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# Lipid Oxidation Inhibition by Natural Tocopherols Increases the Nutritional Value of Tuna Salami

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## ABSTRACT

**Introduction**
The quality attributes of fish products deteriorate due to the lipid oxidation during processing and storage. Tuna salami is highly susceptible to oxidation, which can precipitate health hazards and economic losses in terms of inferior product quality.

**Methods**
The effect of adding different natural antioxidants on the lipid oxidation, colour and fatty acid profile of tuna salami after nine days of storage at 4 ºC was assessed. Three different commercial mixes of tocopherols were added during the production of the tuna salami and samples were sliced and refrigerated for 9 days.

**Results**
The lipid oxidation increased from 0.98 to 4.03 mg of malondialdehyde/kg. The oxidation process was inhibited by DLT-100 and VIT-100; however, RNX10 did not blunt the oxidative process. VIT and dose-limiting toxicity (DLT) reduced the total unsaturated fatty acid reduction. The *RNX* did not prevent the reduction of the $a^*$ value.

**Conclusion**
It was concluded that tocopherols supplementation kept the nutritional value of tuna salami mainly by lipid oxidation inhibition.

**Keywords**
Tuna salami; Fatty acids profile; Thiobarbituric acid-reactive substances (TBARS); Antioxidants; Colour changes.

**Abbreviations**
DLT: Dose-Limiting Toxicity; TBARS: Thiobarbituric Acid-Reactive Substances; PUFA: Polyunsaturated Fatty Acids; MDA: Malondialdehyde; ROS: Reactive Oxygen Species; FAME: Fatty Acid Methyl Esters; GC: Gas Chromatography; HPLC: High-Performance Liquid Chromatography; ANOVA: One-Way Analysis of Variance.

## INTRODUCTION

Tuna salami is a new food product developed by a small Portuguese food producer "*Conservas Dâmaso*" (Vila Real de Santo António, Algarve). This product is a type of dry sausage made from tuna meat that is ground up and mixed with oil and spices. Tuna salami is normally sliced thinly when served and often used for sandwich filling (salami sandwich), for pizza toppings and for viand as well. Nowadays, there is a growing interest in tuna products due to the beneficial effects on human health, because of its high polyunsaturated fatty acids (PUFA) content. However, food products with high content of PUFA are highly susceptible to lipid oxidation and therefore the shelf-life of these products are short.[1,2] Therefore, the reduction of lipid oxidation during storage has been of major importance to the food process industries.

Lipid oxidation encompasses a series of reactions between oxygen and unsaturated lipids to form lipid peroxides, often

readily identified by sensory changes, such as generation of off-flavour and off-odours.[2-4] This oxidative spoilage releases secondary products. Malondialdehyde (MDA) is one of the most important products of oxidation that can be followed as a marker of lipid oxidation.[1,5] Simultaneously, heme-pigments (myoglobin and haemoglobin) also oxidize in a coupled lipid-pigment reaction.[6] The myoglobin is a globular heme protein localised in red muscle fibres and the major giver to the colour of muscle.[7-11] Changes in fish colour are usually influenced by intrinsic factors such as muscle pH, redox potential, metmyoglobin reductase activity, oxygen-consuming reactions, and susceptibility to lipid oxidation. However, as extrinsic factors such as light exposure and storage temperature also produce changes in the fish colour. Therefore, colour changes are also an important factor that influences quality and acceptability by the consumers of tuna products (colour quality attribute strongly influences consumers' preferences). In order to quantify colour changes objectively, the visual observing components needed to be taken into account are the object, light, and the observer.[12] Colour can be measured by a tristimulus colorimeter, represented by three coordinates in the colour scale. The L parameter represents lightness (L=0 black; L=100 white), $a^*$ (red to green) and $b^*$ (blue to yellow).[12-14]

Vacuum packages are normally used in this type of fish food products in order to prevent lipid oxidation and colour changes and consequently increase the products' shelf-life. However, once the package is opened, the contact with the oxygen triggers immediately the lipid oxidation process. The lipid oxidation of fatty acids like omega-3 can be reduced by adding natural or synthetic antioxidants. This process extends the products' shelf-life and moreover enhancing the health benefits of food products.[2] Vitamin E can be found naturally in some foods, which can be added to others and is available as a dietary supplement. Vitamin E is the collective name for a group of fat-soluble compounds with distinctive antioxidant activities, the tocopherols, that stops the production of reactive oxygen species (ROS) formed when fat undergoes oxidation.[15]

The aim of this work was to study the effect of adding different natural antioxidants (tocopherols mix with and without rosemary extract) on the lipid oxidation and colour changes of tuna salamis during nine days at 4 ºC exposure to the air (real situation at consumers home).

