

# Effects of various antioxidants on oxidative stability of anchovy (*Engraulis encrasicolus*) oil

## Çeşitli antioksidanların hamsi (*Engraulis encrasicolus*) yağının oksidatif stabilitesi üzerine etkileri

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**Abstract:** The aim of study was to investigate four commercial available antioxidants (groups A (300 mg propyl gallate (PG)+10 mg rosemary extract (RE)/1000 mg), B (240 mg butylated hydroxy anisole (BHA)+80 mg PG+80 mg citric acid (CA)/1000 mg), C (120 mg BHA+120 mg PG+50 mg CA)/1000 mg), D (150 mg butylated hydroxytoluene (BHT)+100 mg BHA+10 mg PG)/1000 mg) used to evaluate oxidation during the storage in fish oil. Antioxidants were added to the fish oil to determine which ones were most effective in preventing oxidation, and fish oil was stored in the amber bottles at room temperature (20 °C) for 90 days. The control group samples were stored under the same conditions and antioxidant was not added. To determine the effect of antioxidants, the recommended by the manufacturer dose of commercial antioxidant (1000 mg kg<sup>-1</sup> fish oil) was used in the experimental groups. The formation of the primary and secondary oxidation products in fish oil storage trial was examined by conducting the peroxide value (PV) and p-anisidine value (AV) analyses. The total oxidation value (TOTOX) was calculated based on the PV and AV measurements. Minor changes were observed in the PV of the fish oil during the first 30 days. In the study, antioxidant added samples (groups B, C, D > 5 meq kg<sup>-1</sup>) were oxidized on the 45<sup>th</sup> day; on the other hand, both control and group A oxidized on the 75<sup>th</sup> day. A possible prooxidative effect was seen for some of the antioxidants. There was a very little change secondary oxidation of fish oil and no significant effects of all four antioxidant groups on the changes of AV (<20) during the storage period (P>0.05). In addition TOTOX was calculated under GOED (<26) limit during the storage for 90 days. At the end of the study, control samples were not significantly different from the other samples with antioxidant-added. Due to the results obtained at the end of the 90-day study, it was found that none of the antioxidants were used efficiently in this study.

**Keywords:** Fish oil, antioxidants, lipid oxidation, stabilization

**Öz:** Bu çalışmanın amacı, balık yağında depolanma sırasındaki oksidasyonu değerlendirmek için kullanılan dört ticari antioksidanı (A (300 mg propil gallerat (PG)+10 mg biberiye ekstraktı (BE)/1000 mg, B (240 mg bütül hidroksi anisol (BHA)+80 mg PG+80 mg sitrik asit (SA)/1000 mg, C (120 mg BHA+120 mg PG+50 mg SA)/ 1000 mg, D (150 mg bütül hidroksi toluene (BHT)+100 mg BHA+10 mg PG)/1000 mg) incelemektir. Antioksidanlar, hangilerinin oksidasyonun önlenmesinde en etkili olduğunu belirlemek için balık yağına eklenmiş ve balık yağları, 90 gün boyunca oda sıcaklığında (20 °C) amber şişelerde saklanmıştır. Kontrol numuneleri aynı koşullar altında muhafaza edilmiş ve antioksidan eklenmemiştir. Antioksidanların etkisini belirlemek için, deney gruplarında üretici firma tarafından önerilen ticari antioksidan dozu (1000 mg kg<sup>-1</sup> balık yağına) kullanılmıştır. Balık yağı depolama denemesinde birincil ve ikincil oksidasyon ürünlerinin oluşumu, peroksit değeri (PV) ve p-anisidin değeri (AV) analizleri yapılarak incelenmiştir. PV ve AV ölçümlerine dayanarak toplam oksidasyon değeri (TOTOX) hesaplanmıştır. İlk 30 gün boyunca balık yağının peroksit değerlerinde küçük değişiklikler gözlemlenmiştir. Çalışmada, antioksidan takviyeli örnekler B, C, D > 5 meq kg<sup>-1</sup>), 45. günde okside olmuş, ancak kontrol ve A grubu 75. günde okside olmuştur. Bazı antioksidanlar için olası bir prooksidatif etki görülmüştür. Balık yağının sekonder oksidasyonunda küçük değişiklikler gerçekleşmiş ve depolama süresi boyunca dört antioksidan grubunun da AV değişiklikleri üzerinde önemli bir etkisi olmamıştır (p>0.05). İlaveten TOTOX 90 günlük depolama boyunca GOED (< 26) limiti altında hesaplanmıştır. Çalışmanın sonunda kontrol örnekleri ile antioksidan ilaveli örnekler arasında önemli fark bulunmamıştır (P>0,05). 90 günlük çalışmanın sonunda elde edilen sonuçlara göre antioksidanların hiçbirinin verimli bir şekilde kullanılmadığı tespit edilmiştir.

