The effectiveness of the use of antioxidant formulations in the storage of fat from the Pacific sardines Sardinops melanostictus

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1. INTRODUCTION
Fish fat is important as it contains essential fatty acids and fat-soluble vitamins [1–4]. Polyunsaturated fatty acids (PUFAs) of the omega-3 family have biological activity and play a positive role in human metabolism, reducing the risk of developing noncommunicable diseases [5–7]. The fat obtained from the Pacific sardine Sardinops melanostictus has a high content of PUFAs [8]. The main composition of the PUFAs is omega 3 fatty acid (72.3–73.6%), among which docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) predominate.

It is known that during storage, under the influence of various factors (air, oxygen, temperature, light, and humidity), oxidative processes occur in highly unsaturated fish oil, which is carried out according to a free-radical mechanism [9,10]. As a result of the action of oxygen on fatty acids in the composition of triacylglycerols and free fatty acids (FFAs), hydroperoxides are formed, which in turn break down into aldehyde and free radicals. At the same time, hydrolytic reactions occur in fish oil, which leads to the breakdown of triacylglycerols into glycerol, accumulating FFAs and hydrolysis intermediates (mono- and diacylglycerols). The amount of FFA increases in oxidation and hydrolytic decomposition, peroxides and aldehydes toxic to the human body accumulate, and fat-soluble vitamins are destroyed, decreasing the amount of PUFAs. These changes lead to a decrease in fish oil’s organoleptic properties and nutritional value, a deterioration in quality indicators, and a limitation of its shelf life and use [11,12]. Oxidized fish oil, when used in nutrition, poses a risk to human health in the form of oxidative stress or oxidative damage [13].

To slow down the processes of oxidation and hydrolytic cleavage of fish oils and increase their stability and shelf life, antioxidants are used that can interrupt the reactions of free radical oxidation and reduce the accumulation of lipid peroxidation products. The most effective are synthetic antioxidants such as butylatedhydroxyanisole, tert-butyl hydroquinone, and butylated hydroxytoluene [14,15]. Despite the relatively high efficiency of lipid stabilization, synthetic antioxidants can harm the human body.

Different mechanisms of action characterize antioxidant drugs; hence, using their combinations in fish oil is more effective than using one
type of antioxidant [16,17]. Therefore, to increase the stability of fish oil with a high content of PUFAs during storage, it is relevant to develop synergistic compositions of antioxidants of both natural and artificial origin.

This study aims to evaluate the effects of antioxidants and their synergistic mixtures on the oxidative and hydrolytic processes of Pacific sardines’ fat Sardinops melanostictus during storage.

2. MATERIALS AND METHODS

2.1. Examined Material

The object of the research was the fat of the Far Eastern sardines S. melanostictus obtained from a chilled crushed fish. The fat production technology from the fish included heat treatment at 90°C for 30 min, centrifugation, filtration, and drying [18].

Various substances used in the technology of food and feed production were used as antioxidant preparations: α-tocopherol acetate and food additive – “betulin,” 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyvinyl), and salicylic acid amide. The selected antioxidant compounds were injected into the resulting fat from the sardine according to the manufacturer’s recommendations in the following quantities per weight of fat: ethoxyvinyl 0.14%, α-tocopherol acetate 2.0%, betulin 0.3%, and salicylic acid 0.0015%. The synergistic mixtures included antioxidants in the following ratios: ethoxyvinyl:α-tocopherol acetate (50:50), ethoxyvinyl:betulin (50:50), ethoxyvinyl:betulin:salicylic acid amide (69.9:30:0.1), and ethoxyvinyl:α-tocopherol acetate:salicylic acid amide (69.9:30:0.1).

2.2. Determine the Effects of Antioxidants on Lipid Stability

The effects of antioxidants on fat stability were determined using the accelerated method. Experimental fat samples (with antioxidants) were stored in glass containers in a thermostat for 45 days at 45°C. For comparison, a control variant, antioxidant-free fat, was stored and investigated at the same time [19].

2.3. Assessment of the Physicochemical Parameters for Evaluating the Quality of Fish Oil

The evaluation of physicochemical parameters to ascertain the quality of fish oil adhered to the prescribed techniques of the American Oil Chemists’ Society (AOCS) [20]. The fat density (g/L) was measured using a glass pycnometer. The refractive index was measured using the refractometric method at 20°C. The moisture and volatile chemical content were measured by vacuum drying using the AOCS Ca 2d-25 standard.

