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# INHIBITION OF LIPID PEROXIDATION OF FROZEN MACKEREL BY PRE-STORAGE ANTIOXIDANT SUPERFICIAL TREATMENT

S. G. DRAGOEV
*University of Food Technology, Technological Faculty, Technology of Meat and Fish Department, BG - 4002 Plovdiv, Bulgaria*

## Abstract

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With the help of TBARS the antioxidative effect of rosemary extract, rutin, quercetin, sodium erythrobate, BHT, α-tocopherol and a blend, containing rosemary extract, quercetin concentrate and sodium erythrobate on the lipid peroxidation of frozen mackerel, stored 360 d at -18 $^0$C has been studied. It is determined that the best inhibition of the lipid peroxidation of frozen mackerel is achieved when its surface is treated with solution of the composition of rosemary *(Rosmarinus officinalis)* extra$\tilde{\approx}$, quercetin concentrate from flower buds of Japanese acacia (*Sophora japonica)* and sodium erythrobate in concentration 200 mg.kg$^{-1}$ fish. According TBARS values of frozen mackerel stored 360 d at -18$^0$ C the antioxidants tested are arranged in the following descending row: 200 mg.kg$^{-1}$ natural antioxidants composition > 200 mg.kg$^{-1}$ BHT > 30 mg.kg$^{-1}$ BHT ≈ 200 mg.kg$^{-1}$ rosemary extract 30 mg.kg$^{-1}$ natural antioxidants composition > 200 mg.kg$^{-1}$ quercetin > 200 mg.kg$^{-1}$ sodium erythrobate > 200 mg.kg$^{-1}$ α-tocopherol > 30 mg.kg$^{-1}$ α-tocopherol 30 mg.kg$^{-1}$ sodium erythrobate > 30 mg.kg$^{-1}$ rosemary extract 200 mg.kg$^{-1}$ rutin 30 mg.kg$^{-1}$ quercetin > 30 mg.kg$^{-1}$ rutin > control sample without antioxidants.

*Key words:* antioxidants, inhibition, lipid peroxidation, frozen fish
*Abbreviations:* ANOVA - analysis of variance; BHA -butylated hydroxyanisol; BHT - butylated hydroxytoluene; EDTA – ethylenediaminetetraacetic acid; NDGA – nordehydroguiaretic acid; MDA – malondialdehyde; TBARS – 2-thiobarbituric acid reactive substances; TBHQ - tertiary butylated hydroxyquinone; DTPA - 2-ethylene 3-aminopentaacetic acid; THBP – three hydroxybutirophenone; STPP - sodium three polyphosphate; $d^{20}_{20}$ - relative density; $\Pi_D^{20}$ - coefficient of refraction

## Introduction

During the storage of deep frozen fish oxidative processes are initiated and developed as shown by Eun et al. (1994). These processes cause deterioration of the fish sensory properties and are characterized with lipid peroxidation primary and secondary products accumulation, some which harmful for the human health (Dragoev et al., 1998). As a representative of the high-fat salt water fish, the frozen mackerel is one of the fish species in which the processes of the lipid peroxidation are developed most inten-

*E-mail addresses: stefan_dragoev@yahoo.co.uk or logos2000lt@gmail.com*

sively (Dragoev et al., 1998). That is why in the last decade different possibilities for natural antioxidants application have been searched for, aiming at the inhibition of the lipid oxidation in frozen fish. Special attention is paid to some natural phenol compounds (Pazos et al., 2005a; 2006). To decrease the oxidation secondary products levels Banerjee (2006) suggests the muscle lipoxygenase to be inhibited by treatment of the frozen mackerel with polyphenols of the green tea. This author uses for the same purpose synthetic antioxidants, also, as BHA, BHT, esculetin, caffeic acid, ascorbic acid, and EDTA.

Other researchers have looked for synergism between phospholipids and α-tocopherol (Ohshima et al., 1993). It is estimated that 0.1 $g.kg^{-1}$ of caffeic acid, hydroxytyrosol, and propyl gallate could regenerate endogenous α-tocopherol from its oxidized forms resulting in an antioxidant synergy consistent with the reduction of lipid oxidation observed in fish muscle supplemented with phenolic compounds (Pazos et al., 2005b).