**Anahtar kelimeler:** Balık yağı, antioksidanlar, yağ oksidasyonu, stabilizasyon

## INTRODUCTION

Fish meal and fish oil are used as feed ingredient in aquaculture. Small pelagic and demersal species having no economic value are preferred for the production of fish meal and oil. Fish used for obtaining fish oils for commercial production today are herring, cod, sardine, anchovy, menhaden, horse mackerel, sharks and dolphins (Kasbo, 2011; Boran, 2004). Fish oil is the major source of unsaturated omega-3 fatty acids ie. eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA)

(Fakir and Waghmare, 2015). However, due to their high level of unsaturation these omega-3 PUFA are extremely susceptible to oxidative spoilage (Morales et al., 2015).

The content of EPA and DHA in fish oil is dependent on the type of fish, the fish diet, sea water temperatures and geographic location of the catch (Kasbo, 2011). Due to the fish oil structure and environmental factors such as enzyme, light, metal ions, temperature it oxidizes rapidly during the processing and storage (Baek, 2012; Palupi et al., 2016). Oxidation of fish oil produces undesirable flavors. It may also

reduce the nutritional quality and safety of the oils (Eritsland, 2000; Korkut et al., 2007). Thus, studies that are intended to prevent oxidation in fish oil and prolong its shelf life are required.

There are several methods to protect the oil from oxidation. The most common methods are use of metal inactivators, minimizing exposure to air, heat and light, minimizing the loss of naturally occurring antioxidants and adding additional antioxidants (Fennema, 2008).

Antioxidants are chemical compounds which have been shown to prevent and delay oxidative deterioration in oil. These chemical compounds have an effect at the beginning of oxidative and auto oxidative processes and thus inhibiting oxidation which leads to the formation of the reaction products (Fennema, 2008).

Many studies have been carried out so far to protect fish oil against oxidation. Natural and synthetic antioxidants were used in these studies. Some researchers focused on antioxidative effects of rosemary extract (Hraš et al., 2000). Morales et al., (2015) compared the effects of natural and synthetic antioxidants on oxidation in sardine oil and the following researchers tested the effects of synthetic antioxidants on oxidative stability, in herring oil (Carvajal et al., 2014; Baek, 2012), mackerel oil (Fakir and Waghmare, 2015), sardine oil (Chandrasekar et al., 2016) and cod liver oil (Kasbo, 2011), respectively. However, there have not been any studies carried out to investigate the effects of the addition of newly released commercial antioxidants in the market prepared with the addition of different antioxidant combinations in anchovy oil obtained from the Black Sea region on oxidative stability. In this study, four different (A (300g PG+10 g RE)/1000 g, B (240 g BHA+80 g PG+80 g CA)/1000 g, C (120 g BHA+120 g PG+50 g CA)/ 1000 g, D (150 g BHT+100 g BHA+10 g PG)/1000 g) commercial antioxidants were added to fish oil obtained from anchovy fish and their resistance to oxidation in the storage conditions was tested.

## MATERIALS AND METHODS

### Materials

Anchovy oil was obtained from Sürsan Incorporated Company (Samsun). Fatty acid composition of anchovy fish oil is analyzed in EcoSmyrna Laboratories (by gas chromatography). Fatty acid composition of anchovy oil is shown in Table 1.