## MATERIALS AND METHODS

### Antioxidants and Tuna Salami

Tuna salami was supplied by *Conservas Dâmaso*. Three different commercial mixes of tocopherols were added during the production of the tuna salami: DLT-100 (100 mg of tocopherols/100 g of sample) and VIT-100 (100 mg of tocopherols/100 g of sample), from different suppliers (not identified) and RNX 10 (10 mg of tocopherols+3.89 rosemary extract/100 g of sample). The supplier provided one lot of each DLT, VIT and RNX samples. Tuna salami samples were sliced (thickness=2 mm) and stored in a refrigerator (4 °C) and unpacked (forcing the oxidation process and simulating the consumer's utilization) and analysed. Tuna salami without antioxidant was used as a control sample.

### Chemical Composition

Tuna salami samples were analysed for fatty acid composition, thiobarbituric acid-reactive substances (TBARS) and colour. All measurements were performed in triplicate and samples were obtained from cross sections of full-size salami.

**Fatty acid composition determination:** Fatty acid methyl esters (FAME) of tuna salami were performed according to Lepage & Roy.[16] 300 mg tuna salami were homogenised with 5 mL acetyl chloride-methanol mix (98% acetyl chloride, *Acros*; methanol, *Panreac*) for 30 seconds and placed in a water bath at 80 ºC for 1 h and cooled to room temperature. Then, 1 mL of water for high-performance liquid chromatography (HPLC) (*Fisher Scientific*) and 2 mL of *n*-heptane (*Fluka*) were added and samples were homogenised for 1 minute and centrifuged at 1500 g for 5 minutes. All samples were analysed in gas chromatography (GC) (Finnigan Trace GC Ultra, Thermo Electron Corporation, Waltham, MA, USA).

**TBARS determination:** The TBARS values in tuna salami were determined according to the procedure described by Vyncke,[17] Lemon,[18] Robles-Martinez[19] with slight modifications. Fifteen grams of tuna salami were mixed with 30 mL of 75% trichloroacetic acid solution (TCA with propyl 3,4,5-trihydroxybenzoate, *Merck*), trichloroacetic acid *(Schlarlau*), ethylenediaminetetraacetic acid (EDTA, *Panreac*) for 2 minutes in homogeniser; sample was then transferred to an Erlenmeyer by filter (*Whatman* n.º1) and centrifuged at 2000 rpm for 5 min. The extract of this solution was removed into 1-5 mL and fixed volume until 5 mL of the 75% TCA solution; adding 5 mL of TBA reagent (4,6-dihydroxy-2-mercapto pyrimidine, *Acros*) and maintained in boiling water bath for 40 min. The calibration curve was determined by same proportions and reagents. The sample was replaced by the standard 1,1,3,3-tetraethoxypropane (TEP, *Acros*). Finally, absorbance was read at 530 nm in a spectrophotometer (Helios α, Thermo Electron Corporation, Waltham, MA, USA). The results were expressed as milligrams of MDA per kg of product. For each assay were used three different samples of tuna salami with triplicate measures.

**Colour measurement:** Colour was evaluated in terms of $L^*$, $a^*$ and $b^*$ values using a tristimulus colorimeter (Chroma Met er CR-400, Konica Minolta, Inc., Ramsey, NJ, USA). Measurements were performed in the Commission Internationale de bÉclairage (CIE) $L^*$, $a^*$, $b^*$ system, using illuminant D65 and 2º observer. The colorimeter was calibrated against standard white tile ($L^*$ 95.37; $a^*$ 13.10 and $b^*$ 7.94). The colour behaviour of tuna salami was described for $L^*$, $a^*$ and $b^*$ values. Measurements were taken in triplicates with ten readings each (ten slices of each salami were measured at three distinct positions on each slice).

### Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyse data among samples, followed by Dunnett test (when applicable) to discriminate significant differences between samples and controls. These analyses were performed using GraphPad Prism 5 for Win-

dows. Results are presented as mean±standard error of the mean (SEM). The significance level was inferred at $p<0.05$ for all statistical tests.

## RESULTS AND DISCUSSION

Secondary oxidation products are a suitable index of lipid oxidation due to the fact that they are odour-active and stable compounds, in comparison with primary products (hydroperoxides) which are colourless, flavourless, and usually labile compounds.[20] One of the most extensively employed methods to detect oxidative deterioration in foodstuffs is the TBA test. This procedure is based on the formation of MDA), during the autoxidation of PUFAs, followed by reaction with TBA to form a pink complex that is measured spectrophotometrically.[21,22] The extent of oxidation is stated as the TBA value and is reported as milligrams of MDA equivalents per kilogram of sample or as micromoles of MDA equivalents per gram of sample. Nevertheless, TBA is not selective to MDA and can also react with many other compounds such as aldehydes, carbohydrates, amino acids and nucleic acids leading to overestimation and variability in the results attained by the TBA method.[23] For this reason, this method is also known as thiobarbituric acid reactive substances (TBARS) test. The oxidative stability of vacuum packaged tuna salamis was evaluated during 60 days at room temperature and exposure to artificial light. Non-significant differences ($p>0.05$) in TBARS levels were found during the storage time of samples, showing an oxidative stability of packaged tuna salami (data not shown). This all agree with the view that the vacuum packages prevent the lipid oxidation of this type of food product. Moreover, no significant differences were obtained for pH and moisture content of the samples during the storage time (data not shown).

To study the effect of adding different natural antioxidants on the lipid oxidation mimicking the consumer's use, tuna salami samples were sliced and refrigerated unpacked (4 ºC) during those 9 days.

The fatty acid composition of tuna salami slices for days 0 and 9 is shown in Table 1. Fatty acids composition is strongly influenced by the fat quality used during the food processing and may vary during products storage. Additionally, adding antioxidants can also influence the initial fatty acid composition. In this study, supplementation of tuna salami with DTL-100 and VIT-100, generally did not affect the fatty acid composition, when compared to control for day 0 ($p>0.05$). Interestingly, RNX10 characteristics resulted in a low C18:1, n-9 fatty acid content for both days 0 and 9, when compared to control, DLT-100 and VIT-100 (Table 1).

The fatty acid profile is also highly marked by the presence of monounsaturated fatty acid (MUFA). Oleic acid (18:1) is one of the most abundant MUFA and frequently abundant in this type of product. Comparatively to the control, DTL-100 showed an increase in oleic acid content, from 71.86% to 79.65% with the statistically significant difference ($p<0.05$) for day 9, meaning that DLT-100 can prevent lipid oxidation. Similarly, C22:6, n-3 presented higher values for day 9 for samples treated with DLT-100 (6.09%) but also for RNX10 (11.45%). Furthermore, control samples showed the highest unsaturated fatty acids content variation ($p<0.05$) when compared with the samples supplemented, revealing the importance of the addition of antioxidants to prevent their oxidation. Besides this, no significant differences were found for lipid content between days 0 and 9. Nevertheless, one can be concluded differently that commercial mixes of tocopherols, can lead to different antioxidant activity.

