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RELATIONSHIP ANTIOXIDANT CONCENTRATION AND VOLUME HEADSPACE ON THE RANCIDITY OF FISH OIL DURING STORAGE

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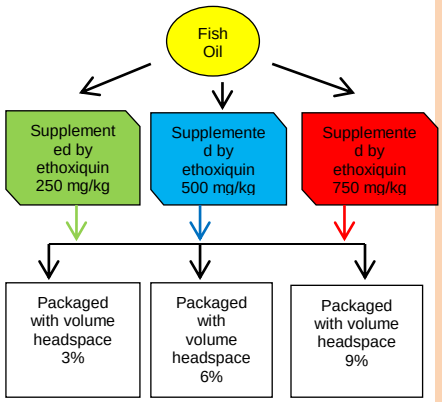
Graphical abstract



Abstract

Fish oil was packed in a dark glass bottle with different headspace volume (3 %, 6 % and 9 %) and stored for 8 wk at room temperature. Ethoxiquin as an antioxidant was added in different concentration, 250 mg kg^{-1}, 500 mg kg^{-1} and 750 mg kg^{-1}. Fish oil which was treated with ethoxiquin, 250 mg kg^{-1} and 500 mg kg^{-1}, became rancid in 5 wk and 6 wk, respectively. However, the lower the percentage of headspace volume, the more stable the fish oils against oxidation. The concentration of ethoxiquin, 750 mg kg^{-1}, prevented rancidity of fish oil that was packed in the bottle with volume of headspace 3 % and 6 %. The highest ethoxiquin concentration (750 mg kg^{-1}) which was combined with the lowest headspace volume (3 %) resulted in the most stable fish oil from rancidity during 8 wk of storage.

Keywords: Ethoxiquin, fish oil, rancidity, storage, volume headspace

1.0 INTRODUCTION

Fish oil is one of the best sources of dietary polyunsaturated fatty acids (PUFA), especially the n-3 PUFA family including eicosapentaenoic acid [EPA or (20:5 n-3)], docosapentaenoic acid (DPA or 22:5 n-3) and docosahexaenoic acid (DHA or 22:6 n-3). [1, 2]. However, due to its high degree of unsaturation, fish oil is simply spoiled in two major ways, oxidative spoilage and hydrolytic spoilage [3]. Hydrolytic and oxidative reactions of fish oil during processing, heat treatment and in the final products during subsequent storage, are among the basic processes causing the production of hydroperoxides [4], free fatty acids [5] and rancidity in food products [6]. Therefore, the addition of antioxidant is important for the preservation of these products during processing and storage.

Synthetic antioxidants have been widely used as it is effective. Ethoxyquin is also known as Santoquin or Santoflexinol. Pure ethoxyquin (EQ; 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; CAS number (1-53-2) is light yellow liquid, but it turns to brown if it is exposed to oxygen [7]. It also tends to polymerize on the exposure to light and air. EQ is also described as mercaptan like odor. As a nonpolar substance, EQ is soluble only in

organic solvents. Presently, ethoxyquin is used primarily as an antioxidant in canned pet food and in feed intended for farmed fish or poultry. However, because EQ is used as a feed antioxidant it can be also found in other products intended for human consumption like fish meal, fish oils, and other oils, fats, and meat.

An acceptable daily intake (ADI) of EQ for human is (0 to 0.005) mg kg^{-1} BW (Body Weight) [8, 9]. There are many rules on ethoxiquin (EQ) addition into feed. According to Council Directive 70/524/EEC replaced by Council Regulation (EC) No. 1831/2003, the maximum concentration of EQ allowed was 150 mg kg^{-1} feed. Furthermore, International Maritime Organization (IMO) suggested that fishmeal must be stabilized to prevent spontaneous combustion during overseas transport and storage by adding EQ at least 100 mg kg^{-1}. However, Joint FAO/WHO Expert Committee on Food Additives (JECFA), the former European Commission's Scientific Committee for Food (SCF) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) reduced the acceptable daily intake (ADI) of EQ from (0.06 to 0.005) mg kg^{-1} BW based on reproductive toxicity in a multi–generation study on dogs [8], although EQ may protect against cancer caused by chemicals [31–33]. Another guide was from the company of EQ (Novus International, Inc.) who suggested 750 ppm (equal to 750 mg kg^{-1}) EQ addition could prevent oxidation of fish oil [9]. Therefore, in this study we applied the addition of EQ, (250, 500, and 750) mg kg^{-1}.

