

## The Effect of Anchovy Fish Supplementation on the Level of N-3 LC-PUFA in Egg Yolk

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### ABSTRACT

Since the recommended daily intake of n-3 LC-PUFA is rarely met, interest in food enrichment has been increasing. It is known that dietary supplementation could alter the level and type of PUFA in the egg. Hence, the present study focused on the enrichment of egg yolk by the addition of 10% anchovy fish to the chicken diet. Based on gas chromatography analysis, dried and pre-dried anchovy from Indonesia contained a considerable amount of total eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which was 60.10 g and 68.80 mg/100 g, respectively. After 24 days of anchovy supplementation, DHA-rich anchovy fish oil diet caused a significant increase of DHA but not EPA in egg yolk. Hens fed with anchovy could produce eggs with a higher amount of total EPA and DHA, which was up to 155.98-201.53%, as compared to control eggs. Furthermore, the sensory profile of control and enriched eggs was also evaluated. There was no significant difference in texture, aroma, flavor, and appearance between control and enriched eggs. In conclusion, this study indicated that anchovy fish supplementation could increase the level of EPA and DHA in egg yolk without causing any sensory changes in the yolk.

**Keywords:** Anchovy, DHA, Egg yolk, Enrichment, EPA

### INTRODUCTION

Recent nutritional trends show that interest in enriched food products with omega-3 polyunsaturated fatty acids (n-3 PUFA) has been increasing. Due to their biological effects, long chain (LC) n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have gained more attention than the shorter chain n-3 PUFA  $\alpha$ -linolenic acid (ALA). Adequate consumption of EPA and DHA has been proven to provide various health benefits, including the promotion of visual and neural development in fetus and young children, the reduction of cardiovascular disease and the attenuation of inflammation and some cancers (Lemahieu et al., 2015). Although proven to be beneficial for health, the amount of EPA and DHA obtained from the conversion of ALA in human body is not efficient. Hence, human needs to obtain n-3 LC-PUFA through their diet (Komprada, 2012).

Fatty fishes and chicken eggs are known to be rich in n-3 LC-PUFA. Atlantic salmon, which is known as gold

standard for omega-3 fatty acid, contains 0.321% EPA and 1.115% DHA (FDC ID: 173686, USDA). Meanwhile, European raw anchovy contains 0.538% EPA and 0.911% DHA (FDC ID: 174182, USDA) (USDA, 2020). Even with the low-fat content, white fish such as milkfish was reported to have 0.36% EPA and 1.17% DHA (Sugata et al., 2019). Although fishes are rich in n-3 LC-PUFA, they are not consumed as much as eggs. Eggs are usually consumed as food or used as food ingredient. Thus, as compared to the fishes, eggs enriched with n-3 LC-PUFA could be one of the best options to fulfil the recommended daily intake of n-3 LC-PUFA.

Each chicken egg commonly contains 1.5 mg of EPA and 47.5 – 51.0 mg of DHA (Kovalcuks, 2014). Based on Food Data Central (USDA), every 100 g of egg yolk contains 11 mg EPA and 114 mg DHA (FDC ID: 783929). However, various feeding strategies have been applied to increase the level of PUFA in eggs. Egg yolk composition, such as EPA and DHA content, is closely

related to the lipids consumed by the laying hens. Enrichment of eggs with different sources of n-3 PUFA such as DHA-rich microalgae (Ao *et al.*, 2015), ALA-rich flaxseed (Ehr *et al.*, 2017), and DHA-rich fish oil (Lawlor *et al.*, 2010) has been widely investigated. Previous study had been reported that ALA in plants, such as flaxseed, was effective to increase linolenic acid (LNA, 18:3 n-3) in eggs, but not EPA and DHA (Ehr *et al.*, 2017). Meanwhile, fish oil could enrich both EPA and DHA levels in eggs (Ao *et al.*, 2015). However, Bruneel *et al.* (2013) found that enrichment of eggs with n-3 PUFA could increase the level of DHA, but it was not effective on EPA level.

The most of n-3 PUFA sources used in previous studies had relatively expensive prices and were difficult to obtain in Indonesia, hence, the use of those ingredients for this study is not feasible. Indonesia is an agricultural country with high amount of fish production. In 2017, the production of anchovy fish, which is known as “ikan teri”, in Indonesia reached about 3% of the world production of anchovy (MMAFRI, 2018). Moreover, to the best of authors’ knowledge, enrichment of egg yolk with anchovy fish as n-3 PUFA source has not been widely studied. Therefore, this research aimed to investigate the effect of anchovy fish supplementation on the level of n-3 LC-PUFA in egg yolk.