**Table 1.** Fatty acid composition of anchovy oil used in the study

Fatty Acids		%
14:00	myristic acid	8.46
16:00	palmitic acid	26.20
18:00	stearic acid	4.51
18:01	oleic acid	22.90
18:3 -3	$\alpha$ -linolenic acid	1.31
20:3 $\omega$ -3	eicosatrienoic acid	0.30
20:5 $\omega$ -3	eicosapentaenoic acid (EPA)	11.30
22:5 $\omega$ -3	docosapentaenoic acid	0.59
22:6 $\omega$ -3	docosahexaenoic acid (DHA)	15.30
<b><math>\omega</math>-3 Total</b>	<b><math>\omega</math>-3 Total</b>	<b>28.80</b>
18:2 $\omega$ -6	linoleic acid	1.31
18:3 $\omega$ -6	gamma linolenic acid	0.28
20:2 $\omega$ -6	eicosadienoic acid	0.23
20:3 $\omega$ -6	eicosatrienoic acid	0.27
20:4 $\omega$ -6	arachidonic acid	0.25
<b><math>\omega</math>-6 Total</b>	<b><math>\omega</math>-6 Total</b>	<b>2.82</b>
18:1 Trans	trans oleic acid	0.04
18:2 Trans	trans-linoleic acid	0.48
18:3 Trans	trans linolenic acid	0.05
23:0 Trans	trans tricosanoic acid	0.00
	<b>Total</b>	<b>93.78</b>

Anchovy oil, tocopherol content was analyzed in Süleyman Demirel University Applied Basic Sciences and Technologies Research Laboratory (with HPLC RF-10AXL Fluorescence detector). Tocopherol content of anchovy oil is shown in Table 2.

**Table 2.** Tocopherol content of anchovy oil (ppm) used in the study

	$\alpha$ -tocopherol	$\beta$ -tocopherol	$\gamma$ -tocopherol	$\delta$ -tocopherol
Anchovy Oil	71.59	<0.10	0.37	<0.005

Antioxidants; Miradox L PV 301, Miradox L AP 248, Miradox L AP 1212 were supplied Miavid Company (Germany) and Oxy-Nil Eqzero Plus Liquid was supplied Nutriad Company (United Kingdom).

The antioxidants presented are propyl gallate (PG), rosemary extract (RE), butylated hydroxyanisole (BHA), citric acid (CA), butylated hydroxytoluene (BHT).

The contents of the antioxidants used in the experiment are as follows:

Group Control no antioxidant added

Group A (Miradox L PV 301) (300.000 mg PG +10.000 mg RE) kg<sup>-1</sup>

Group B Miradox L AP 248 (240.000 mg BHA + 80.000 mg PG + 80.000 mg CA) kg<sup>-1</sup>

Group C Miradox L AP 1212 (120.000 mg BHA + 120.000 mg PG + 50.000 mg CA) kg<sup>-1</sup>

Group D Oxy-Nil Eqzero Plus Liquid (150.000 mg BHT + 100.000 mg BHA + 10.000 mg PG) kg<sup>-1</sup>

### Sample preparation

This study was carried out in the laboratories of Skretting Incorporated Company. Fish oil was weighed and placed in separate plastic containers.

Commercial antioxidants were added to fish oil at the recommended rate (1000 mg kg<sup>-1</sup>) by the manufacturer. The antioxidants weighed in petri dishes were added to the fish oil and mixed with a plastic-tipped beater for 5 minutes to be homogenized. The sample without antioxidants was used for the control group. The fish oil was then filled into the amber bottles, each of 100 ml with a screw cap. In this study a total of 70 amber bottles was used. The bottles were stored in a dark room at 20±1 °C.

### The determination of oxidative stability

Fish oil samples were measured by the use of peroxide value (PV), p-anisidine value (AV) and free fatty acid value (FFA). Samples were analyzed every two weeks during the trial. All samples were tested in duplicate.

### The peroxide value (PV)

The primary oxidation of fish oil was determined by measuring the peroxide value. GOED has defined a limit of PV 5 meq kg<sup>-1</sup> for accepted quality of oil (GOED, 2019).

