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Impact of Sweet Orange Peel Essential Oil on the Nutrient and Oxidative Stability of Aquafeed During the Storage Condition

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ABSTRACT

The main goal of this study was to assess the qualitative composition of essential oil (P), a natural extract produced from wasted orange peels in the citrus processing sector. Additionally, the study aimed to investigate the alterations in fish feeds when this essential oil is incorporated and stored. The essential oil was extracted from the orange peels provided for the study using the hydrodistillation method and the Clevenger apparatus. The study determined the volatile components of the oil with 100% accuracy. The primary constituent was identified as D-limonene, comprising 59.27% of the total composition. Subsequently, experimental feed groups were established by including the essential oil in the feed rations at ratios of ‰0 (P0), ‰0.5 (P5), ‰1 (P10), and ‰3 (P30). Under storage conditions, the feeds were stored in feed sacks for 60 days. Periodic samples were collected during storage and subjected to nutritional, microbiological, structural, and oxidation tests. The results of the study show that P had no protective effect in fish diets against the growth of yeasts, molds, and other mesophilic aerobic bacteria ($p > 0.05$). It was shown that the nutritional values varied over time during storage. However, this variation was not substantially correlated with the amount of P in the diets ($p > 0.05$). There was no discernible impact of the addition of P on the structural characteristics of the feed grains. Nevertheless, the inclusion of P substantially impeded the process of lipid oxidation in the diet ($p<0.05$). After the two-month storage period, it was shown that adding at least 1 ‰ of P to the fish meals prevented the peroxide generated from oxidation in the feed from exceeding acceptable levels.

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Introduction

In the aquafeed industry, the shelf life of feed is an important criterion (Bhilave, 2018). Fish feeds are inherently prone to spoilage despite attempts by manufacturers to extend their shelf life. Some of the components of fish feed have specific chemical properties that make feed storage difficult. Therefore, feeds prepared for fish can be considered perishable products, and these spoilages bring many economic losses (Robb et al., 2013; O'Keefe & Campabadal, 2015). For this purpose, various additives are added to fish feeds to maintain feed quality and protect fish health. These additives optimize and maintain feeds' physical and chemical properties (Encarnação, 2016; Dawood et al., 2018). Until recent years, synthetic additives were prominent among the preservative additives added, but limitations and bans have been imposed on the use of such additives due to problems such as affecting the natural microbiota and leaving residues (Gümüş, 2019; Kesbiç, 2019). Accordingly, interest in replacing synthetic additives with natural plant-based alternatives has increased and scientists have focused on safe dietary supplements and additives (Chakraborty and Hancz, 2011; Hermund, 2018). Phytogenics, in this particular situation, refers to a relatively new category of feed additives that have recently gained attention in the aquaculture sector. They consist of naturally occurring bioactive chemicals from various plants (Encarnação, 2016). Phytogenics are products widely acknowledged as safe (Liu et al., 2011; Sutili et al., 2018). According to Encarnação (2016), essential oils are the primary type of photogenic substance utilized in aquafeeds. Essential oils possess significant potential in terms of their antibacterial and antioxidant capabilities. Based on their specific active components, they can serve as substitutes for chemical preservatives and antibiotics, particularly in combating foodborne infections. Furthermore, essential oils possess highly promising antioxidants for safeguarding food items' longevity and nutritional quality. This is achieved by diminishing lipid oxidation, as documented by Bayaz (2014).

P is one of these phytonegics and its use in fish nutrition has become trendy recently (Kesbiç, 2019). P is extracted from the peel of orange fruit, one of the citrus industry products. The solids remaining after juice production are considered citrus waste and citrus waste ranges from about 50% to 70% of processed fruit, depending on the technology adopted and the fruit variety (Zema et al., 2018). These wastes are generally low in pH and high in water and organic matter. The volatile oils in the waste could have toxicity that can harm soil microbiota. These characteristics limit the disposal of citrus waste due to their economic and environmental impacts (Calabrò et al., 2016; Zema et al., 2018). Efficient waste processing and conversion into an economic product is paramount in countries that process citrus fruits industrially. P comprises 30 to 60 volatile compounds, mainly D-limonene (Ruiz and Flotats, 2014; Acar et al. 2015; Gültepe, 2018). It has been reported in previous studies that when these compounds are added to feed, they promote growth, improve general health values, and even provide resistance to diseases for many breeding species (Gültepe, 2018; Kesbiç, 2019; Shabana et al., 2019; Vicente et al., 2019). However, almost all of the research on additives to feeds has been on the benefits of additives to the metabolism after fish consumption (Sutili et al., 2018). Furthermore, another primary task of additives to fish feed is to keep the quality criteria within acceptable limits from the production of the feed to the time it is offered for fish consumption (Jobling et al., 2011).

In this frame, the primary aim of this study is to evaluate the advantages and potential of incorporating P on aquafeed quality, which has been previously demonstrated to be harmless to fish and to have beneficial metabolic effects when consumed, into fish feed as a substitute for synthetic products commonly employed in the industry.