The antioxidant properties of sodium tripolyhosphate, propyl gallate, ascorbic acid, and erythrobic acid during the storage of raw mackerel and lake trout have been investigated (Weilmeier and Regenstein, 2004). It was determined that the addition of sodium tripolyhosphate with other free radical quenchers did not enhance the anti-oxidant effect in mackerel. Advantages of both citric acid and ascorbic acid were discussed to extend the shelf life of medium- and high-fat content fish species during frozen storage (Aubourg et al., 2004). Greek researchers have focused their attention on the natural rosemary (*Rosmarinus officinalis*) extract application (Vareltzis et al., 1997). It was determined that fillets and minced horse mackerel (*Trachurus trachurus*) and Mediterranean hake (*Merluccius mediterraneus*) treated with rosemary (*Rosmarinus officinalis*) extract contained significantly (*$P<0.05$) less MDA compared with controls during frozen storage at -18°C (Vareltzis et al., 1997). It has been established that the rosemary extract is more effective for minced fish protection from lipid oxidation in comparison with quercetin (Montero et al., 2005).

Becker et al. (2007) tried to find the synergism and antagonism between quercetin and other chain-breaking antioxidants in lipid systems of increasing structural organization. The results have shown that rutin was only effective as an antioxidant in the liposomes where it showed clear synergism with quercetin.

However, due to safety concerns, interest in natural antioxidants with synergistic effect has intensified (Shahidi, 2000). To address the demand by consumers, mixed tocopherols, herbal extracts such as rosemary and sage, as well as tea extracts have been commercialized for food and nutraceutical applications (Shahidi, 2000).

For preservation of the quality of the muscle in frozen fish (Tseng et al., 2005) have proposed pre-storage anti-oxidant dipping treatment. The review of the accessible references shows that during the storage of frozen, rich in fats fish (as Mediterranean mackerel) is necessary the effect of different antioxidants to be studied and the optimal concentration for their application to be estimated.

The aim of the current investigation is to determine the effect of the pre-storage anti-oxidant superficial treatment with natural, synthetic antioxidants and blend, developed by us, containing Rosemary and Japanese acacia extracts and sodium erythrobate on the lipid peroxidation inhibition of frozen mackerel, stored 360 d at -18°C.

## Materials and Methods

### *Materials*

The Mediterranean mackerel (*Scomber scombrus*), used in the experiment is purchased from "Happy Lady" Ltd, Varna. The fish weights 373 - 428 g and is supplied from Greece chilled and covered with ice slurry in ratio fish: ice = 1: 1.

In this experiment we investigated the effect of series natural antioxidants as rosemary *(Rosmarinus officinalis)* extract, rutin, quercetin, α-tocopherol, sodium erythrobate and a blend, developed by out team containing rosemary extract, quercetin powder concentrate of flower buts of Japanese acacia (*Sopho-*

*ra japonica)* and sodium erythrobate, as well as one of the strongest considered synthetic antioxidants BHT.

The liquid rosemary *(Rosmarinus officinalis)* extract is 280 – 300 $g.kg^{-1}$ undistillated alcohol extract and is supplied by "Aromena" Ltd, Sofia. The extract is corresponding to the indicators of the Technological Documentation TC 3267 - 2000 of the company producer and is a liquid with a solid phase presence, yellow-brown colour, a smell characteristic for the rosemary, in the form of 550 – 600 $g.kg^{-1}$ in distilled alcohol extract. Its dry substance is 60.4–69.0$g.kg^{-1}$. Flavonoids content is 21.2 – 26.4 $g.kg^{-1}$. Coefficient of refraction is $\Pi_D^{20}$ = 1.3605 – 1.3650, relative density $d^{20}_{20}$ = 0.9664 – 0.9845.

Buthylated hydroxytoluene and purified rutin and quercetin were purchased by E. Merck (Darmshtadt, Germany).

Sodium erythrobate was purchased from F.I.A. Food Ingredients Anthes GmbH (Teising, Germany).