The PV of the fish oil were measured according to a modification of the method of the American Oil Chemist's Society Official method Cd 8b-90 (AOCS 2017b). Approximately 5 g of fish oil was weighed into a 250 ml Erlenmeyer flask. To this flask was added 18 ml of acetic acid/chloroform mixture (3/2) (v/v) and the contents were continuously stirred in order to fully dissolve the fish oil. 0.5 ml potassium iodide (KI) solution was added and the flask was allowed to stand for 1 min with occasional shaking. Distilled H<sub>2</sub>O (30 ml) was added to the flask and was titrated against 0.01 N sodium thiosulphate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) using 2 ml of 1% starch indicator. Blank samples were determined by titration of samples which did not contain fish oil. The PV was calculated as follows:

$$PV \text{ (meq O}_2\text{ kg}^{-1}\text{ oil)} = (S - B) * N * 1000 / \text{sample weight} \quad (1)$$

where, S is sample titre (ml); B is blank titre (ml) and N is Normality of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

### The p-Anisidine value (AV)

The measurement of the AV is a common method for determining the level of secondary oxidation products in oils. GOED has been defined a limit of AV 20 for accepted quality of fish oil (GOED, 2019).

p-Anisidine value was determined according to AOCS Cd 18-90 (AOCS 2017a). This method determines the amount of aldehydes (principally 2-alkenals and 2,4-dienals) in animal

and vegetable fats and oils, by reaction in an acetic acid solution of the aldehydic compounds in the oil and the p-anisidine, and then measuring the absorbance at 350 nm.

The anisidine values were calculated as in Eq. (2)

$$pAV = (25 * 1.2 A_s - A_b) / m \quad (2)$$

A<sub>s</sub> is the absorbance of oil after reaction with p-anisidine, A<sub>b</sub> is the absorbance of oil in isooctane, m is the weight of anchovy oil used for analysis (g).

### The total oxidation value (TOTOX)

The total oxidation values (TOTOX) of the oil samples were used in this study as an indication of overall oxidative stability. TOTOX was defined as the addition of both the peroxide and anisidine value;

$$TOTOX \text{ value} = (2 PV + p-AV) \quad (3)$$

PV: Peroxide value  
AV: p-anisidine value

The GOED monograph states TOTOX of 26 as the limit for oxidative status (GOED, 2019).

### The free fatty acid (FFA)

The formation of FFA is an important measure of food rancidity. Determination of FFA, expressed as percentage of oleic acid, were done by acidimetric titration after adding ethanol and using phenolphthalein as an indicator, following AOAC (1990) method. Hertrampf and Piedad-Pascual (2000) has defined a limit of FFA 5 % for accepted quality of oil.

$$FFA = (M_1 * 0.25 * 28.2) / M_2 \quad (4)$$

Free fatty acid = (FFA) (as oleic acid %)  
M<sub>1</sub>: amount of sodium thiosulfate consumption (ml)  
M<sub>2</sub>: sample amount (gr)

### Statistical analysis

Statistical analysis comprised a one-way ANOVA using the probability level of 0.05. The significant differences between the means of parameters were determined using Duncan's test. All statistical analyses were performed using SPSS 14.0 for Windows (SPSS INC. Chicago, IL, USA). Each replicate is expressed as mean ± SE. All experiments were replicated two times.

## RESULTS AND DISCUSSION

### The effect on peroxide value (PV)

Peroxides are the first compounds formed when polyunsaturated fatty acids oxidized, and this is the first step of lipid oxidation. The level of primary oxidation products, hydroperoxides, can be measured by using PV as a method

(Kasbo, 2011). Changes in peroxide values of anchovy oil during 90 days of storage are shown in Table 3.

Table 3. Changes in peroxide value of anchovy oil during 90 days of storage

Days	Groups				
	Control	A	B	C	D
Initial	3.93±0.06 <sup>BC</sup>	3.93±0.06 <sup>BC</sup>	3.93±0.06 <sup>E</sup>	3.93±0.06 <sup>C</sup>	3.93±0.06 <sup>E</sup>
15th Day	4.23±0.13 <sup>ABC</sup>	4.33±0.09 <sup>AB</sup>	4.53±0.03 <sup>AC</sup>	4.85±0.04 <sup>AB</sup>	4.70±0.38 <sup>CDE</sup>
30th Day	3.66±0.04 <sup>AC</sup>	3.92±0.17 <sup>ABC</sup>	4.22±0.12 <sup>bcCDE</sup>	4.65±0.01 <sup>AB</sup>	4.43±0.01 <sup>abDE</sup>
45th Day	3.97±0.14 <sup>BC</sup>	4.42±0.35 <sup>bcB</sup>	5.12±0.17 <sup>abB</sup>	5.61±0.17 <sup>AA</sup>	5.69±0.43 <sup>AB</sup>
60th Day	2.64±0.02 <sup>AD</sup>	3.48±0.05 <sup>BC</sup>	3.00±0.02 <sup>CF</sup>	3.69±0.06 <sup>AC</sup>	3.13±0.00 <sup>CF</sup>
75th Day	4.44±0.27 <sup>hABC</sup>	4.28±0.07 <sup>BB</sup>	4.11±0.04 <sup>hDE</sup>	5.11±0.05 <sup>BA</sup>	5.33±0.29 <sup>ABC</sup>
90th Day	5.32±0.67 <sup>AA</sup>	5.74±0.25 <sup>AA</sup>	5.59±0.16 <sup>AA</sup>	5.92±0.24 <sup>AA</sup>	6.14±0.00 <sup>AA</sup>