Material and Method

Extraction and Analysis of Essential Oils from Orange Peel

The hydrodistillation method was used to extract essential oil from orange peels using a Clevenger apparatus. Gas chromatography/mass spectrometry (GC/MS) analyzed the extracted essential oil. Instrument configurations, mobile gas, interface, and ion source injection methods were applied according to Acar et al. (2019). The column's oven temperature ramp program was programmed as Kesbic (2019) recommended. The peaks obtained were identified by comparison with the W9N11 library. Peaks with a similarity rate above 95% were considered as identified (Adams, 1997).

Preparation of Experimental Feeds

The experimental diets were prepared at Çanakkale 18 Mart University, Faculty of Aquaculture Laboratories to contain 48% crude protein and 23% crude fat by using fish meal as the main protein source and fish oil as the main lipid source. P was supplemented into the feed rations at ‰0 (P0), ‰0.5 (P5), ‰1 (P10) and ‰3 (P30), respectively. Experimental feed formulations are presented in Table 1.

Storage and Sampling

The experimental feeds were stored in permeable feed sacks at 25±2°C, 50±5% humidity, and darkness in an acclimatization cabinet simulating storage conditions. Sampling was performed every 15 and 30 days. Nutrient analyses were performed at 30-day intervals, oxidation-based analyses at 15-day intervals, and microbiological and structural analyses at the end of the experiment.

Proximate Analysis

Samples taken for nutrient analysis were frozen in liquid nitrogen and homogenized by grinding with a porcelain mortar and mortar. Crude ash (942.05, AOAC, 1998) and moisture (934.01, AOAC, 1998) analyses were performed by gravimetric method. Kjeldahl method was used for crude protein content ((N × 6.25) 955.04, AOAC, 1998). Crude fat content was determined by the alcohol extraction method (954.02, AOAC,1998).

Analyse of the Fatty Acids Profile

The extracted oil from proximate analysis determined the fatty acid profile. The sample preparation involved mixing 20 mg of the oil with 1 mL of 1 N NaOH in methanol. After that, the mixture was heated at a temperature of 110°C for 15 minutes to expedite the transesterification process, after which 1 mL of a solution containing 14% BF_3 in methanol was introduced. Subsequently, 1 mL of n-hexane was introduced, and the amalgamation was vigorously mixed for 1 minute before adding 3 mL of saturated sodium chloride solution. The resulting liquid remaining after sedimentation was utilized as the sample solution. The oil samples were examined for their fatty acid content using the Shimadzu GC-MS QP 2010 ULTRA instrument, manufactured by Shimadzu in Kyoto, Japan. The carrier gas utilized in the device was helium with a purity of 99.99%. The device employed an RTX-2330 capillary column with dimensions of 60 m length, 0.25 mm diameter, and 0.20 µm particle size. The temperature of the column furnace was set to 100°C, the injection temperature was set to 250°C, the interface temperature was set to 250°C, the ion source temperature was set to 200°C, the pressure was set to 90 kPa, and the injection volume was set to 1 µL. Oven temperature program: 5 minutes at 100°C, followed by a linear increase from 100°C to 240°C at a rate of 4°C per minute, and finally 15 minutes at 240°C.

Monitoring Radical Scavenging Activities and Peroxide Formation of Experimental Feeds

Radical scavenging activities (RSA) were performed according to the method of Beta et al. (2005). Peroxide analysis was performed using the AOCS Official Method Cd 8b-90 (AOCS, 2017).

Statistical Analyses

IBM SPSS Statistics 23 program was used for statistical analyses. The conformity of the parameters to normal distribution was determined by Shapiro Wilks test. Kruskal Wallis test was used for intergroup comparisons of parameters that did not show normal distribution and Dunn's test was used to determine the group causing the difference. Friedman test was used for intra-group and total comparisons of the parameters that did not show the normal distribution, and the Wilcoxon sign test was used to determine the period causing the difference.

Table 1. Formulation of experimental feeds

%	P0	P5	P10	P30
Fish meal[1]	59	59	59	59
Soybean meal[2]	18	18	18	18
Wheat flour [3]	1	1	1	1
Corn Starch [4]	1	1	1	1
Vit. Min Mix.[5]	4	4	4	4
Fish oil[6]	17	16.95	16.90	16.70
P[7]	0	0.05	0.10	0.30
Nutrient content *				
Crude Protein	48.01	48.01	48.01	48.01
Crude Lipid	23.08	23.08	23.08	23.08
Crude Ash	9.70	9.70	9.70	9.70
Crude Cellulose	1.90	1.90	1.90	1.90
Nitrogen-free Extract (NFE)	17.31	17.31	17.31	17.31