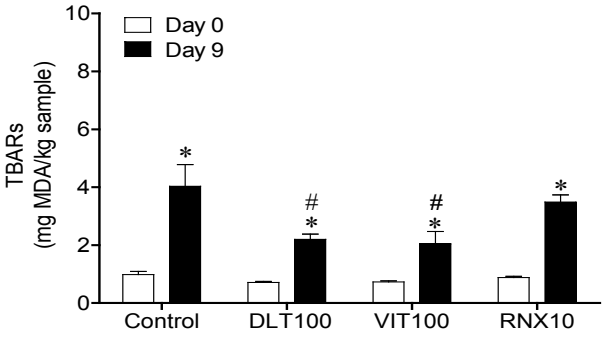
As mentioned before, TBARS assay is one of the most widely used indices of food quality as a critical index in lipid oxidation. Figure 1 presents the TBARS values of sliced tuna salami in the absence or in the presence of three different commercial mixes of tocopherols. Results showed clearly that lipid oxidation occurs during the storage time in tuna sausages products as observed in

**Table 1.** *Fatty Acid Composition (% of Total Fat) of Slices of Tuna Salami (Day 0 and Day 9; Stored at 4 °C) in the Absence or in the Presence of Three Different Commercial Mixes of Tocopherols, DLT-100 (100 mg of Tocopherols/100 g Sample), VIT-100 (100 mg of Tocopherols/100 g of Sample), RNX10 (10 mg of Tocopherols+3.89 Rosemary Extract/100 g of Sample)*

| Fatty Acid | Samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Control | DLT 100 | DLT 100 | VIT 100 | VIT 100 | RNX 10 | RNX 10 |
| | Day 0 | Day 9 | Day 0 | Day 9 | Day 0 | Day 9 | Day 0 | Day 9 |
| 16:0 | 14.63±1.12 | 14.46±1.68 | 13.88±1.73 | 12.26±0.30 | 11.80±0.19 | 14.46±2.07 | 21.54±0.68# | 21.60±0.57# |
| 18:1, *n*-9 | 73.55±2.66 | 71.86±0.41 | 76.41±3.00 | 79.65±0.79# | 78.67±0.53 | 74.16±4.10 | 63.76±1.10# | 62.53±0.83# |
| 20:4, *n*-6 | 0.66±0.07 | 0.65±0.10 | 0.43±0.18 | 0.45±0.18 | 0.77±0.01 | 0.78±0.16 | 0.75±0.02 | 1.38±0.44 |
| 20:5, *n*-3 | 1.43±0.25 | 1.43±0.31 | 1.19±0.19 | 1.02±0.05 | 1.01±0.06 | 1.34±0.29 | 2.07±0.08# | 1.98±0.10 |
| 22:6, *n*-3 | 8.66±1.31 | 5.38±1.58 | 7.09±1.07 | 6.09±0.35 | 7.16±0.27 | 8.59±1.63 | 11.07±0.36 | 11.45±0.39# |
| Σ Unsaturated | 84.31±1.04 | 79.32±1.52* | 85.12±1.65 | 87.20±0.31# | 87.60±0.21 | 84.87±2.23# | 77.65±0.66# | 77.34±0.61 |
| Σ *n*-3 | 10.09±1.55 | 6.81±1.28 | 8.28±1.25 | 7.11±0.39 | 8.17±0.32 | 9.93±1.90 | 13.15±0.44 | 13.43±0.49# |
| *n*-3+*n*-6 | 10.75±1.62 | 7.47±1.18 | 8.71±1.38 | 7.56±0.48 | 8.94±0.33 | 10.72±2.01 | 13.89±0.45 | 14.81±0.37# |
| *n*-3/*n*-6 | 15.15±0.67 | 11.72±2.87 | 12.69±1.30 | 9.68±0.18* | 10.67±0.33# | 13.16±1.89 | 17.62±0.43 | 11.68±3.02* |
| DHA/EPA | 6.11±0.16 | 4.73±1.49 | 5.99±0.08 | 5.94±0.12 | 7.12±0.37# | 6.54±0.71 | 5.35±0.06# | 5.80±0.13* |

Values are means±SEM of 3 to 5 independent experiments. Significantly different from control values (#$p< 0.05$) or from day 0 (*$p< 0.05$).

