in the presence of traces of water) may be formed by self-assembly of lipid components (such as phospholipids) or peroxidation products (hydroperoxides, aldehydes and ketones). Currently there is insufficient evidence to support the hypothesis that these partnership structures are the sites where lipid peroxidation occurs. Polar antioxidants, instead of being located in the air–oil as previously believed, are preferentially located at the interface of these colloidal structures (e.g., oil–water interface) and are thus more efficient in inhibiting the peroxidation, than nonpolar that is dissolved in the lipid phase. This theory was supported by the fact that polar antioxidants are unable to decrease the surface tension, they succeeded in reducing the interfacial tension [21]. On the basis of these considerations, more comprehensive studies are required to better understand the behaviour of MLT and structural analogues during lipid peroxidation in different media.

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