* The nutritional contents of the prepared feeds were calculated according to the analysis results of the raw materials used; [1]% fish meal: Dry matter 91%, crude protein 65%, crude fat 10%, crude ash 15%, crude cellulose 1%; NFE 10%; [2]% Soy Protein: Dry matter 90%, crude protein 52%, crude fat 0.5%, crude ash 4.6%, crude cellulose 7%, NFE 42.9; [3] Wheat Flour: Dry matter 86%, crude protein 10.8%, crude fat 1.7%, crude ash 2%, crude cellulose 2.8%, NFE 85.5; [4] Corn Starch: Crude protein 0.3%, crude fat 0.1%, crude cellulose 1.5%, NFE 99.6; [5] Vitamin/Mineral Premix: vit. A, 18.000 IU; vit. D3, 2500 IU; vit. E, 250 m g/kg; vit. K3, 12 m g/kg; vit. B1, 25 mg; vit. B2, 50 mg; vit. B3, 270 mg; vit. B6, 20 mg; vit. B12, 0.06 mg; vit. C, 200 mg; folic acid, 10 mg; calcium D-pantothenate, 50 mg; biotin, 1 mg; inositol, 120 mg; choline chloride, 2000 mg./Fe, 75.3 mg; Cu, 12.2 mg; Mn, 206 mg; Zn, 85 mg; I, 3 mg; Se, 0.350 mg; [6] Fish Oil: Crude oil 100%; [7] Orange Peel Essential Oil

Table 2. Volatile Compounds of orange peel essential oil

Compound	Retention Time (Min.)	%
α- Pinene	9.168	2.01
Myrcene	11.526	2.91
β- Myrcene	11.610	2.71
Octanal	12.002	3.07
Δ^3- carene	12.197	2.74
Linalyl acetate	12.460	1.73
D-Limonene	13.360	59.27
1-Octanol	15.071	2.54
Linalool	16.204	6.04
α -Terpineol	19.493	2.49
Decanal	19.956	1.23
Citronellol	20.843	1.52
Neral	21.306	1.35
Citral	22.393	2.29
Others		8.1
Total		100.00

Table 3. Changes in % moisture levels within and between groups during the storage period

Moisture (%)	P0 (n=3)	P5 (n=3)	P10 (n=3)	P30 (n=3)	p[1]
0.day	9.52±0.01 (9.5)	10.01.15±0.01 (10)	8.97±0.01 (9)	9.81±0.01 (9.8)	0.072
30.day	7.65±0.01 (7.6)	7.66±0.01 (7.7)	7.24±0.01 (7.2)	7.79±0.01 (7.8)	0.184
60.day	7.78±0.01 (7.8)	7.79±0.01 (7.8)	7.89±0.01 (7.9)	7.83±0.01 (7.8)	0.072
p[2]	0.135	0.135	0.135	0.135	
0.-30.day p[3]	-	-	-	-	
0.-60.day p[3]	-	-	-	-	
30.-60.day p[3]	-	-	-	-	

Data presented Mean±SD (Median) [1]Kruskal Wallis Test [2]Friedman Test [3]Wilcoxon Sign Test *$p<0.05$

Results

Volatile Compounds of Orange Peel Essential Oil

The identifiable volatile components of P used in our study are presented in Table 2. According to the results of the analysis, a total of 14 components with a content of 1% and above were identified. These constituents constituted 91.9% of the total oil and the remaining constituents with 1% or less constituted 8.1%.

Changes in Nutritional Content of Experimental Feed in Storage Condition

The % moisture levels of the experimental feeds were evaluated within and between groups as a result of storage time (Table 3). At the end of the study, no statistical significance was found between the groups.

Table 4. Changes in % crude ash levels within and between groups during the storage period

Crude Ash (%)	P0 (n=3)	P5 (n=3)	P10 (n=3)	P30 (n=3)	p[1]
0.day	8.07±0.05 (8.1)	7.97±0.16 (7.9)	8.47±0.26 (8.5)	7.95±0.52 (8)	0.129
30.day	8.85±0.11 (8.9)	8.81±0.14 (8.9)	8.44±0.33 (8.6)	8.83±0.27 (8.9)	0.264
60.day	8.54±0.02 (8.5)	8.45±0.15 (8.4)	8.9±0.46 (8.8)	8.65±0.26 (8.5)	0.459
p[2]	0.050	0.050	0.717	0.097	
0.-30.day p[3]	-	-	-	-	
0.-60.day p[3]	-	-	-	-	
30.-60.day p[3]	-	-	-	-	

Data presented Mean±SD (Median) [1]Kruskal Wallis Test [2]Friedman Test [3]Wilcoxon Sign Test *$p<0.05$

Table 5. Changes in % crude protein levels within and between groups during the storage period

Protein (%)	P0 (n=3)	P5 (n=3)	P10 (n=3)	P30 (n=3)	p[1]
0.day	48.64±0.42 (48.7)	48.6±0.82 (48.8)	48.66±0.16 (48.8)	48.53±0.24 (48.5)	0.922
30.day	45.42±0.38 (45.5)	45.74±0.9 (46.1)	46.16±1 (46.6)	45.87±0.97 (45.8)	0.617
60.day	40.7±0.25 (40.8)	40.53±0.37 (40.7)	40.34±0.36 (40.3)	41.79±1.76 (41.6)	0.369
p[2]	0.050	0.050	0.050	0.050	
0.-30.day p[3]	-	-	-	-	
0.-60.day p[3]	-	-	-	-	
30.-60.day p[3]	-	-	-	-	