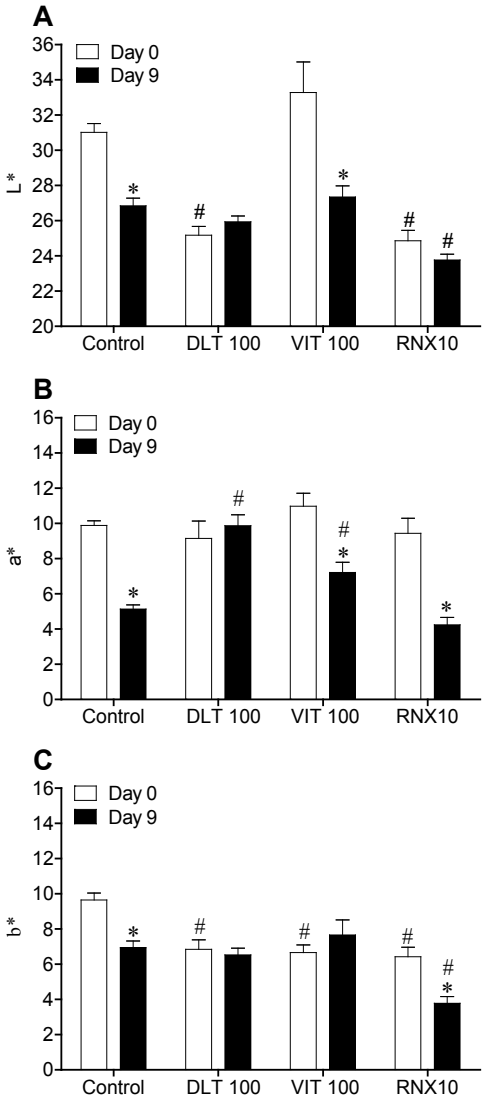
**Figure 1.** *TBARs Values (mg of MDA/kg of sample) of Slices of Tuna Salami (Day 0 and Day 9; Stored at 4 °C) in the Absence or in the Presence of Three Different Commercial Mixes of Tocopherols, DLT-100 (100 mg of Tocopherols/100 g Sample), VIT-100 (100 mg of Tocopherols/100 g of Sample), RNX10 (10 mg of Tocopherols+3.89 Rosemary Extract/100 g of Sample). Each Column is the Mean of 6 to 14 Separate Experiments; Vertical Lines Indicate SEM. Significantly Different from Control Values (#p<0.05) or from Day 0 (*p<0.05)*




control. In this study, all samples (at time zero) showed lower values of TBA than the proposed limits (5 mg of malonaldehyde equivalents kg-1 of tissue). The level of lipid oxidation of freshly caught fish is typically between 3 and 5 mg of MDA equivalents per kilogram flesh.[2] However, according to Fernández & Rodríguez,[24] these values are insufficient for the sensorial detection of rancidity.[21] It was also observed that, TBA greatly increased during the 9 days of storage in all samples ($p< 0.05$) (Figure 1). These results indicated that, although the tocopherols should reduce lipid oxidation supplementation was not enough. Nevertheless, samples treated with VIT-100 and DLT-100 antioxidants present lower level of lipid oxidation than control on day 9 (lower level of TBARS – 2.1969 and 2.0441 for DLT-100 and VIT-100, respectively). Previous studies have found that Rosemary extracts present efficient antioxidant activity. Besides this, in the present work, for tuna salami supplemented it seemed that the prevention of lipid oxidation was not increased by the presence of rosemary extract. This can be explained due to a number of active compounds that can act as antioxidants and are present in VIT-100 and DLT-100; therefore, it is possible that synergism occurs. Additionally, it can be also explained by the higher content of tocopherols compared to RNX10 (100 mg of tocopherols/100 g of sample and 10 mg of tocopherols/100 g of sample, respectively).

The most important indices by which consumers evaluate the freshness and quality of foods are colour and flavour. The colour is commonly used by the consumer as an indication of the freshness of the product. In general, food colour should remain unaltered upon the addition of additives and during storage. Colour parameters are presented in Figure 2. In Figure 2A small changes on $L^*$ value can be observed for samples treated with DLT-100 and RNX10 at day 0 and day 9, respectively, showing that samples became darker. The dark colour formation in tuna salamis can be explained by the browning and the exposure to the light. Papadima & Bloukas[26] also referred that high-levels of fat resulted in higher $L^*$ values in Greek sausages.[22] Furthermore, sample darkening coincides with higher oxidation values; whereas, low TBARS are nor-