The peroxide content and primary oxidation levels were assessed using AOAC Official Method 965.33 [21], and the results were documented based on peroxide values (PVs). The degree of hydrolytic alterations in the oil was assessed using the AOCS Official Method Cd 3d-63 [22] and reported according to acid values (AVs). The oil oxidation degree was calculated using the AOCS Official Method Cd 18-90 [23], which measures the presence of secondary oxidation products, specifically aldehydes, expressed as para-anisidine values (p-AVs). The overall rate of oil oxidation was determined by calculating the TOTOX values, which include measurements of PV and p-AV. The relationship between p-AV and PV was used to make these calculations.

2.4. Determination of the Composition of Fatty Acids

The oil fatty acid composition was analyzed using the AOCS Official Method Ce 1h-05 with modifications [24], and the fatty acid methyl esters were prepared following the AOCS Official Method Ce 2-66 [25]. Gas chromatography analysis was done using a Hewlett-Packard model 5890 II gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with a flame ionization detector and a Supelcowax 10 column (30 m × 0.25 mm × 0.25 mm). The temperature of the injector was held constant at 240°C while running in split mode at a ratio of 1:25. The hydrogen flow rate was adjusted to 1 mL/min while maintaining an oven temperature of 220°C. The temperature of the detector was adjusted to 240°C. The peaks were identified by comparing their retention durations with those of a FAME reference standard.

The concentration of FFAs was measured by acidometric titration using a 0.1 M solution of NaOH, following the AOCS Official Method Ca 5a-40. The results were quantified as a percentage of oleic acid.

2.5. Determination of Vitamin Content

High-performance liquid chromatography (HPLC) analysis of vitamin A was performed according to the method described by Nadeeshani et al. [26]. Vitamin A was analyzed in triplicate by injecting 20 µL of samples and standards to HPLC (Thermo Scientific™ Dionex UltiMate™ 3000 standard system, UK). At the same time, a C18 column (4.6 × 250 mm, 5 µm) (Agilent ZORBAX Eclipse Plus™, USA, 5 µm, 4.6 × 250 mm) was used with a linear gradient of methanol at a constant flow rate of 1 mL/min and UV detection at 325 nm. All procedures were carried out under subdued light conditions.

Vitamin D3 content was analyzed using a published stability-indicating method and an HPLC Agilent 1100/1200 Series instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector and ChemStation data acquisition system. In brief, a reversed-phase column Gemini C18 100 × 3.0 mm, 3 µm particle size column (Phenomenex, Torrance, CA, USA) at 40°C was used with a mobile phase of acetonitrile–water (99:1, v/v) at a flow rate of 1 mL/min. The detection was carried out at 265 nm [27].

2.6. Statistics

All data are presented as mean ± standard deviation for parametric variables or the median (interquartile range) for nonparametric variables and as number (%) for categorical variables. P-values were derived from a paired Student’s t-test. The results were analyzed using the computer programs “Microsoft Excel” 2014 and Statistica 7.0.

3. RESULTS

This study utilized five samples of fat from the sardine S. melanostictus, harvested on various production dates.

3.1. Characteristics of the Initial Indicators Regarding Fat Quality From Sardine S. melanostictus

The fat obtained from S. melanostictus sardine for this study displayed a translucent, pale yellow hue without any noticeable foreign scent or flavor. Table 1 shows the physicochemical characteristics of the fat from the sardine species S. melanostictus. The parameters consist of the refractive index at 20°C and density, which is determined by comparing the mass of fat at 20°C to the mass of the same volume of water at 4°C. The values were similar to those of vegetable oils. Furthermore, the PV, AV, ranitidine value (p-AV), and moisture and volatile content were determined to be at a low level, suggesting excellent fat quality and the lack of secondary oxidation products.
To assess the influence of antioxidants on fat samples’ long-term stability, lipid oxidation, and hydrolysis markers were measured at 5-day intervals.

PV is a key measure of fat oxidation, indicating the speed at which peroxides and hydroperoxides are formed. Then, peroxides decompose into aldehyde and ketone substances, measured by the anisidine number (p-AV), which indicates the presence of secondary oxidation products that cause rancidity. Figure 1 illustrates the variations in PV in sardine fat samples of *S. melanostictus* when antioxidants are present.