Data presented Mean±SD (Median) [1]Kruskal Wallis Test [2]Friedman Test [3]Wilcoxon Sign Test *$p<0.05$

Table 6. Changes in % crude lipid levels within and between groups during the storage period

Lipid (%)	P0 (n=3)	P5 (n=3)	P10 (n=3)	P30 (n=3)	p[1]
0.day	23.23±0.6 (23)	23.02±0.12 (23)	22.97±0.15 (23)	23.06±0.2 (23.1)	0.933
30.day	21.35±0.69 (21.6)	20.79±2.39 (22)	20.71±1.04 (20.3)	21.41±1.85 (22.2)	0.715
60.day	18.52±1.01 (18.9)	16.2±1.34 (15.7)	17.05±4.46 (15.9)	14.75±1.96 (15.4)	0.270
p[2]	0.050	0.050	0.097	0.050	
0.-30.day p[3]	-	-	-	-	
0.-60.day p[3]	-	-	-	-	
30.-60.day p[3]	-	-	-	-	

Data presented Mean±SD (Median) [1]Kruskal Wallis Test [2]Friedman Test [3]Wilcoxon Sign Test *$p<0.05$

However, in all groups, there was a tendency for a decrease in % moisture values within the group, this decrease was not statistically significant (p>0.05).

The research assessed the variations in crude ash levels over time and based on the additive amount. The findings are shown in Table 4. Despite the observed increasing trends, the % ash readings were consistent and there was no statistically significant alteration (p>0.05). The experimental feeds were prepared to contain 48% crude protein and although there was a tendency to decrease between the % protein levels at the end of the storage period (Table 5.), it was determined that there was no statistically significant change in the time-dependent evaluations between and within the groups (p>0.05). The % lipid levels of the experimental feeds (Table 6.) were evaluated within and between the groups in a time-dependent relationship. According to the results of the analysis, there was no statistically significant difference between the groups. Although a decreasing trend was observed in % lipid values with time, this decrease was not found to be statistically significant (p>0.05). The fatty acid profiles of the experimental feeds were also monitored throughout the storage study (Table 7.).

Radical Scavenging Activities and Peroxide Formations of Experimental Feeds

The radical scavenging activity of P against DPPH radical is given as % RSA in Table 8. In the analyzes performed during the two-month storage period, it was determined that the antioxidant capacity decreased statistically in all groups depending on time (p<0.05). In addition, there was no statistical difference in %RSA depending on the supplementation level (p>0.05) in the measurements performed until the 30th day, while it was determined that %RSA increased statistically depending on the supplementation level (p<0.05) in the 45th and 60th day measurements.

Peroxide value (PV) analysis is another method used for the detection of oxidation and the results of our present study are given in Table 9. As a result of the PV analysis of the current study, a significant increase in PV values was determined in all groups except P30 group feeds (p<0.05).

Discussion

Essential oils extracted from the fruit peels of many trees belonging to the citrus family have been used as additives to the feeds of many fish species and some positive results have been recorded (Kesbiç et al., 2020; Acar et al., 2019). However, there is no research on the effects of supplementation of these oils on feed quality. Essential oils obtained from citrus fruits have extremely important bioactive contents and can be extracted from the wastes of many industries, especially the fruit juice industry. However, it is of great importance to determine the quality of the obtained oils and to add these results to the products. The composition of citrus essential oils is subject to variation based on characteristics such as variety, seasonality, geographical origin, and fruit maturity.

Table 7. Fatty acid profile of experimental diets during the storage time

Fatty Acid (%)		P0			P5		
		0	30	60	0	30	60
C8:0	(Caprylic acid)	0.034	0.075	0.066	0.027	0.12	0.26
C10:0	(Capric acid)	0.05	0.12	0.085	0.04	0.24	0.51
C12:0	(Lauric acid)	0.22	0.29	0.25	0.22	0.46	0.81
C13:0	(Tridecylic acid)	0.16	0.25	0.21	0.15	0.43	0.81
C14:0	(Myristic acid)	12.52	11.46	11.95	12.92	11.91	11.68
C16:0	(Palmitic acid)	35.75	39.98	40.13	36.26	37.76	34.96
C17:0	(Margaric acid)	1.42	1.64	1.58	1.41	1.74	1.99
C18:0	(Stearic acid)	6.70	7.99	8.40	6.63	7.61	8.62
C20:0	(Arachidic acid)	1.31	1.31	1.35	1.28	1.52	1.92
C21:0	(Heneicosylic acid)	0.18	0.27	0.22	0.17	0.49	0.93
C22:0	(Behenic acid)	0.43	0.53	0.50	0.42	0.71	1.12
C23:0	(Tricosylic acid)	0.145	0.26	0.20	0.137	0.45	0.87
C24:0	(Lignoceric acid)	0.27	0.58	0.52	0.26	0.70	1.08
∑ Saturated Fatty Acids		59.18	64.755	65.46	59.924	64.14	65.56
C16:1	(Palmitoleic acid)	12.27	10.11	10.33	12.29	10.02	8.86
C18:1	(Oleic acid)	18.24	15.82	15.97	18.00	15.35	13.57
C24:1	(Nervonic acid)	1.09	1.27	1.13	1.07	1.19	1.29
C20:1	(Eikosenoic acid)	0.90	0.77	0.76	0.87	0.96	1.22
∑ Monounsaturated Fatty Acids		32.5	27.97	28.19	32.23	27.52	24.94
C18:2	(Linoleic acid)	2.21	2.66	2.44	2.17	2.73	3.12
C18:3	(Alpha-linolenic acid)	0.55	0.48	0.41	0.53	0.67	1.03
C20:3	(Eicosatrienoic acid)	0.09	0.15	0.11	0.08	0.33	0.67
C20:4	(Arachidonic acid)	0.51	0.46	0.45	0.50	0.63	0.95
C20:5	(Eicosapentaenoic acid)	1.75	1.00	0.85	1.60	1.16	1.21
C21:5	(Heneicosapentaenoic acid)	0.17	0.18	0.098	0.16	0.31	0.43
C22:6	(Docosahexaenoic acid)	3.05	2.34	1.91	2.81	2.51	2.08
∑ Polyunsaturated Fatty Acids		8.33	7.27	6.26	7.85	8.34	9.49
∑ Fatty Acids		100	100	100	100	100	100