**Figure 2.** *Colour Parameters L (A), a* (B) and b* (C) of Slices of Tuna Salami (Day 0 and Day 9; Stored at 4 °C) in the Absence or in the Presence of Three Different Commercial Mixes of Tocopherols, DLT-100 (100 mg of Tocopherols/100 g Sample), VIT-100 (100 mg of Tocopherols/100 g of Sample), RNX10 (10 mg of Tocopherols+3.89 Rosemary Extract/100 g of Sample). Each Column is the Mean of 30 Separate Experiments; Vertical Lines Indicate SEM. Significantly Different from Control Values (#p<0.05) or from Day 0 (*p<0.05)*



mally associated with higher L* values.[6] However, in this study, this correlation was not observed. The VIT treatment was the only that did not change the L* colour at day 0. After 9 days the L* value was similar in all the samples. However, the DLT and RNX presented the same darkening parameter at day 0 and day 9 (Figure 2A).

The occurrence of lipid oxidation in tuna salami-like products can be shown by the decrease of a* value (product less red). The difference of red tint over the time occurs due to oxidative process of transfer of myoglobin to metmyoglobin, which is associated with the reduction of reddish pigmentation.[6] The negative change of this parameter influences the acceptability of the product by the consumer, which is of major concern to food producers. Figure 2 presents the a* values at days 0 and 9, for samples treated with and without antioxidants. The a* value showed a significant ($p$<0.05) decreasing trend with time (Figure 2B), except for samples treated with DLT, that non-significant differences ($p$>0.05) were observed after the 9 days period. However, the a* value reduction was also lower on the VIT samples. These results confirm the occurrence of lipid oxidation in control samples and are well correlated with TBARS values. During the dry processing of tuna muscle, changes in colour, from bright red to dark red, are the result of the conversion of myoglobin and oxymyoglobin oxidation to brown metmyoglobin1.[2,6] The view that VIT and DLT supplementation partial and total the decrease on reddish colour, respectively, are in line with the TBARS observation and the stability of unsaturated fatty acids levels. Moreover, the results are in agreement with those reported by Sánchez et al[27] who reported that myoglobin and oxymyoglobin oxidation to brown metmyoglobin is associated with reduction in reddish colour and lower a*. Dark fish, like tuna, contain considerable amounts of myoglobin and haem, which makes them susceptible to discoloration and lipid oxidation.[28] In respect to day 0, non-significant differences were observed among samples which mean that no colour change is developed by the addition of antioxidants. Moreover, non-significant differences were observed among samples treated with RNX and control.

The b* value showed changes between days 0 and 9, for samples treated with RNX and for the control i.e. samples became more blue as time increased. According to Bozkurt,[29] browning reactions explained those results. Pérez-Alvarez et al[30] who reported a decrease in b* value for Spanish sausages, refers that it is correlated with oxygen consumption by microorganisms. Samples treated with DLT and VIT present stability over time in respect to b* value

## CONCLUSION

The antioxidants added to tuna salami demonstrated effects in delaying both lipid oxidation and darkness of the samples. Moreover, the reduction of the unsaturated fatty acids levels observed in the control samples was by the presence of VIT and DLT antioxidants. During 60 days, the tuna salami preserved on a vacuum package did not show significant changes in lipid oxidation levels, pH, and colour. However, the experiment with slices of tuna salami (mimicking the consumer experience) evidenced a significant increase in the lipid oxidation after 9 days at 4 ºC. This evidence was accompanied by decrease in unsaturated fatty acids and decreases on the red colour of the tuna salami. The supplementation with DLT (100 mg of tocopherols/100 g sample) and VIT-100 (100 mg of tocopherols/100 g of sample) inhibited the lipid oxidation, the reduction of the reddish colour and also the unsaturated fatty acids reduction. On the other hand, the 10 fold reduction of tocopherols on the RNX antioxidant samples, which is constituted by rosemary, being the factor of differentiation to the others, showed higher values of darkness in slices during the period of storage. Moreover, the prevention of the oxidation process and the reduction of the reddish colour were not observed with the RNX supplementation.It is concluded that tocopherols supplementation (100 mg/100g of sample) enhance the nutritional value of tuna salami mainly by lipid oxidation inhibition.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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