The control group, which did not have antioxidants, showed a faster oxidation process than the experimental groups, as indicated by the rapid rise in PV. Following 45 days, the control group’s peroxidizability value (PV) increased to 25.3 mmol O₂/kg of fat, indicating significant oxidative alterations. Including antioxidant compounds significantly reduced the increase in PV. The antioxidant formulations consisting of ethoxyvine + α-tocopherol acetate + salicylic acid amide and ethoxyvine + betulin + salicylic acid amide showed the highest effectiveness in preventing the accumulation of peroxide and hydroperoxide. After 45 days, the PV values of these formulations were 2.14–2.21 times lower than the control, indicating a significant slowdown in oxidation.

The statistical analysis produced regression equations, as shown in Table 3, that accurately describe the lipid oxidation of *S. melanostictus* sardine fat. These equations were validated using approximation coefficients that approach 1, confirming the precision of the equations.

Similarly, the path metric measures the buildup of secondary oxidation products, which closely reflects the trends observed in PV. The fat samples enriched with antioxidants showed decreased p-AV values compared to the control, especially in three-component antioxidant formulations, indicating effective protection against oxidation.

The AV measures the extent of fat hydrolysis during storage. The control sample’s antioxidant activity (AV) increased by a factor of 2.12 after 15 days and 4.51 after 45 days. In contrast, the samples stabilized with antioxidants exhibited less significant increases in AV. The antioxidant combinations consisting of ethoxyvine + α-tocopherol acetate + salicylic acid amide and ethoxyvine + betulin + salicylic acid amide showed the lowest AV values, which suggests that they are highly effective in inhibiting hydrolysis.

The regression equations for AV changes closely mirrored the trends observed in p-AV during statistical analysis, strengthening the lipid degradation evaluation’s reliability.

### 3.3. Changes in Quality Parameters of Sardine *S. melanostictus* Fat Throughout Storage

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3.4. Modifications in Fatty Acid Composition During Fat Storage in the Presence of Antioxidants

Table 4 displays the data on the fatty acid composition of fat samples obtained from *S. melanostictus* sardines after 45 days of experimental storage.

Throughout the trial duration, the control sample, which did not receive any antioxidant treatment, showed noteworthy elevations in FFAs (15.8-fold rise) and saturated fatty acids (specifically myristic acid 2-fold increase, and palmitic acid 17% increase) compared to the...
original fat sample. The control fat sample had a significant decline in the levels of PUFAs, including a 3.4-fold reduction in EPA (22:6 n-3) and an approximately 5-fold decrease in DHA (20:5 n-3). This indicates that there was active degradation of PUFAs.

During storage, the presence of antioxidants in fat samples slowed down the degradation of PUFAs. When α-tocopherol acetate, betulin, and ethoxyvine were present separately, the levels of EPA and DHA fell by 54%, 50%, and 35–38%, respectively. Nevertheless, using a blend of...
antioxidant compounds led to a reduction in the level of PUFA compared to those using individual antioxidants. For example, when a fat sample containing a mixture of ethoxyvin, betulin, and salicylic acid amide was tested, it showed a drop in EPA and DHA content of approximately 27–33%. However, when the fat sample had a mixture of ethoxyvin, α-tocopherol acetate, and salicylic acid amide, the decrease was 30%.

3.5. Variations in the Levels of Fat-Soluble Vitamins in Fat Samples Throughout Storage

The data in Table 5 demonstrate the changes in the concentrations of vitamins A and D3 in fat samples during the storage experiment. It was noted that the control fat sample saw a 40% decline in the initial vitamin A concentration and an 88% fall in vitamin D3. The presence of individual antioxidants led to a further decrease in the vitamin levels in fish oil samples. Significantly, including ethoxyvin in the fat led to the least vitamin loss. In addition, antioxidant combinations consisting of three components exhibited the greatest preservation of vitamins A and D3 in fish oil samples during the storage period.

4. DISCUSSION

This study investigates the effects of natural antioxidants (α-tocopherol acetate and betulin) and synthetic antioxidants (ethoxyvin and salicylic acid amide) on the breakdown of lipids in fish oil during storage. The antioxidant capacity was assessed by subjecting fat to accelerated aging by utilizing individual antioxidants and their mixtures. This work used sardine S. melanostictus fat, renowned for its elevated levels of omega-3 PUFA [8].