The basic mechanisms of free radical induced lipid oxidation can be characterised by three distinctive steps: initiation, propagation and termination reactions. This phenomenon can be influenced by both intrinsic and extrinsic factors, such as fatty acid composition, concentration of pro-oxidants, endogenous ferrous iron, myoglobin, enzymes, pH, temperature, ionic strength and oxygen consumption [10]. In term of oxygen consumption, in this research, the samples were treated with different headspace of packaging. We argued that higher volume of headspace would provide more oxygen which would be highly consumed by fish oil samples. The absorption of light by naturally occurring or synthetic pigments is principally related in food products, directly exposed to the light. Certain food colorants have been studied in relation with oxidation of lipids [11, 12]. The most appropriate way to protect a fat rich product is to remove all kinds of light exposure [13]. Several studies have shown that the 400 nm to 500 nm regions are the most harmful part of the visible spectral region with regard to photo–oxidation [14, 15]. It was proven by numerous studies which showed that greater the light transparency of edible films, the greater was the photo-oxidation of the product packed in it [16, 17]. Therefore, in this work, the fish oil was packaged in the dark bottle and kept in the room with minimum light. The main purpose of this study was to investigate the stability of fish oil which was prepared with different antioxidant concentration and packaging headspace, then, kept for 8 wk. For evaluation of oil stability and monitoring of deterioration during storage, commonly used methods are peroxide value (PV), free fatty acid (FFA), and iodine value (IV). In term of product from hydrolytic rancidity, the investigation was also conducted for mositure content of fish oil.

2.0 EXPERIMENTAL

2.1 Materials

Fish oil and ethoxiquin were kindly provided by Feed Division Japfa Comfeed PT. Suri Tani Pemuka, Sidoarjo, Indonesia. All chemicals used in this study were analytical grade. Purified water was used for the preparation of all solution. All experiments and analysis were carried out in triplicate.

2.2 Preparation and Sampling of Samples

Fish oil used in this study provided characteristics: peroxide value (PV) 1.30 meq kg^{-1}, free fatty acid (FFA) 0.706 %, iodine value (IV) 165 meq kg^{-1}, moisture content 0.18 %, color and odor were normal. For this work, 30 L of fish oil was divided into 9 L of 250 mg kg^{-1} EQ treatment, 9 L of 500 mg kg^{-1} EQ treatment, 9 L of 750 mg kg^{-1} EQ treatment, and the rest 3 L as a control. The fish oil containing different concentrations of ethoxiquin (250 mg kg^{-1}, 500 mg kg^{-1}, and 750 mg kg^{-1}) were placed in the brown colored glass bottle with different headspace of packaging (3 %, 6 %, and 9 %). The 9 L of every EQ treatment was divided into 3 L of 3 %, 3 L of 6 %, and 3 L of 9 % of volume headspace of packaging. The percentage of the headspace volume based on Muchtadi [21] was 6 %. The headspace volume 3 % and 9 % were used as comparison to 6 %. All samples were packed in the closed brown paper box without sealed and was stored in a room with a controlled temperature of 5 °C for 8 wk. Therefore, the effect of light and temperature were minimized. Every week, the fish oil from each bottle was taken around 0.35 L for analysis of PV, FFA, IV, and moisture content. The analysis were performed in duplicate. Fish oil stability was determined by measuring the peroxide value (PV) iodine value (IV), free fatty acid (FFA), and moisture content which were conducted every week during 8 wk of storage.

2.3 Analysis of Peroxide Value (PV)

Peroxide value (PV) of all samples was measured according to the AOAC method with slight

modifications [18, 19]. Fish oil samples (2.00 g) were dissolved in 30 mL of acetic acid – chloroform solution (3:2, v/v). Then, 1 mL saturated solution of potassium iodide (KI) was added. The mixture was shaken by hand for 1 min and was kept in the dark for 5 min. After the addition of 75 mL distilled water, the mixture was titrated against 0.022 M sodium thiosulphate ($Na_2S_2O_3$) until the yellow color disappeared. Subsequently, 0.5 mL of starch indicator (1 %) was added where titration was continued until the blue color of the mixture disappeared. The blank was analyzed under similar conditions and PV samples were expressed as mEq kg^{-1}.