α-Tocopherol was supplied by Sigma Chemical Company Ltd. (St. Louis, USA, Deisenhofen, Germany).

Our blend of natural antioxidants (Dragoev et al., 2004), containing three compounds:

1) 357.1 $g.kg^{-1}$ liquid rosemary extract, combined with

2) 523.8 $g.kg^{-1}$ quercetin powder concentrate of flower buts of Japanese acacia (*Sophora japonica)*, with quercetin content 53.33 %, and

3) 119.1 $g.kg^{-1}$ sodium erythrobate

***The setting of the experiment***

The mackerel is stored under liquid ice till the moment of its antioxidants treatment prior the freezing. The heads, internal organs and blood clots of the fish are not separated and it is not filleted. The fish is divided into 15 samples. The first sample is the control one. It is not superficially treated by spraying with antioxidants solution. The fourteen control samples are preliminary treated with antioxidants solutions with concentrations of 30 and 200 $mg.kg^{-1}$, respectively. Every sample is divided into five portions. All portions of samples are packaged in polymer bags and cardboard boxes prior to freezing.

The first portion is investigated as starting raw material – unfrozen fish (1 d). The rest samples are fast frozen (front of crystallization 0.5 - 3 $cm.h^{-1}$), at the temperature of the chilling air -40 ± 1°C, after which are stored 360 d at -18°C till the moment of analysis. Samples for laboratory analyses are taken on 90, 180, 270 and 360 d from the experiment start. Immediately before each analysis the samples are defrosted to 0 - 1°C. The defrosted mackerel is cleaned, filleted and milled in meat grinder with griddle apertures diameter 5 mm. On 90, 180, 270 and 360 d of the third experiment the change of the TBARS values is traced.

***Determination of TBARS***

Acid-extraction method of Schmedes and Holmer (1989) is used. TBARS are presented as g $MDA.kg^{-1}$ fish. The absorption of the samples was been estimated using 1 cm long container by UV-VIS spectrophotometer PYE UNICAM, Model 8800 (Perkin Elmer, UK), combined by integral scheme for diffusion reflection.

***Statistical analysis***

Data were analyzed using the Microsoft Excel program, Version 5.0 (SPSS Inc., Chicago, IL, USA). All determinations were carried out in three triplicate and data were subjected to analysis of variance (ANOVA). ANOVA was made with the General Linear Models (GLM) with a significant level of $P \leq 0.05$ (Draper and Smith, 1998). The Fischer's test with significant difference at $P \leq 0.05$ was used to compare sample means. Significant differences between means less than 0.05 were considered statistically significant (Kenward, 1987).

## Results

The effect of the type and concentration of the antioxidant added on the lipid peroxidation inhibition in frozen mackerel is presented at Table 1. It is determined that when all antioxidants are applied in concentration of 200 $mg.kg^{-1}$ less TBARS are accumu-

**Table 1**

**Effect of the type and concentration of the antioxidant added on the lipid peroxidation development during the 360 d storage of frozen mackerel (Scomber scombrus) at -18°C**