## MATERIALS AND METHODS

### Source of n-3 Polyunsaturated Fatty Acids

Indonesian anchovy used in this study was *Stolephorus* sp. (based on certification from Indonesian Institute of Sciences). There were two kinds of anchovy obtained from the market: dried and fresh. Dried anchovy was fresh anchovy that had been dried before reached the market, hereinafter referred to as dried anchovy. Meanwhile, fresh anchovy was raw anchovy that was available in the market without going through the drying process. Prior to experiment, fresh anchovy was sun-dried, hereinafter referred to as pre-dried anchovy.

### Lipid extraction from anchovy fish

To analyze n-3 PUFA levels, the lipid fraction of anchovy was extracted according to Bligh and Dyer (1959). One hundred gram of dried anchovy was homogenized with chloroform/methanol/water (2:2:1.8, v/v/v) as solvents. The mixture was then filtrated and centrifuged at 1000 rpm for 10 min. The lipid fraction was collected from chloroform layer at the bottom and then

filtered. Afterwards, the solvents were evaporated at 50°C with agitation at 120 rpm. The lipid fraction must be converted to methyl esters (FAME) prior to analysis using gas chromatography (Harynuk *et al.*, 2006). Fifteen milligrams of fish oil were methylated with one ml of 14% BF<sub>3</sub>-MeOH in boiling water for 7 min. After cooling, hexane (1 ml) and water were added to the mixture, followed by vigorous shaking. Phase layers would be formed, and FAMES can be found in hexane layer on the top.

### Animals' diets and egg collection

Prior to experiment, nine laying hens (28 weeks of age, Hisex Brown) from Rizky Farm (Bogor, Indonesia) were fed with commercial chicken feed. The laying hens were placed individually in cage without any environmental control and divided into three groups of three hens. The control group continued to receive 120 g commercial feed per day, while the treated groups received the commercial feed supplemented with anchovy fish (10% w/w). In other word, treated group received a mixture of 12 g anchovy fish and 108 g commercial feed every day (total feed: 120 g). The group of hens fed with dried anchovy hereinafter referred to as treatment group A, while another group fed with pre-dried anchovy hereinafter referred to as treatment group B. Feed and water were given ad libitum. For each group, laid eggs were collected at either day 20-21 (almost the end of supplementation period) and at either day 23-24 (end of supplementation period). All collected eggs were stored at 4°C until analysis.

### Lipid extraction from chicken egg yolk

The extraction of lipid fraction from egg yolk was carried out according to Kovalcuks (2014). One part of egg yolk was added with two part of the mixture of hexane and isopropanol (70:30). The mixture was homogenized using a magnetic stirrer for 30 minutes at room temperature and then filtered using Whatmann filter paper number 1. The solvent was evaporated using a rotary evaporator at 80°C and a speed of 30 rpm. The lipid fraction was then methylated prior to analysis using gas chromatography. A modified method by Morrison and Smith (1964) was used to form methyl esters fatty acid (FAME) from egg yolk lipid. Twenty-five microliters of lipid were methylated with 2 ml of BF<sub>3</sub>-MeOH 14% at 60°C for 10 minutes. Water (1 ml) was added to the solution, and then FAMES were extracted with hexane (1 ml). FAMES were then dried over sulfate anhydrous.

### **N-3 Polyunsaturated Fatty Acids in anchovy and egg yolk**

FAMEs were analyzed by gas chromatography (Agilent 7890A Series GC System) equipped with a 5975C Mass Selective Detector. The separation was performed on a fused-silica capillary column (0.25  $\mu$ m; 30 m x 0.25 mm i.d.; DB-wax; Agilent Technologies, USA). The flow rate of the helium carrier gas was 2,69 ml/ min. The injection was operated in pulsed splitless mode at a temperature of 260°C, while the detector was set at 280°C. For analysis, one microliter of FAME was automatically injected (Agilent 7693, Agilent Technologies, USA). The column oven was set to maintain a temperature of 50°C for 1 min, then risen 25°C/ min to 200°C, and 2°C/ min to 250°C, followed by a plateau of 250°C for 20 min. The mass spectrometer was operated in selected ion monitoring (SIM) mode.

### **Preparation of Sensory evaluation**

Thirty volunteers were involved in consumer acceptance test to assess the difference between control and enriched eggs after hard-boiled. Eggs were placed in boiling water for 10 min. After cooled, shells were removed, and each egg cut into quarters. No salt was used. There were four categories for the assessment, including appearance, texture, flavor, and aroma, which scored based on hedonic scale (1-extremely disliked; 6- extremely liked). Appearance was specified to the color of egg yolk, assuming more lipid content would give thicker yolk color, which was considered as more attractive. Texture was defined as the overall measure of oral sensations associated with placing food in the mouth. Flavor was emphasized at the savory degree of the egg, assuming higher oil content would give tastier flavor. Aroma was referred to the presence of “fishy” odor, meaning less preference in aroma indicates more “fishy” odor from the sample.