During the experimental storage, there was a noticeable breakdown of lipids in fish oil without antioxidants (control sample) due to oxidation. This was demonstrated by the large increases in PV, AV, and p-AV, as well as a decrease in PUFA levels. The decline in quality is ascribed to the active processes of oxidation and hydrolysis [9-12], with higher temperatures speeding up the peroxidation of lipids [29].

The inhibitory effect of synthetic ethoxyvin on fish oil oxidation was greater than that of individual natural antioxidants (α-tocopherol acetate and betulin). This is consistent with previous studies that have shown that synthetic antioxidants have a greater ability to prevent oxidation [30-32]. Tocopherols operate as antioxidants by inhibiting the formation of lipid radicals and slowing down the chain events of autooxidation [29,33]. However, their efficiency decreases once the process of oxidation begins. Therefore, it is possible that α-tocopherol acetate alone, when taken at the prescribed levels, may not be sufficient for achieving optimum inhibition. Betulin, a naturally occurring molecule, exhibits antioxidant effects in fatty meals [4,34]. However, its mechanism of action is still not fully understood. Ethoxyvin’s antioxidant mechanism involves the interaction with peroxide radicals, forming molecular products and inactive antioxidant radicals. This process stops the oxidation chains [32].

The combination of two antioxidants (ethoxyvin + α-tocopherol acetate or ethoxyvin + betulin) exhibited synergistic effects due to various antioxidant interactions, surpassing the solo contributions of each antioxidant [29,31]. It is worth mentioning that mixes of three antioxidants, which include salicylic acid amide, showed the strongest combined inhibition of autooxidation. This resulted in improved stability of fats and preservation of fat-soluble vitamins during storage. This information is supported by Figures 1–3 and Table 4. When salicylic acid amide is coupled with other antioxidants, it synergizes, enhancing the overall antioxidative action [35]. The results of this study align with prior research [32], which emphasizes the capacity of salicylic acid amide to eliminate hydroperoxides by as much as 70–75% without producing any free radicals.

The initial fish oil samples exhibited elevated concentrations of vitamins A and D3 (Table 5). The stability of vitamins in fats and oils decreases as secondary lipid oxidation products accumulate [18,36]. A significant decrease in vitamin content was seen in samples without antioxidants, resulting in increased PV, AV, and ranitidine value (p-AV). Although the individual antioxidants had a small positive impact on preserving vitamins, the synthetic ethoxyvin had the most significant effect. Prior research [32] has shown that bicomponent antioxidant combinations improve the stability of fat-soluble vitamins during storage. When salicylic acid amide was used with other antioxidants, it effectively prevented fat oxidation and lipid peroxidation accumulation. This resulted in improved stability of vitamins in fish oil samples during storage experiments.

5. CONCLUSIONS

During the experimental aging of Pacific sardine S. melanostictus fat without antioxidants, there were significant increases in PV, AV, and p-AV. These increases were accompanied by a notable decline in omega-3 PUFA by 3.4–5 times and fat-soluble vitamins by 40–80%. These findings indicate the presence of vigorous oxidative processes and lipid degradation.

A slight reduction in the oxidation of fats, degradation of PUFAs, and loss of vitamins was seen when specific antioxidants (α-tocopherol acetate 2%, betulin 0.3%, ethoxyvin 0.14%) were present. The synthetic antioxidant ethoxyvin had superior antioxidant effectiveness. The combination of two antioxidants, α-tocopherol acetate and ethoxyvin, along with betulin and ethoxyvin, at a concentration of half the usual amount, resulted in a synergistic effect. This impact improved the stability of fish oil during storage. The addition of salicylic acid amide as a synergist showed strong antioxidant activity in mixtures of antioxidants (α-tocopherol acetate + ethoxyvin + salicylic acid amide, betulin + ethoxyvin + salicylic acid amide), resulting in improved preservation of fats during storage.

Following sardine S. melanostictus fat preservation, the omega-3 PUFA content remained at 70–73% of its initial level. Similarly, the retention
rates for vitamin A and D3 were 83.2–85.2% and 51.2–56%, respectively. Salicylic acid amide in a synergistic antioxidant composition for preserving highly unsaturated fish oils is an efficient method for preventing oxidative reactions and minimizing the degradation of omega-3 PUFAs and fat-soluble vitamins during storage.

6. AUTHORS’ CONTRIBUTIONS
All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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8. CONFLICT OF INTEREST
The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS
This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY
All the data is available with the authors and shall be provided upon request.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY
The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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