| Sample TBARS, mg MDA. kg$^{-1}$ | Antioxidant concentration, mg/kg | Period of storage at minus 18°C | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 d | | 90 d | | 180 d | | 270 d | | 360 d | |
| | | Average value | SD (n-1) | Average value | SD (n-1) | Average value | SD (n-1) | Average value | SD (n-1) | Average value | SD (n-1) |
| Sample without antioxidant | 0 | 0.151 | 0.031 | 0.809 | 0.042 | 0.933 | 0.059 | 4.687 | 0.109 | 7.225 | 0.173 |
| Rosemary extract | 30 | 0.151 | 0.031 | 0.800 *a* | 0.043 | 0.894 *a* | 0.061 | 3.948 | 0.089 | 6.472 | 0.164 |
| Rosemary extract | 200 | 0.151 | 0.031 | 0.746 *b* | 0.049 | 0.803 *b* | 0.049 | 1.693 | 0.091 | 3.996 | 0.62 |
| Rutin | 30 | 0.151 | 0.031 | 0.806 | 0.051 | 0.932 | 0.057 | 4.459 | 0.095 | 7.001 | 0.155 |
| Rutin | 200 | 0.151 | 0.031 | 0.799 | 0.046 | 0.900 | 0.051 | 4.102 | 0.101 | 6.521 | 0.175 |
| Quercetin | 30 | 0.151 | 0.031 | 0.789 | 0.044 | 0.896 | 0.046 | 3.967 | 0.094 | 6.598 | 0.168 |
| Quercetin | 200 | 0.151 | 0.031 | 0.751 | 0.051 | 0.866 | 0.054 | 3.235 | 0.086 | 4.356 | 0.163 |
| Sodium erythrobate | 30 | 0.151 | 0.031 | 0.801 *c* | 0.048 | 0.889 *c* | 0.055 | 3.982 | 0.104 | 5.398 | 0.159 |
| Sodium erythrobate | 200 | 0.151 | 0.031 | 0.767 | 0.043 | 0.877 | 0.062 | 3.761 | 0.087 | 4.875 | 0.152 |
| α–tocopherol | 30 | 0.151 | 0.031 | 0.808*d* | 0.049 | 0.901*d* | 0.060 | 4.606 | 0.099 | 5.333 | 0.150 |
| α–tocopherol | 200 | 0.151 | 0.031 | 0.794 *e* | 0.055 | 0.894 *e* | 0.058 | 4.526 | 0.089 | 5.092 | 160 |
| Butylated hydroxitoluen | 30 | 0.151 | 0.031 | 0.810 *f* | 0.052 | 0.857 *f* | 0.046 | 2.316 | 0.096 | 3.950 | 0.164 |
| Butylated hydroxitoluen | 200 | 0.151 | 0.031 | 0.802 *g* | 0.054 | 0.790 *g* | 0.055 | 1.419 | 0.094 | 3.184 | 0.177 |
| Blend of antioxidants | 30 | 0.151 | 0.031 | 0.796 *h* | 0.049 | 0.869 *h* | 0.056 | 2.333 | 100 | 4.025 | 0.165 |
| Blend of antioxidants | 200 | 0.151 | 0.031 | 0.739 *i* | 0.045 | 0.783 *i* | 0.047 | 1.357 | 0.088 | 3.093 | 0.158 |

Mathematic-statistical conclusions: *All compared average values of the sensory ratings in rows are statistically different with the exception of these marked with letter index: a, b, c, d, e, f, g, h u i, which are not statistically significantly differ*

lated, while in the most of cases when antioxidants in concentration of 30 mg. kg$^{-1}$ antioxidant is used it is not effective.

On 360 d least free MDA is estimated in the samples treated with 200 mg.kg$^{-1}$ composition of natural antioxidants. Similar results, but with little higher TBARS values are measured in the samples treated respectively with: 200 mg.kg$^{-1}$ BHT, 30 mg.kg$^{-1}$ BHT and 200 mg.kg$^{-1}$ rosemary extract. The highest MDA concentrations except in the control sample are estimated in the samples treated with 30 mg.kg$^{-1}$ rutin, 30 mg.kg$^{-1}$ quercetin, 200 mg.kg$^{-1}$ rutin and 30 mg.kg$^{-1}$ rosemary extract.

## Discussion

The results obtained by our team are similar to the ones reported by Ramanathan and Das (1992), who have researched the influence of some polyphenole natural products on the lipid peroxidation in chilled Canary mackerel (*Scomberomorus comersoni*) and Eun et al. (1993), who have investigated the effect of 100 mg.kg$^{-1}$ BHA, BHT, NDGA, TBHQ, pyrogallol, EDTA, STPP, DTPA, THBP, ethoxycine, α–tocopherol, β–carotene, rosemary extract, sodium ascorbate and sodium erythorbate on the enzyme lipid peroxidation in American channel sheat-fish (*Ictalurus*

*punctatus*). According Ramanathan and Das (1992) all studied polyphenole natural antioxidants except rutin (200 and 300 mg.kg$^{-1}$) and α–tocopherol (30 mg.kg$^{-1}$) are effective. Our results show that for the lipid peroxidation inhibition of deep frozen mackerel for 360 d ineffective are rutin and α-tocopherol in both experimented concentrations (30 and 200 mg.kg$^{-1}$), as well as quercetin, rosemary extract and sodium erythrobate in the low concentration (30 mg.kg$^{-1}$).