\* Values are presented as mean ±SE, n=2

<sup>a,b</sup> Values that contain different superscript lowercase letters in the same row are significantly different (P<0.05)

<sup>A,B</sup> Values that contain different superscript uppercase letters in the same column are significantly different (P<0.05)

The PV of the fish oil, stored at 20 °C, changed insignificantly till the 30<sup>th</sup> day during the storage in all the experimental groups (P>0.05). Control samples were not distinguished from samples with added antioxidants until the 45<sup>th</sup> day. For the first time the B (5.12±0.17 meq kg<sup>-1</sup>), C (5.61±0.17 meq kg<sup>-1</sup>) and D (5.69±0.43 meq kg<sup>-1</sup>) groups exceeded over the limits of GOED (PV 5 meq kg<sup>-1</sup>) on the 45<sup>th</sup> day. However, there was a limited increase in control (3.97±0.14 meq kg<sup>-1</sup>) and group A (4.42±0.35 meq kg<sup>-1</sup>). The effects of antioxidants are dependent on the concentration. Too high concentration of antioxidants may change the action of antioxidants to work as prooxidants (Kasbo, 2011). This result could be due to the possible effects of prooxidative effects of antioxidants as stated by Kasbo (2011). PV values of all fish oil samples decreased at 60<sup>th</sup> day. The decrease in the PV of oil samples, as stated by Hras et al., (2000) may be attributed to a decline in the rate of hydroperoxide formation in associated with a rise in the production of the secondary oxidation products.

The control and the group A remained below the PV 5 meq kg<sup>-1</sup> throughout 75 days. It has been determined that all groups have exceeded the limit of PV 5 meq kg<sup>-1</sup> on the 90<sup>th</sup> day. As of 90<sup>th</sup> day, the control samples did not distinguish from samples with antioxidant added.

At the beginning of the study, the tocopherol level of anchovy oil was measured as 71.59 ppm. It can be considered that the α-tocopherol naturally found in anchovy oil might have protected fish oil from oxidation. This situation could explain why the PV of control group was lower than the groups supplemented with antioxidant throughout the trial. Similar to our results, Morales et al., (2015) stated that the low concentrations of α-tocopherol (50 ppm) in sardine oil together with ascorbyl palmitate was effective in eliminating the hydroperoxides. This is due to the destruction ability of α-

tocopherol with non-radical processes like the elimination and reduction of hydroperoxides or hydrogen donation. It was reported in the same study that the high levels of α-tocopherol demonstrate prooxidative behaviors and thus accelerating the oxidation of fish oil (Kulas and Ackman, 2001; Drusch et al., 2008; Frankel, 2005).

Throughout the trial, it was revealed that there was lower PV in the group (A) in which PG and rosemary extract were added when compared to the other groups supplemented with antioxidants. However, the group A exhibited similar PV with the control group but it could not demonstrate a better performance than the control group. It was reported by Tsimidou et al., (1995) and O'Sullivan et al., (2005) that when rosemary was added to the mackerel oil and cod liver oil, there was a similar decrease in oxidation.

### The effect on p-Anisidine values (AV)

AV is an indicator of the formation of secondary oxidation products such as aldehydes and ketones formed by the decomposition of peroxide and hydroperoxides. AV can be used as a rough estimator of future storage stability of freshly processed oil (Frankel, 2005). Changes in p-anisidine values of anchovy oil during 90 days of storage are shown in Table 4.