Fatty Acid (%)		P10			P30		
		0	30	60	0	30	60
C8:0	(Caprylic acid)	0.075	0.186	0.167	0.028	0.061	0.094
C10:0	(Capric acid)	0.14	0.37	0.3	0.05	0.1	0.12
C12:0	(Lauric acid)	0.36	0.65	0.55	0.13	0.14	0.16
C13:0	(Tridecylic acid)	0.28	0.62	0.52	0.17	0.18	0.2
C14:0	(Myristic acid)	13.43	12.7	12.66	13.23	11.72	11.54
C16:0	(Palmitic acid)	35.79	35.31	38.2	37.07	40.16	40.94
C17:0	(Margaric acid)	1.49	1.81	1.92	1.47	1.64	1.64
C18:0	(Stearic acid)	6.70	7.03	7.56	6.87	7.93	8.09
C20:0	(Arachidic acid)	1.39	1.72	1.61	1.33	1.32	1.32
C21:0	(Heneicosylic acid)	0.30	0.71	0.57	0.18	0.25	0.26
C22:0	(Behenic acid)	0.53	0.90	0.80	0.44	0.51	0.52
C23:0	(Tricosylic acid)	0.26	0.65	0.54	0.15	0.24	0.25
C24:0	(Lignoceric acid)	0.35	0.80	0.76	0.27	0.57	0.61
∑ Saturated Fatty Acids		61.095	63.456	66.157	61.388	64.821	65.744
C16:1	(Palmitoleic acid)	11.95	10.24	9.81	12.49	10.13	7.86
C18:1	(Oleic acid)	17.52	14.96	14.65	18.04	15.67	15.32
C24:1	(Nervonic acid)	1.00	1.18	1.18	1.06	1.15	1.11
C20:1	(Eikosenoic acid)	0.94	1.15	0.99	0.89	0.75	0.74
∑ Monounsaturated Fatty Acids		31.41	27.53	26.63	32.48	27.7	25.03
C18:2	(Linoleic acid)	2.77	2.69	2.53	1.98	2.63	2.69
C18:3	(Alpha-linolenic acid)	0.58	0.85	0.66	0.45	0.45	0.44
C20:3	(Eicosatrienoic acid)	0.18	0.50	0.39	0.08	0.14	0.14
C20:4	(Arachidonic acid)	0.55	0.84	0.71	0.49	0.45	0.44
C20:5	(Eicosapentaenoic acid)	1.25	1.34	0.94	1.10	0.91	0.93
C21:5	(Heneicosapentaenoic acid)	0.18	0.35	0.27	0.12	0.14	0.11
C22:6	(Docosahexaenoic acid)	1.99	2.47	1.83	1.82	2.57	2.45
∑ Polyunsaturated Fatty Acids		7.5	9.04	7.33	6.04	7.29	7.2
∑ Fatty Acids		100	100	100	100	100	100

Table 8. % Radical scavenging activities (RSA) of experimental feeds during the storage condition.