### **Sensory evaluation methodology**

Each panelist was given two hard-boiled eggs, the first one was the control egg and the other one was the enriched egg. Both control and enriched egg were blind-coded. Each panelist was asked to evaluate these blind-coded eggs by giving a hedonic scale. Assessment was calculated by the following:  $\Sigma$  (number of responses  $\times$  hedonic scale) / total panelist, with total number of all responses = total panelist.

### **Statistical analysis**

The results were statistically analyzed using Minitab 15 (Minitab Inc., USA) software. All data were stated as the mean  $\pm$  Standard Deviation (SD) with statistical significance at  $p < 0.05$ .

## **RESULTS AND DISCUSSION**

The productive age of laying hens ranges from 28 to 97 weeks and the weight of egg yolk produced could increase with the age of hens. In general, the weight of egg yolk increases proportionally to its total lipid content (Ahn et al., 1997). However, Lawlor et al. (2010) reported that the addition of microencapsulated fish oil (20 g/kg) in chicken diet could decrease the weight of egg yolk by 5.29%. Since the consumption of n-3 LC-PUFA might decrease serum triglycerides in the hens, less lipids are available for yolk formation (Fraeye et al., 2012).

Dried anchovy contained approximately 3.33–3.59% lipid, meaning there was 0.40–0.43 g lipid in 12 g anchovy (10% w/w supplementation). Thus, the percentage of additional lipid for treatment groups was approximately 0.33–0.36% (in 120 g chicken feed). According to Khosravinia et al. (2014), adding too much fish oils to chicken feed could increase the concentration of urea and uric acid in chickens. Through the addition of 2% fish oil, Lawlor et al. (2010) reported that DHA content in chicken eggs could be increased by 54.48%. Meanwhile, Lemahieu et al. (2015) showed that supplementation of 0.68 % fish oil resulted in enrichment efficiency of n-3 LC-PUFA of more than 55%.

The results of this study showed that anchovy contained more DHA than EPA. According to Codabaccus et al. (2012), fatty fishes tend to use dietary EPA for  $\beta$ -oxidation, but not DHA. This might explain why EPA was not present in excess, while DHA can be found at a relatively high level in anchovy. The level of total EPA and DHA in dried and pre-dried anchovy was 60.10 and 68.80 mg/100 g, respectively. Dried anchovy was already in dried form when it was on the market; hence, the fish might have been stored for some time. On the other hand, pre-dried anchovy was still fresh when it was on the market and was dried immediately without long storage period. Crypian et al. (2017) reported that the level of PUFAs could decrease during storage because PUFAs are highly susceptible to oxidation. This might also be the case with dried anchovy.

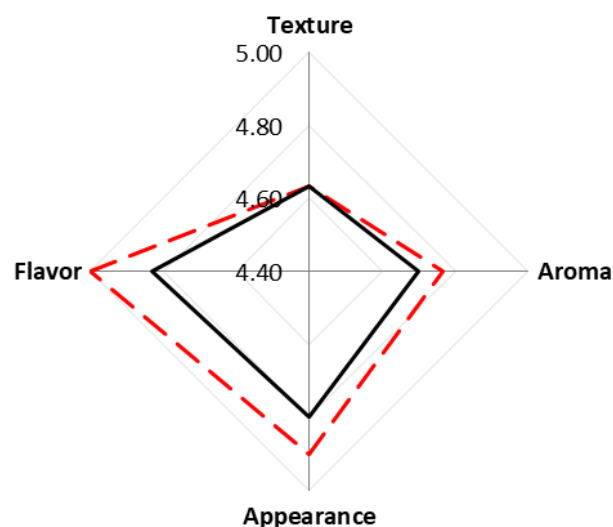
After anchovy supplementation, n-3 LC-PUFA in egg yolks was examined using GC-MS. Table 1 shows that daily enrichment with 0.33–0.36% anchovy fish oil increased the level of EPA and DHA in egg yolk, but only DHA content increased significantly ( $p < 0.05$ ). In the end of supplementation period, the addition of dried and pre-dried anchovy increased EPA and DHA in egg yolk by 155.98% and 201.53%, respectively. Since total EPA and DHA content in pre-dried anchovy was higher than dried anchovy, it was expected that treatment group B produced

eggs with higher increase of total EPA and DHA as compared to treatment group A. Other studies reported that the addition of 2% fish oil in chicken diet could increase the DHA content in eggs by 64% (Lawlor et al., 2010) or up to 300% (Gonzalez-Esquerria and Leeson, 2001). In accordance with Lawlor et al. (2010), EPA-rich and DHA-rich fish oil diet caused significant increase ( $p < 0.05$ ) of DHA, but not EPA, in egg yolk. Since most EPA is converted to DHA before it is deposited, the major n-3 LC-PUFA in eggs is DHA (Cachaldora et al., 2008).