Table 4. Changes in p-anisidine value of anchovy oil during 90 days of storage

Days	Groups				
	Control	A	B	C	D
Initial	6.61±0.25 <sup>AD</sup>	6.61±0.25 <sup>BC</sup>	6.61±0.25 <sup>AC</sup>	6.61±0.25 <sup>AB</sup>	6.61±0.25 <sup>AC</sup>
15th Day	6.68±0.38 <sup>AD</sup>	6.44±0.46 <sup>AC</sup>	6.75±0.54 <sup>AC</sup>	6.65±0.50 <sup>AB</sup>	7.30±0.04 <sup>BC</sup>
30th Day	6.69±0.02 <sup>AD</sup>	6.85±0.21 <sup>BC</sup>	6.97±0.22 <sup>AC</sup>	6.75±0.10 <sup>AB</sup>	7.02±0.28 <sup>BC</sup>
45th Day	6.77±0.34 <sup>CD</sup>	7.44±0.13 <sup>AB</sup>	7.21±1.04 <sup>BC</sup>	7.12±0.58 <sup>AB</sup>	6.64±0.31 <sup>AC</sup>
60th Day	7.57±0.09 <sup>BC</sup>	7.57±0.03 <sup>AB</sup>	7.95±0.07 <sup>ABC</sup>	8.18±0.07 <sup>AB</sup>	7.81±0.06 <sup>BC</sup>
75th Day	8.01±0.01 <sup>AB</sup>	8.02±0.09 <sup>AA</sup>	8.59±0.07 <sup>AB</sup>	9.58±1.03 <sup>AA</sup>	8.05±0.02 <sup>AB</sup>
90th Day	8.48±0.02 <sup>AA</sup>	8.06±0.51 <sup>AA</sup>	8.84±0.13 <sup>AA</sup>	7.84±0.04 <sup>AB</sup>	9.09±0.86 <sup>AA</sup>

\* Values are presented as mean ±SE, n=2

<sup>a,b</sup> Values that contain different superscript lowercase letters in the same row are significantly different (P<0.05)

<sup>A,B</sup> Values that contain different superscript uppercase letters in the same column are significantly different (P<0.05)

Small changes were recorded for AV which was found to be 6.61 ± 0.25 (P> 0.05) at the beginning of the experiment but there was a difference between the groups on the 60<sup>th</sup> day (P <0.05). At the end of the experiment, the lowest values were observed in the group C (7.84±0.04) and the AV of all groups were found to be similar (P>0.05). At the end of the experiment, the AV remained below the GOED (<20) limits in all groups. Fakir and Waghmare (2015) in their study added synthetic antioxidants in mackerel oil and AV showed small differences throughout the storage and the addition of antioxidant did not have an effect on this parameter (P>0.05). This result revealed that the secondary oxidation was very little throughout the storage. These results are compatible

with our results regarding the AV. Wang et al., (2011) determined that in addition to the concentration and type of the antioxidant added to the fish oil and the temperature of the environment was effective on the fish oil oxidation.

### The total oxidation value (TOTOX)

PV and AV change over time as hydroperoxides are produced and decomposed in fish oil. TOTOX gives complete information about the oxidative state of the oil, combines the history of the oil (AV) with the present status (PV). TOTOX considers both primary and secondary oxidation products (Kasbo, 2011). Changes in total oxidation value of anchovy oil during 90 days of storage are shown in Table 5.