2.4 Analysis of Iodine Value (IV)

The iodine value (IV) in the oil samples was determined by the Wijs method, as described in the AOAC official method [18]. First, fish oil samples (0.2 g) were dissolved in 15 mL of cyclohexane – acetic acid solution (1:1, v/v). The mixture was added with 25 mL Wijs solution [Iodinechloride (ICl) in acetic acid] and was kept in the dark for 1 h. Then, the mixture was added with 20 mL 15 % KI solution and 150 mL distilled water. The mixture was gradually titrated against 0.1 M $Na_2S_2O_3$ solution with continuously vigorous shaking until the dark brown color disappeared. The blank was analyzed under similar conditions. The IV was expressed as g of iodine absorbed per 100 g sample (g I_2 100 g $sample^{-1}$).

2.5 Measurement of Free Fatty Acids (FFA)

Free fatty acids, as oleic acid percentages in oil samples, were determined using an alkali titration method according to the AOAC official method [18]. First, fish oil samples (7 g) were dissolved in 50 mL neutralized ethanol. Then, the mixture was titrated against sodium hydroxide (0.25 M) using phenolphthalein solution as an indicator while shaking the content mixture. The mixture was titrated until permanent faint pink color developed and persisted for more than 1 min. Percentage of free fatty acids was expressed as % FFA oleic acid volume mL of 0.25 M NaOH used.

2.6 Determination of Moisture Content

Moisture content of fish oil samples was determined by Ohause MB 45 at a temperature of 135 °C. Samples weight for this analysis was 5 g. The moisture content was expressed as percentage (%).

2.7 Data Analysis

Statistical analysis was performed using Microsoft excel. The data was analyzed by analysis of variance (ANOVA). Duncan's (Duncan Multiple Range Test) comparison test was used to determine the differences between the samples ($p < 0.05$).

3.0 RESULTS AND DISCUSSION

3.1 Peroxide Value

Hydroperoxides, known as primary oxidation products, were determined by peroxide value (PV) analysis. This increase in PV was attributed to the formation of lipid hydroperoxides [20]. Lipid hydroperoxides is the primary oxidation product produced as a result of lipid oxidation. It may break down into non-volatile and volatile secondary products, which will deteriorate the quality of the oil. This is an indicator of the initial stage of oxidative changes. The presence of hydroperoxide in the oil can be determined based on the oxidation of iodine ion with hydroperoxide [18].

According to Joint FAO/WHO Standards Programme Codex Committee on Fats and Oils 23rd Session 2013, the standard of Peroxide Value was ≤ 5 meq kg^{-1} [30], and the initial PV of fish oil which was used in this study was 1.30 meq kg^{-1}. Therefore fish oil in this study had a good quality due to its low PV. Based on our statistical analysis using analyze of variance ($p < 0.05$), it was clear that there were interaction between antioxidant concentration and storage period also headspace volume of packaging and period of storage. However, interaction between ethoxiquin concentration and headspace volume of packaging, during 8 wk of storage was not significant difference (data was not shown).

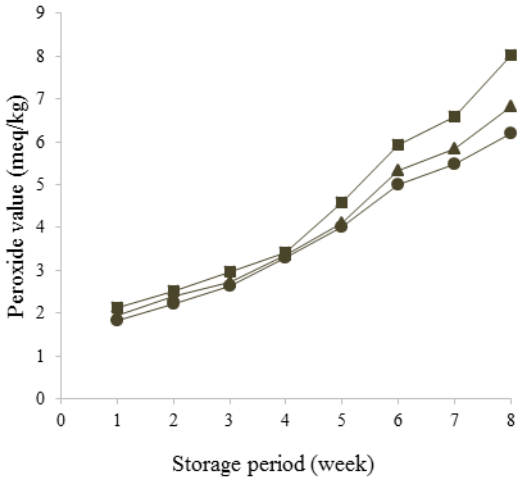


Figure 1 Peroxide value (PV) (meq kg^{-1}) of fish oil during 8 wk of storage. The fish oils supplemented with different concentrations of ethoxiquin (250 mg kg^{-1}) (■), 500 mg kg^{-1} (▲), and 750 mg kg^{-1} (•) and packaged with headspace volume