**Table 1.** The increase of EPA and DHA in the chicken eggs at the start (day 1) and at the end (day 23-24) of supplementation period

Day	Description	Yolk weight (g/egg)	Total lipid (g/egg)	Increase of DHA content per egg (%)	Increase of EPA content per egg (%)	Increase of total EPA+DHA (%)
0	Control	14.05 ± 0.59 <sup>a</sup>	2.15 ± 0.43	-	-	-
20-21	Treatment group A	15.63 ± 0.87 <sup>b</sup>	2.57 ± 0.39	70.08 ± 5.28 <sup>a</sup>	6.31 ± 3.30 <sup>a</sup>	65.89 ± 5.15 <sup>a</sup>
	Treatment group B	15.43 ± 1.00 <sup>a</sup>	2.60 ± 0.50	162.31 ± 8.05 <sup>b</sup>	65.92 ± 7.09 <sup>b</sup>	155.98 ± 7.85 <sup>b</sup>
23-24	Treatment group A	14.79 ± 0.19 <sup>a</sup>	2.18 ± 0.11	201.76 ± 7.71 <sup>c</sup>	113.29 ± 22.53 <sup>c</sup>	195.90 ± 7.56 <sup>c</sup>
	Treatment group B	14.47 ± 0.32 <sup>a</sup>	2.14 ± 0.52	213.27 ± 6.90 <sup>c</sup>	34.55 ± 5.63 <sup>d</sup>	201.53 ± 6.64 <sup>c</sup>

Note: The table shows the data from eggs produced by hens fed with commercial feed (control), commercial feed supplemented with 10% dried anchovy (treatment group A), and commercial feed supplemented with 10% pre-dried anchovy (treatment group B). Day indicates the period of anchovy supplementation. Values with the different letter in the same column are significantly different ( $p < 0.05$ ).



	Control	Treatment
Texture	4.63	4.63
Aroma	4.70	4.77
Appearance	4.80	4.90
Flavor	4.83	5.00

**Figure 1.** The overall assessment of control (—) and enriched eggs (---)

Although enrichment period showed a good correlation with the increase of n-3 LC-PUFA in egg yolk, in this study, the supplementation was limited for 24 days. Further studies need to be done to investigate the effect of longer enrichment period on the increase of EPA and DHA content in egg yolk, or even in chicken meat. Since about 60% of egg production costs comes from chicken feed, the addition of 10% anchovy will increase the feed cost. However, the price of omega-3 enriched eggs on the market is also double as compared to bulk eggs. Despite the more expensive price, modern society has high interest in food enrichment with n-3 PUFA due to its health benefits. Thus, the use of anchovy is expected to give little or no burden on chicken farmers.

N-3 LC-PUFA can be oxidized easily and give undesirable off-flavors in food products. Since fish oil supplementation could increase the level of lipid in egg yolk, higher extent of lipid oxidation might occur (Fraeye et al., 2012). The amount of lipid in the eggs is strongly correlated with the “fishy” taste and odor, hence, the amount of fish used for feeding strategies needs to be confined. Gonzalez-Esquerria and Leeson (2001) found that western consumers could not accept eggs produced

from inclusion of more than 1.5% of fish oil. In this study, to analyze the acceptance of panelist to the eggs enriched with 0.33–0.36% anchovy fish oil, sensory evaluation was done based on organoleptic profile of control and enriched eggs (Figure 1). Overall assessment on texture, aroma, flavor and appearance of both control and enriched eggs indicated that even though the panelist might not be able to distinguish between control and enriched eggs ( $p \geq 0.05$ ), the enriched eggs were more preferred.

## CONCLUSION

This study showed that the level of n-3 polyunsaturated fatty acids in egg yolk could be increased by the supplementation of anchovy fish in chicken diet. The supplementation of docosahexaenoic acid (DHA)-rich anchovy fish for 24 days in chicken diet caused significant increase of docosahexaenoic acid, but it was not effective on eicosapentaenoic acid in egg yolk. Furthermore, the evaluation of sensory profile of control and enriched eggs showed that there was no significant difference on texture, aroma, flavor and appearance between control and enriched eggs.

## DECLARATIONS

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### Competing interests

The authors have declared that no competing interest exists.

### Authors' contributions

TTJ formulated the concepts. AA, AD, YT and SCD collected the data and drafted the manuscript. DR<sup>1</sup> supported data collection. MS, HV and DR<sup>2</sup> analyzed the data and prepared the figures, tables. MS finalized the manuscript.

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