The results obtained can be explained with the natural flavonoids nature. The rutin (quercetin-7-rutinozide) contains as disaccharide residuum rutinoze (L-ramnoglucose), attached to the hydroxyl group at $C_3$. The quercetin (5, 7, 3', 4'-tetrahidroxyflavonole) is aglicon of several natural glycosides. The quercetin and rutin have stronger antioxidative activity with the increase of their concentration in the product and reach their optimum at 1000 – 2000 mg.kg$^{-1}$ (Banerjee, 2006).

α-Tocopherol is synthetic chinone, inhibiting the lipid peroxidation by attaching free radicals to its double conjugated linkages (Pazos et al., 2005$^{B}$). The effect of α-tocopherol increases with augmentation of its concentration. Over particular concentration so called "inversion" occurs in its action, explained with the speed increase of hydroperoxides disintegration into free radicals. Therefore recommended concentration of the α-tocopherol is 200 - 500 mg.kg$^{-1}$ fats (Ohshima et al., 1993). Moderate inhibiting effect shows 100 mg.kg$^{-1}$ α–tocopherol or rosemary extract, too (Eun et al., 1993). Average expressed activity show concentrations of 200 mg sodium erythrobate.kg$^{-1}$ and 200 mg quercetin.kg$^{-1}$. Many substances possess the feature to link heavy metals with changeable valence into chelate complexes. Thus they deactivate them. Indirectly they show antioxidant or synergetic action in the fish lipids oxidation process. The ascorbic acid and its dehydrogenated form constitute oxidative-reduction system capable to accept and give hydrogen atoms. Instead of L-ascorbic acid or in combination with it L-ascorbate, potassium L-ascorbate, erythrobic (D-isoascorbic) acid and sodium erythrobate are used, also (Weilmeier and Regenstein, 2004). According Weilmeier and Regenstein (2004) the fat fish should be dipped in solution containing 1000 - 3000 mg.l$^{-1}$ L-ascorbic acid.

Sodium erythrobate in concentration of 100 mg.kg$^{-1}$ activates the lipid peroxidation, while higher concentrations of these substances show inhibition effect (Eun et al., 1993). Hundred mg.kg$^{-1}$ L-ascorbic acid reveals as pro-oxidant in steam blanched and micro waved fish during the one week storage of frozen at -20$^{O}$C mackerel (Ramanathan and Das, 1992). Relatively low concentrations (20 - 30 mg.kg$^{-1}$) of exogenous ascorbate addition are ineffective and even act pro-oxidative, when the ascorbate is applied independently. Unlike the real antioxidants which possess inhibition activity even on partially oxidized fats, ascorbates and erythrobates can give catalytic action. Dissociating hydrogen the ascorbic acid can be converted into dehydro ascorbic acid in convertible reaction. When restituted the latter oxidizes other metabolite products increasing the absorption capability of ferrous ions ($Fe^{3+}$), which biological accessibility is low and they are reduced into $Fe^{2+}$. According Aubourg et al. (2004) only concentrations higher than 50 mg.kg$^{-1}$ can show some antioxidative activity.

Strongly expressed activity show rosemary extract in 200 mg.kg$^{-1}$ concentrations and 30 mg.kg$^{-1}$ BHT and natural antioxidants composition. The rosemary extract increases its antioxidative activity with the increase of its concentration in the product. It is more active than the α–tocopherol and goes back only to BHT. Antioxidative activity of the rosemary extract is due to its content of phenol compounds with flavonoid nature and especially to the phenol diterpenes (Vareltzis et al., 1997). Their antioxidative effect is imparted basically to the presence of carnosol and carnosolic acid pursued by 12-metoxycarnosine acid, 7-metoxyrozmanole, rozmanole, 7-metoxyepirosmanole and 12-metoxy-γ-lactone **(**Montero et al., 2005). The rosemary extracts increase their antioxidative activity with the increase of the concentration from 100 to 300 mg.kg$^{-1}$ **(**Montero et al., 2005). Put in dozes of 4000 mg.kg$^{-1}$ increase the oxidative stability approximately twice in comparison with the effect, achieved by addition of tocopherols, BHT or grounded rosemary (Vareltzis et al., 1997). Rosemary extracts