**Table 5.** Changes in total oxidation value of anchovy oil during 90 days of storage

Days	Groups				
	Control	A	B	C	D
Initial	14.48±0.00 <sup>abDE</sup>	14.48±0.00 <sup>abDE</sup>	14.48±0.00 <sup>abEF</sup>	14.48±0.00 <sup>abD</sup>	14.48±0.00 <sup>abD</sup>
15th Day	15.13±0.12 <sup>cd</sup>	15.10±0.63 <sup>abDE</sup>	15.80±0.48 <sup>cdDE</sup>	16.36±0.59 <sup>abcd</sup>	16.69±0.80 <sup>abc</sup>
30th Day	14.01±0.11 <sup>bdE</sup>	14.69±0.56 <sup>abE</sup>	15.41±0.47 <sup>abDE</sup>	16.05±0.08 <sup>cd</sup>	15.88±0.26 <sup>cd</sup>
45th Day	14.71±0.61 <sup>bdE</sup>	16.27±0.58 <sup>abBC</sup>	17.46±0.71 <sup>abB</sup>	18.34±0.92 <sup>abA</sup>	18.01±1.17 <sup>abC</sup>
60th Day	12.86±0.04 <sup>de</sup>	14.53±0.14 <sup>de</sup>	13.95±0.03 <sup>ef</sup>	15.56±0.05 <sup>cd</sup>	14.07±0.05 <sup>bd</sup>
75th Day	16.88±0.55 <sup>abC</sup>	16.58±0.04 <sup>ab</sup>	16.81±0.01 <sup>abC</sup>	19.80±1.13 <sup>abA</sup>	18.72±0.59 <sup>abB</sup>
90th Day	19.12±1.04 <sup>ba</sup>	19.54±0.01 <sup>baA</sup>	20.02±0.45 <sup>baA</sup>	19.69±0.51 <sup>baA</sup>	21.38±0.84 <sup>baA</sup>

\* Values are presented as mean ±SE, n=2

<sup>a-b</sup> Values that contain different superscript lowercase letters in the same row are significantly different (P<0.05)

<sup>A-B</sup> Values that contain different superscript uppercase letters in the same column are significantly different (P<0.05)

The TOTOX of the fish oil was calculated as 14.48 at the beginning of the trial. Insignificant increases were observed from the first day to the 15<sup>th</sup> in all groups but there were not significant differences between the groups (P>0.05). On the 30<sup>th</sup> and 45<sup>th</sup> days, TOTOX continued to increase and except for the group D, all groups exhibited similarities (P>0.05). On the 60<sup>th</sup> day, based on the decreases in peroxide values, very clear changes in TOTOX caused significant differences among the groups (P<0.05). It was also verified by O'Sullivan et al., (2005) that the decrease of PV occurred due to the reduction of hydroperoxides. On the 75<sup>th</sup> day, the TOTOX increased in all the groups (P>0.05). At the end of the storage, on the 90<sup>th</sup> day the TOTOX in group D was considerably higher than the control group (P<0.05); however, it was found similarly in the other groups (P>0.05). For 90<sup>th</sup> day storage, the TOTOX was calculated below the limits of GOED (26) just like the PV and AV.

Similar to our findings, the results shown by Kasbo 2011 the added different commercial antioxidants stated that due to the unclear results, the most efficient antioxidants and concentrations were not obtained. However, O'Sullivan et al., (2005) carried out a study in which they added natural

antioxidants in cod (*Gadus morhua*) and white pollack (*Pollachius pollachius*) oils and they reported that the TOTOX in the groups supplemented with antioxidants was quite lower than the control group (P<0.05). According to their findings, they stated that the natural antioxidants added in the fish oil were quite successful in stabilizing. The results obtained from the different studies reveal that the effectiveness of the antioxidants depend on such factors as the types of fish oil, storage temperature, and synergic effect of the antioxidants (Baek, 2012; Kasbo, 2011; Wang et al., 2011; O'Sullivan et al., 2005).

### The effect on free fatty acids (FFA)

FFA formation is due to the hydrolysis of triglycerides; this process may be promoted by the reaction of oil with moisture (Iqbal and Bhanger, 2007). Lipid rancidity gives in increase in the number of effects such as hydrolysed and oxidation rancidity. In particular, polyunsaturated acids easily oxidized by air, producing peroxide which breaks down into aldehydes and ketones. The production of aldehydes and ketones causes unpleasant taste (Wang et al., 2011). Changes in free fatty acid value of anchovy oil during 90 days of storage are shown in Table 6.