% RSA	P0 (n=8)	P5 (n=8)	P10 (n=8)	P30 (n=8)	p^1
0. day	19.38±4.61 (19.5)	22.27±9.59 (20.1)	22.1±10.94 (17.7)	19.05±3.26 (18.2)	0.952
15. day	12.24±1.92 (12.7)	15.6±4.6 (14.9)	16.35±4.64 (16)	15.63±4.11 (13.9)	0.133
30. day	7.96±2.78 (7)	10.77±3.12 (9.7)	11±4.04 (10.6)	11.9±3.34 (11.1)	0.101
45. day	7.7±2.18 (7.8)	8.86±2.18 (8.5)	9.4±1.6 (9.6)	11.63±1.29 (11.7)	0.003*
60. day	2.84±2.68 (2.3)	3.51±1.9 (3.7)	4.51±1.61 (4.8)	6.43±2.65 (5.6)	0.049*
p^2	<0.001*	<0.001*	<0.001*	<0.001*	
0.-15. day p^3	0.021*	0.021*	0.326	0.093	
0.-30. day p^3	0.012*	0.012*	0.012*	0.012*	
0.-45. day p^3	0.012*	0.012*	0.012*	0.012*	
0.-60. day p^3	0.012*	0.012*	0.012*	0.012*	
15.-30. day p^3	0.012*	0.017*	0.069	0.036*	
15.-45. day p^3	0.017*	0.025*	0.012*	0.036*	
15.-60. day p^3	0.012*	0.012*	0.012*	0.012*	
30.-45. day p^3	0.889	0.327	0.484	0.674	
30.-60. day p^3	0.036*	0.012*	0.012*	0.017*	
45.-60. day p^3	0.012*	0.012*	0.012*	0.017*	

Data presented Mean±SD (Median) [1]Kruskal Wallis Test [2]Friedman Test [3]Wilcoxon Sign Test *p<0.05

Table 9. Peroxide formation of experimental feeds during the storage condition.

Peroxide Value (meq O_2/kg)	P0 grubu (n=3)	P5 grubu (n=3)	P10 (n=3)	P30 (n=3)	p^1
0. day	1.8±0.54 (1.5)	1.42±0.09 (1.4)	1.23±0.22 (1.2)	2.34±0.25 (2.3)	0.057
15. day	1.91±0.61 (1.9)	2.27±0.64 (1.9)	2.43±0.53 (2.2)	4.28±1.55 (5.2)	0.183
30. day	3.48±1.24 (3.6)	3.71±1.32 (4.4)	4.97±1.55 (5.8)	6.66±3.8 (8.2)	0.442
45. day	15.61±3.99 (15.4)	11.75±3.96 (11.6)	9.19±2.19 (8)	3.89±3.9 (3.9)	0.041*
60. day	33±2.51 (34.3)	29.18±3.23 (28.9)	19.59±2.29 (20)	17.46±4.39 (19.8)	0.029*
p^2	0.022*	0.017*	0.017*	0.066	
0.-15. day p^3	0.593	0.109	0.109	-	
0.-30. day p^3	0.109	0.109	0.109	-	
0.-45. day p^3	0.109	0.109	0.109	-	
0.-60. day p^3	0.109	0.109	0.109	-	
15.-30. day p^3	0.109	0.109	0.109	-	
15.-45. day p^3	0.109	0.109	0.109	-	
15.-60. day p^3	0.109	0.109	0.109	-	
30.-45. day p^3	0.109	0.109	0.109	-	
30.-60. day p^3	0.109	0.109	0.109	-	
45.-60. day p^3	0.109	0.109	0.109	-	

Data presented Mean±SD (Median) [1]Kruskal Wallis Test [2]Friedman Test [3]Wilcoxon Sign Test *p<0.05

Nevertheless, it is possible to delineate a few shared traits (Ruiz and Flotats, 2014). Based on the findings from the present investigation, it was concluded that D-limonene had the greatest concentration at a rate of 59.27%. In their analysis of prior research on P, Velázquez-Nuñez et al. (2013) found that the D-limonene ratio was discovered to be 96.62% in their study. In their study, Espina et al. (2011) determined that the D-limonene ratio in the oil obtained from orange peel was 85.5%. A previous study involved extracting the oils from 12 distinct types of *Citrus sinensis* and it was observed that the ratios of D-limonene ranged from 73.9% to 97% (Geraci et al., 2017). Multiple investigations have consistently found that D-limonene is the primary constituent of P (Moufida & Marzouk, 2003; Acar et al., 2015; Duman & Amir, 2016).

Supplementing feed additives to feed is crucial for preserving the nutritional integrity of the feed throughout storage (Hossain et al., 2024). To ensure the quality standards of aquafeeds, it is essential to control the moisture content within specific thresholds. Preserving industrial dry aquafeeds gets challenging when the moisture content above 10%. According to a research by Kop and Korkut (2002), the presence of microorganisms in the aquafeed is likely if the moisture content in the raw material and end product exceeds 14.5-15.5%. The present study not shown any statistically significant variation in the moisture content of the feeds throughout the storage period, even at the conclusion (p>0.05). Nevertheless, while observing a decline in moisture levels across all groups, this tend did not reach statistical significance (p>0.05). The study carried out by Hossen et al. (2011) analyzed the variations in moisture content of meals during storage. However, in contrast to the current study, there was a noticeable upward trend in moisture levels towards the conclusion of the storage period, although this rise did not reach statistical significance. The disparity between the two experiments is believed to stem from the correlation between the relative humidity of the storage enviroment and the moisture content of the feeds. Simultaneously, while the ash level of meals may differ depending on the specific item, it can be asserted that there exists a negative correlation between ash and moisture. The ash content of