The graphic (Figure 1) shows that the lower antioxidant supplemented on the fish oil promoted higher rate on peroxide value. It was similar trend with the previous study of Horn *et al*. [22] which showed that higher concentration of caffeic acid, ascorbyl palmitate or gamma-tocopherol as an antioxidants in fish oil–enriched energy bars resulted in lower peroxide value. In addition, the fish oil–enriched energy bars added with 300 mg kg^{-1} caffeic acid, 300 mg kg^{-1} ascorbyl palmitate, and gamma-tocopherol 220 mg kg^{-1} provided 21 meq kg^{-1}, 19 meq kg^{-1} and 12 meq kg^{-1} peroxide value, respectively, after 8 wk of storage. To compare, in this study, the fish oil supplemented with 250 mg kg^{-1} EQ after 8 wk of storage had 7.957 meq kg^{-1} peroxide value. However, different fish oil used in the research provided different result of antioxidant supplementation. In this work (Figure 1), fish oil supplemented with 250 mg kg^{-1} and 500 mg kg^{-1} EQ had peroxide value out of the standard at the 6th wk yet fish oil was added with 750 mg kg^{-1} EQ, its PV was out of the limit at the 8th wk of storage.

Figure 2 indicates that fish oil which was packaged with higher headspace had higher peroxide value. Therefore, it is suggested that higher headspace in packaging provided higher oxygen exposure, resulting in higher oxidation rate which produced higher peroxide compound. Park *et al.* [10] stated that one of the factors which can influence lipid oxidation was oxygen consumption. In this study, fish oil packaged with 3 % headspace volume had peroxide value just over the limit (5.013 meq kg^{-1}) at the 5th wk, yet fish oil packaged with 6 % and 9 % headspace volume provided PV values which were over the limit at the 4th week (Figure 2).

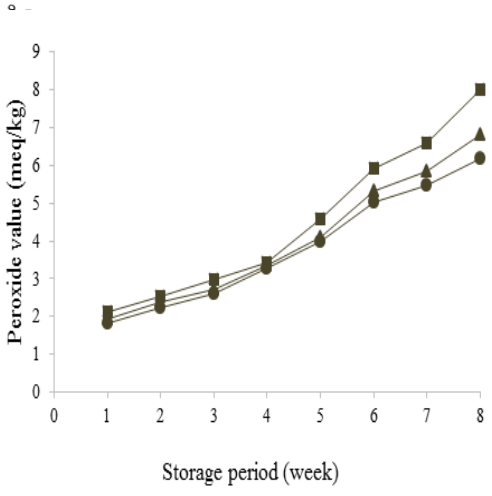


Figure 2 Peroxide value of fish oil during 8 wk of storage. The fish oils packaged with different head space volume 3 % (•), 6 % (▲), and 9 % (■) and supplemented with ethoxiquin as an antioxidant

3.2 Free Fatty Acid Content

Free fatty acid (FFA) value is used as an indicator of fat hydrolysis, specifically, the presence of free fatty acids. FFAs are formed due to the hydrolysis of triglycerides and can be promoted by moisture content [18]. According to guidelines from International Fishmeal and Oil Manufacturers Association (IFOMA), the range of FFA of crude fish oil was (1 to 7) % [28] and in this study, the initial FFA of fish oil samples were 0.706 %. However, because of its high degree of unsaturation, fish oil, like oil from animal and vegetable sources, was easily spoiled in two major ways, oxidative spoilage and hydrolytic spoilage [3].