**Table 6.** Changes in free fatty acid value of anchovy oil during 90 days of storage

Days	Groups				
	Control	A	B	C	D
Initial	1.53±0.02 <sup>cd</sup>	1.53±0.02 <sup>abC</sup>	1.53±0.02 <sup>abA</sup>	1.53±0.02 <sup>abE</sup>	1.53±0.02 <sup>abD</sup>
15th Day	1.48±0.02 <sup>bd</sup>	1.38±0.02 <sup>abC</sup>	0.95±0.18 <sup>bc</sup>	1.24±0.08 <sup>abF</sup>	1.23±0.03 <sup>abE</sup>
30th Day	1.53±0.09 <sup>bcd</sup>	1.40±0.18 <sup>cd</sup>	1.35±0.16 <sup>cd</sup>	2.03±0.02 <sup>abA</sup>	1.86±0.00 <sup>abA</sup>
45th Day	2.48±0.17 <sup>abA</sup>	1.95±0.05 <sup>abC</sup>	1.84±0.14 <sup>ba</sup>	18.2±0.02 <sup>abC</sup>	1.67±0.03 <sup>bc</sup>
60th Day	1.75±0.06 <sup>abC</sup>	1.70±0.03 <sup>ad</sup>	1.76±0.03 <sup>ba</sup>	1.72±0.00 <sup>cd</sup>	1.72±0.02 <sup>abC</sup>
75th Day	1.67±0.01 <sup>abCD</sup>	1.62±0.00 <sup>abABC</sup>	1.71±0.05 <sup>abA</sup>	1.67±0.05 <sup>ad</sup>	1.68±0.04 <sup>ac</sup>
90th Day	1.80±0.02 <sup>ab</sup>	1.95±0.02 <sup>abA</sup>	1.76±0.03 <sup>ba</sup>	1.93±0.00 <sup>abA</sup>	1.79±0.03 <sup>abAB</sup>

\* Values are presented as mean ±SE, n=2

<sup>a-b</sup> Values that contain different superscript lowercase letters in the same row are significantly different (P<0.05)

<sup>A-B</sup> Values that contain different superscript uppercase letters in the same column are significantly different (P<0.05)

FFA values decreased on the 15<sup>th</sup> day of fish oil storage study (ranging from 0.95 to 1.48 %) but on the 30<sup>th</sup> day FFA increased in all the groups (ranging from 1.35 to 2.03 %). The highest FFA contents (2.48 %) were observed in the control group (P<0.05) on the 45<sup>th</sup> day while C and D showed the lowest FFA contents (1.82% and 1.67% respectively) on the same day of the storage. Except for the control sample, the FFA levels of the other groups showed no difference in the 45<sup>th</sup> days. High FFA increases were not detected during the storage between the 60<sup>th</sup> – the 90<sup>th</sup> days. There were no statistically significant differences between the control and with antioxidant added groups. This is explained by the lack of

hydrolysis due to the low moisture content in stabilized oil (Aidos et al., 2002). Throughout the trial, the moisture level was between 0.15 and 0.37% in all the groups and did not reach the upper limit of 1% during the trial. Similarly, the FFA level was between 0.95 and 2.48%, but did not reach 5% higher in all groups. (Hertrampf and Piedad-Pascual, 2000). Similar to our results, Morales et al., (2015) stated that during the storage period, FFA content of oils stabilized with different concentrations of  $\alpha$ -tocopherol did not increase, but a slight decrease occurred. On the other hand, Wang et al., (2011) reported that different concentrations of carnosic acid (CA) and TBHQ provided much better FFA than the control group at two different temperatures (4-30 °C) in the 66-day storage study and the highest FFA was detected in the control group. Researchers reported that although oils have been supplemented with antioxidants, the oils should be stored at low temperatures and that low moisture content should be maintained during the storage.

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## CONCLUSION

The control group and the group A oxidized on the 75th day and PV exceeded 5 meq kg<sup>-1</sup> (<5 meq kg<sup>-1</sup> GOED limits). The groups B, C and D got oxidated in a shorter time on the 45<sup>th</sup> day. AV showed an increase at a small level throughout 90 days but it was below the GOED limits (<20) in all the groups. TOTOX was calculated under GOED (<26) limit during the storage throughout 90 days. FFA values increased slightly during the trial. There are many aspects connected to the oxidation of fish oil and one has to consider each of them when choosing an antioxidant. Factors such as EPA and DHA content of the fish oil, temperature of the setting, light, access to oxygen, and metal ion concentration are effective on the oxidation of oil. Considering the results, the antioxidants used in the experiment were not effective in anchovy oil. It is thought that the new studies should be carried out with these commercial antioxidants in different fish oil and temperature environments.