food is a component of its dry matter content. A reduction in moisture levels will result in an increase in the percentage of ash content in the feed. The moisture and ash measurements acquired in this investigation exhibit a comparable pattern in this regard. Ash refers to the inorganic residue that is left behind after the complete combustion of organic materials. Alternatively, it may be quantified as the aggregate quantity of minerals included in the diet. The ash concentration of food in this particular setting has an impact on several aspects, including the nutritional value, microbiological stability, and process ability of the meal. Hence, it is crucial to ascertain the ash content of food in relation to nutritional analyses (Park and Bell, 2004). The present investigation found that although there were growing tendencies, the % ash values were stable and there was no statistically significant change ($p>0.05$). The findings are consistent with the research conducted by Hossen et al. (2011), since both investigations concluded that the ash levels remained unchanged during the storage period, indicating no major alteration. The variations in ash levels are believed to be caused by diet. Furthermore, contrary to our investigation, a decline in ash levels was seen, maybe linked to the moisture content. Another crucial concern about fish feeds is the protein composition of the feeds. Commercial fish feeds consist of varying ratios of protein (Hossen et al. 2011). Specialized feed formulations are utilized for different fish species due to the species-specific variation in protein requirements in their diet. The focus of our work is high protein concentration feed for members of the carnivorous fish category. It is advised that the protein ratio in feeds for carnivorous fish be between the range of 40-55% (Aragão et al., 2022). The experimental feeds were formulated with a crude protein content of 48%. Upon completion of the storage period, the addition of P at varying rates did not have any statistically significant impact on the protein content of the experimental feeds ($p>0.05$). Nevertheless, it was observed that the protein composition of each experimental group exhibited a decline with time. This phenomenon is believed to occur as a result of protein denaturation during storage and the transformation of protein constituents into NFE. In a study conducted by Solomon et al. (2016), three commercially available fish feeds were kept for a duration of 6 months. The researchers found that there was no statistically significant change ($p>0.05$) in the protein content of the feeds at the conclusion of the storage period. Similarly, Hossen et al. (2011) observed same findings after a storage time of two months. Based on the results from the current investigation and prior studies, it was established that the protein content of the stored meals remained unchanged after a period of two months. The quantity of lipids is another crucial factor to consider when assessing the nutritional quality of feed. It is important to consider the impact of environmental conditions, such as feed quantity, storage temperature and duration, light exposure, oxygen levels, and humidity, on potential nutritional losses in feed. It is widely recognized that several chemical molecules included in feed exhibit reactivity and instability. Hence, organic molecules may undergo denaturation and oxidation when exposed to unfavorable environmental conditions, as stated by Atter et al. (2017). Oxidation, particularly in the presence of oxygen, can lead to nutritional degradation in lipids (Özçelik and Evranuz, 1998). This might result in a reduction in the nutritional characteristics of the diet, leading to an imbalance in nutritional values. It is crucial to sustain the quality of the lipids and ensure that the nutritive values are at their optimal level in the feed sector. Within the scope of our investigation, we assessed the percentage of lipids and determined that there was no statistically significant disparity between the experimental groups ($p>0.05$). While there was a decline in the percentage of lipid values over time, this drop did not reach statistical significance ($p>0.05$). The occurrence is thought to happen as a result of the degradation of ester bonds in oxidized lipids, leading to the production of free fatty acids. This aligns with the progressive rise in PV values over the storage period of time. An important indicator of oxidation is the elevation in free fatty acidity and PV value (Zhang et al., 2018). Several research, like Filipe et al. (2023), Hossen (2011), and O'Keefe and Campabadal (2022), have consistently found that there is a negative correlation between the duration of storage and the amount of lipids in feed. Our investigation also identified a comparable pattern. Despite the scarcity of research in the previous studies about the impact of incorporating essential oils into fish feeds, Filipe et al. (2023) undertook a study to explore this matter. Trout diets were infused with garlic and thyme essential oils and then kept for a duration of two months. The study's findings indicated that the inclusion of aromatic oils had no substantial impact on the total crude lipids of the feeds. Similarly, the utilization of P in this study did not have a significant impact on the lipid content of the experimental feeds.

The fatty acid profile in feed directly affects the lipid composition in the tissues of fish. Deficiency of essential fatty acids can lead to a series of problems such as growth, feed utilization and increased mortality rates in fish (Bayır et al. 2010). Therefore, it is essential to have sufficient levels of essential fatty acids, which are critical for fish, in feeds. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are used to characterize fish oil, are the most important of these essential fatty acids (Filipe, 2023). The fatty acid profiles of the feeds in our study showed differences according to the supplementation rates. However, the statistical significance of these differences is not one of the objectives of this study. A decreasing trend was determined in EPA and DHA fatty acids that make fish oil valuable, but it was observed that this decrease was less in the P-supplemented groups compared to the P0 group. In addition, it has been stated in the literature that as the degree of unsaturation of the oil increases, the oxidation rate increases and oxidation products are formed (Kesbiç et al., 2023). In this context, unsaturated fatty acid content in the P0 group was determined to be higher than the other experimental feed groups. Peroxides are the initial byproducts of the oxidation process of fatty acids (Özden and Gökoğlu, 1997). Hence, it may be asserted that there exists a reciprocal correlation between unsaturated fatty acids and PV values. The P0 group had greater PV values compared to the other groups. Although the PV values exhibited an upward trend over time, there was a concurrent decline in the quantity of unsaturated fatty acids in the fatty acid composition. Due to insufficient data, it was not possible to statistically analyze the association