Hydrolysis and oxidation impact on lipid breakdown, forming free fatty acids or aldehydes and ketones as the end–products. According to ANOVA analysis, there was no interaction between antioxidant concentration versus packaging volume head space and packaging volume headspace versus storage period. However, the interaction was happened between antioxidant concentration and storage period (data was not shown). Result of this work showed that lower antioxidant concentration caused higher percentage of FFA (Figure 3). Hydrolytic and oxidative reactions of fish oil in the final products during subsequent storage among the basic processes causes the production of hydroperoxides [4] and free fatty acids [5]. Therefore, trend of FFA changes was similar to PV.

The percentages of FFA in this work was higher than sardine mince [24], herring oil [25] and higher than that found in lipid sardine [5]. Generally, biochemical compounds (lipids, phospholipids, fatty acids.) vary within species and are also affected by diet, temperature, catching season and the fishing area [26, 25, 27]. Similarly, Bandarra *et al.* [26] have reported a higher FFA level (4.29 %) for lean sardine compared with fatty sardine (1.04 %) caught in Portuguese coast. According to data found, free fatty acid percentage was around 4 %. Such level is in agreement with Bimbo's results [28] who suggested that the FFA content normally range between 2 % and 5 % in crude fish oil.

Gradual increase of FFA of fish oil during storage in this study was similar with the result from Chaijan *et al.* (2006) who worked on sardine lipid during iced storage. It was stated that lipid hydrolysis possibly occurred to a great extent at the end of the period of storage [5]. Interestingly, supplementation of 250 mg kg^{-1} EQ resulted in lower FFA of fish oil than FFA of lipid sardine which treated with iced storage. During similar storage period, iced storage–lipid sardine had 7 % FFA after 15 d of storage [5], yet EQ supplemented-fish oils had FFA around 1.5 % after 2 wk of storage (Figure 3).

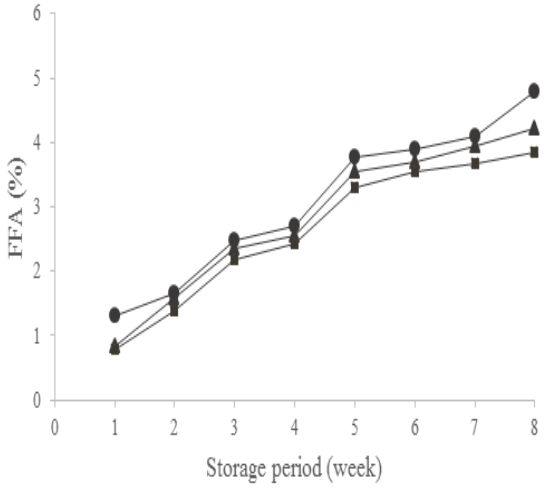


Figure 3 Free fatty acid (FFA) of fish oil samples during 8 wk of storage. Fish oils were supplemented with different concentrations of ethoxiquin: 750 mg kg^{-1} (■), 500 mg kg^{-1} (▲), and 250 mg kg^{-1} (•) and packaged with volume headspace

3.3 Iodine Value

According to ANOVA statistical test, the interactions between the factors were no significant difference. Individual factor which had significant impact was the headspace volume of packaging and the storage time (data was not shown). Therefore, in this discussion, Table 1 and Table 2 were performed in order to show the effect of both individual factors.

Based on Guidelines of Food Grade Fish Oil from International Fishmeal & Oil Manufacturers Association, iodine value (IV) of fish oil depended on fish species, ranging from 95 meq kg^{-1} to 200 meq kg^{-1} [28]. In this study, the initial IV of fish oil before storage was 165 meq kg^{-1}. From Table 1, the data showed that higher headspace volume caused higher iodine value. Headspace of packaging provided oxygen in the bottle and more oxygen consumption promoted more intensive lipid oxidation. Oxidation of PUFA of fish oil resulted in more saturated compound which was indicated by lower iodine value. These PUFAs are prone to lipid oxidation [19].

During storage, the double bonds of these PUFAs are attacked by free radicals [29]. The unsaturated fatty acids react with iodine monochloride and release free iodine. The free iodine can then react with sodium thiosulphate. The trend showed a decrease in iodine value (IV) under storage (Table 2). This decrease is indicative of the increase of rate of oxidation double bonds [18].