phenols contain movable hydrogen atom. Their action is explained with the reaction chain break due to the interaction with active radicals, leading the oxidative chain and their convention into stable molecule products. Phenol antioxidants act due to the lower solidity of the O-H linkage in the antioxidant in comparison with the solidity of the R-H linkage in the oxidized substrate (fatty acids, glycerols, etc.). The lower activity of the free radicals formed in the antioxidant is due to their resonance stabilizing. Electron donor groups (methyl, metoxyl, etc.) at o- and p-position significantly increase their antioxidative activity, and the electron acceptor groups (nitrose, carboxyl, etc.) decrease it.

BHT (2,6-ditertiary-butyl-p-cresol or 2,6-ditertiary-butyl-4-methylphenol) is synthetic phenol, containing movable hydrogen atom. BHT is water insoluble at 25$^{O}$C, and methanol soluble to 250 g.kg$^{-1}$. It is well soluble in fats and oils. Antioxidative activity of BHT increases along with its content in substrate increase (Shahidi, 2000). In high concentrations BHT detains the peroxidation in easily oxidized systems (Shahidi, 2000). Therefore the most strongly expressed activity show concentrations of 200 mg.kg$^{-1}$ BHT and most of all natural antioxidants composition. Combination of quercetin and rosemary extract is used for oxidative stability increase of high pressure thermally treated fish stuff (Montero et al., 2005).

The chain oxidative reactions can be inhibited not only by the chain break speed increase as in the case of the individual phenols and other antioxidants (chinones, amines) action, but also by free radicals formation speed decrease by means of degenerated off set of the chain (Dragoev et al., 1998). It can be achieved by introduction of substances into the oxidizing environment, which is capable to react with the hydroperoxides without free radicals formation (Shahidi, 2000). The mechanism of the anti oxidative action of this type of antioxidants is not chain and their efficiency is significantly lower than the radical antioxidants. If added to the oxidizing system together with phenols they can significantly enhance their action. When two inhibitors are used, significant synergic effect is achieved when one of them breaks lipid peroxide chain reaction and the other destroys the peroxides (Shahidi, 2000). This phenomena is explained by the fact that the two inhibitors not only oppress the main substance oxidation, separately, mutually secure each other from finishing. The inhibitor breaking the chain oppresses hydroperoxides formation and thus protects the other inhibitor – hydroperoxides destroyer, quickly to be spent. On the part of the second inhibitor it destroys the peroxides, decreases the number of the originating chains and thus preserves for longer time the first inhibitor – breaking the chain (Shahidi, 2000). When two or three antioxidants with different mechanism of action are jointly added, strong effect of synergism is exhibited. As synergists in the blend of the natural antioxidants, substances, which are very weak antioxidants, are used. The addition of similar compounds significantly increases the efficiency of other inhibitors action. Such are some polyalkali organic hydroxycarboxyl acids as ascorbic, or sodium erythrobate. When added to the rosemary extract and quercetin concentrate from the blend, sodium erythrobate enhances their effect, playing the role of the hydrogen donor for exhausted natural antioxidants recovery (Dragoev et al., 2004). This explains the most effective lipid peroxidation inhibition of the frozen mackerel in the sample, treated with solution at concentration 200 mg.kg$^{-1}$ natural antioxidants blend.

## Conclusions

The results obtained allow the conclusion to be made that in order lipid peroxidation processes inhibition of frozen mackerel to be achieved in maximum possible degree it is best the fish to be frozen and its surface to be treated in advance with liquid composition containing rosemary *(Rosmarinus officinalis)* extract, quercetin powder concentrate of flower buds of Japanese acacia (*Sophora japonica)* and sodium erythrobate in concentration 200 mg.kg$^{-1}$ fish. On 360 d of the deep frozen mackerel storage at - 18$^{O}$C, treated with antioxidants composition not more than 3.093 mg.kg$^{-1}$ MDA are formed.

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