between PV and unsaturated fatty acids, both in terms of overall analysis and subgroup analysis. To get more accurate and significant outcomes, it is believed that a larger quantity of data should be examined. Based on the collected data and comparisons with existing literature, it was concluded that the addition of P as a preservation additive did not adversely affect the levels of ash, moisture, protein, fat, and fatty acids in the feed.

Fish oil serves as the primary lipid source in the production of fish feed. Fish oil is a substance abundant in polyunsaturated fatty acids (PUFA). Fish oil and its derivatives are vulnerable to oxidative instability (Hasdemir, 2023; Kesbiç et al., 2023). Oxidation is a detrimental state that oils may undergo. For many years, fish diets have been supplemented with synthetic antioxidants including EQ, BHT, and BHA to minimize undesirable losses (Hamre et al., 2010). These antioxidants have been extensively utilized for an extensive duration due to their affordability and minimal cost (Deveci et al. 2016; Hermund, 2018). Currently, the utilization of synthetic antioxidants is limited and even prohibited by several legislative bodies (OJEU, 2003; Hermund, 2018) due to their health hazards and toxicity. The rise in consumer knowledge and recent advancements have led to a shift in preference for natural antioxidant compounds over synthetic antioxidants (Deveci et al., 2016; Gümüş and Gümüş 2019). Essential oils are regarded as a beneficial natural substitute in this context (Al-Khayri, 2023; Tit and Bungau, 2023). Various published research has unequivocally shown the antioxidant effects of these oils. The predominant technique employed to assess the antioxidant capacity of oils is the ability to scavenge 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals. (Kesbiç, 2023; Hasdemir et al. 2023; Zekri et al. 2023). In the current study, it was determined that antioxidant capacity decreased in all groups in a time-dependent manner during two months of storage. Similar results were reported in a study in which four different commercial antioxidants were added to fish oils, the main fat source of fish feeds. In this study, it was reported that the effect of antioxidants was inversely proportional to the storage time and decreased over time (Soydan and Erdoğan, 2019). Although decreases in % RSA values were detected in our current study, it was determined that the concentration-dependent preservation was higher in the P-added groups compared to the control group P0 group and this preservation gained statistical significance on the 45th and 60th days ($p<0.05$). At the end of the storage period, P30 (6.43%) group showed the highest antioxidant capacity, while P0 (2.84%) group showed the lowest antioxidant capacity. In the study conducted by Viuda et al. (2010) in which the antioxidant activity of EO obtained from various aromatic plants was measured, it was determined that the highest protection was obtained at the highest concentration. In addition, in this study, it was stated that essential oils can be an alternative preservative instead of BHT, a synthetic antioxidant. In another study by Yang et al. (2010), the activity of six plant essential oils (lavender, peppermint, rosemary, lemon, grapefruit and frankincense) against DPPH radical was investigated and it was reported that essential oils dose-dependently reduced DPPH radical. Analyzing PV is another method used for the detection of oxidation. Peroxides are the primary oxidation products that occur during the oxidation of lipids and there is a positive correlation between PV and the level of degradation of lipid (Zhang et al., 2021). In the current study, a significant increase in PV values was determined in all groups except P30 depending on time ($p<0.05$). Yıldırım and Çantaş (2020) examined the peroxide values of sea bass and sea bream feeds during storage at two different temperatures for 45 days. During the storage period, peroxide measurements were made at similar periods with our study and it was reported that peroxide values increased in this process. Again, in another study on feed by Solomon et al. (2016), it was determined that PV values increased in all groups at the end of 6 months' storage period. As a result, in our study, although there was an increase in peroxide values depending on time, it was determined that the increasing trend slowed down depending on the P concentration and this situation was statistically significant on the 45th and 60th days ($p<0.05$). At the conclusion of the storage period, the P30 group exhibited the lowest PV value (17.46 meq O_2 g^{-1}), whereas the control group P0 had the highest PV value (33 meq O_2 g^{-1}). For fish oils, it is considered unfavorable for the peroxide value to exceed 20 meq O_2 g^{-1}. Fish oils are prone to peroxidation, which can occur in fish feed. In our study, we found that in order to maintain the PV below the desired level, at least 1 ‰ P should be added to the fish feed.

Conclusion

As a result, it was determined that P did not have a negative effect on the preservation of the nutrient content of fish feeds during the 60 days of storage and even effectively protected the feeds against lipid oxidation, especially after the 45th day. In future studies, it is recommended to investigate the protective effect of P against fish oil oxidation with more detailed methods rather than its effects on fish feed.

Declarations

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