Table 1 Effect of headspace volume of packaging on iodine value of fish oil supplemented with antioxidant ethoxiquin

Headspace volume	Iodine value (meq · kg^{-1})
3 %	124.398[a]
6 %	118.072[b]
9 %	110.909[c]

Same letter on a column indicate no significant difference according to Duncan multiple range test ($p < 0.05$).

Tabel 2 Changes of iodine value of fish oils during 8 wk of storage. Fish oil was supplemented with ethoxiquin and packaged with headspace volume

Storage period (wk)	Iodine value (meq · kg^{-1})
1	149.727[a]
2	131.268[b]
3	121.839[c]
4	119.002[d]
5	113.970[d]
6	107.763[d]
7	102.391[d]
8	96.370[e]

Same letter on a column indicate no significant difference according to Duncan multiple range test ($p < 0.05$).

3.4 Water Content

Result of statistical analysis by ANOVA showed that there were no interactions between the factors. However, the individual factor which had significant impact was ethoxiquin concentration and storage time (data was not shown). Therefore, in this discussion, Table 3 and Table 4 was performed in order to show the effect of both individual factors.

Guidelines of fish oil quality by IFOMA (1998) stated that the range of moisture in fish oil was 0.5 % to 1 % [28]. In this study, the initial water content of fish oil before storage was 0.18 %.

Table 3 Water content of fish oils which were supplemented with different ethoxiquin concentration. The fish oil was packaged with volume headspace and storage for 8 wk

Ethoxiquin concentration (mg · kg^{-1})	Water content (%)
250	0.143[a]
500	0.131[b]
700	0.127[c]

Same letter on a column indicate no significant difference according to Duncan multiple range test ($p < 0.05$).

Table 3 showed that water content of samples decreased with the increase of ethoxiquin concentration. Hydrolysis and lipid oxidation were run at the same time. It was assumed that the more the oxidation reaction occurred, the more the lipid was

hydrolyzed. In this work, it is assumed that higher antioxidant concentration resulted in less lipid oxidation (which was shown in Figure 1) and promoted decreased water content (Table 3). This statement was in an agreement with Stapelfeldt [34] who reported that the rate of lipid oxidation depended on the water activity.

The result in this work (Table 3) did not agree with another work [35] which showed that higher water content was not followed by higher peroxide value. It is argued that the peroxide compound had been rapidly decomposed into secondary compounds, or might interact with other constituents during storage [35].

Tabel 4 Water content of fish oils treated with different headspace volume of packaging and ethoxiquin supplementation during 8 wk of storage

Storage period (wk)	Water content of samples (%)
1	0.100[a]
2	0.120[b]
3	0.138[c]
4	0.161[d]
5	0.162[e]
6	0.155[f]
7	0.128[g]
8	0.106[h]

Same letter on a column indicate no significant difference according to Duncan multiple range test ($p < 0.05$).

Table 4 showed that water content of fish oil samples during storage had two patterns. In the 1st wk until 5th wk, the water content increased from 0.100 % to 0.162 %, then in the rest 3 wk decreased to 0.106 %. It was suggested that in the wk 1 to wk 5, oxidation and hydrolysis reaction of lipid was intensively happened, therefore the moisture content increased in the first 5 wk. Then, in the last 3 wk, some lipid enzime such as lipase used water for the activity which caused the decrease in water content. Lipase activity resulted in higher free fatty acids and MUFA, which were shown by higher FFA (Figure 3) and lower iodine value (Table 3 and Table 4) of fish oil samples.

The result in Table 4 was not similar with Thomsen [36] who reported the increase in water activity during longer period of milk powder storage. Therefore, it is assumed that different product had different characteristic. In this work, the fish oil used contained higher lipid than milk powder. Moreover, other chemical compositions in milk powder, such as lactose and protein, probably influence its water activity during storage.

4.0 CONCLUSION

Oxidative stability of fish oil during storage was influenced by ethoxiquin supplementation and headspace volume of packaging. The higher ethoxiquin concentration which was combined with the lower volume of headspace provided better oxidative stability. The highest EQ concentration (750 mg kg^{-1}) which was combined with the lowest headspace (3 %) result in the most stable fish oil from lipid oxidation and hydrolysis during 8 wk of storage.

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