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Fish Feed Formulation with Microalgae *H. Pluvialis* and *A. Platensis*: Effect of Extrusion Process on Stability of Astaxanthin and Antioxidant Capacity

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Abstract

Astaxanthin is one of the most expensive pigments, commonly used in aquaculture due to its strong coloring and anti-oxidative properties. In this work, fish feed was developed using *Arthrospira platensis* and *Haematococcus pluvialis* which contains Beta-carotene and astaxanthin. The low stability of these molecules at high temperature are the main limitation for the use of carotenoids as a functional food ingredient. Therefore, we evaluate the effects of the food extrusion process on the antioxidant value and stability of these ingredients.

The extrusion process was evaluated on 4 different feeds formulated with microalgae, and it was found that in the formulations with microalgae the antioxidant value increased from 23 to 32% compared to feeds without microalgae. In addition, retention of astaxanthin in extruded feeds was 85-92.7% even after 5 months of storage in dark at 4°C. Also, algal biomass inclusion improved feed water stability, showing a retention of 82-90% of its dry weight after 30 minutes immersion in water, and hence water contamination in the ponds.

Keywords: Astaxanthin; Antioxidant; Extrusion; Fish feed; Microalgae.

Introduction

In fish feed processing, one of the most widely used techniques is extrusion, which improves protein digestibility, destroys toxic factors, inactivates enzyme inhibitors and allows starch gelatinization. However, it involves high temperatures, pressure and mechanical stress, which makes it an aggressive procedure for thermolabile compounds such as carotenoids[1].

Pigmentation in fish is a quality attribute that determines consumer acceptance. Color in the muscle and skin of the fish is given by carotenoids, which must be obtained by feeding because the fish are not able to synthesize carotenoids. For this, is necessary to incorporate carotenoids in the diet of the cultivated species[2]. The possible use of antioxidant ingredients rich in carotenoids, available naturally, such as the use of microalgal biomass in fish feed (*Haematococcus pluvialis*, and *Arthrospira platensis*) are discussed in this article.

Microalgae have been implemented in the nutrition of various marine species, due to their nutritional composition and their immunostimulating and antioxidant properties. *A. platensis* is an important source of phenolic compounds and β-carotene[3]. *A. platensis* does not have a cellulose cell wall, it has mucopolymermurein that is easily digested by the digestive enzymes secreted by fish[4]. *A. platensis* biomass is a rich source of vitamins, essential amino acids, minerals, essential fatty acids (γ-linolenic acid), and antioxidant pigments such as carotenoids and phycocyanin[5]. It can enhance

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the color of flesh in red tilapia[6]. Carotenoids such as astaxanthin and canthaxanthin are commonly used in aquaculture as pigmentation sources[7].

Astaxanthin is a carotenoid modified by oxygenated functional groups, its biological activity as a potent antioxidant has been reported with a 10-fold higher activity than β-carotene and lutein, and 100 times alpha-tocopherol[8,9]. It protects against the oxidation of fatty acids, participates in the improvement of the metabolism in addition to the pigmentation that it gives to the organisms[10]. However, its use in the food industry is limited, since having a highly conjugated and unsaturated structure during food processing becomes unstable and susceptible to degradation, especially when extracted from its biological matrix and when exposed to chemical changes induced by the conditions of the industry[11].

In the wild condition, fish derive astaxanthin through their prey[12]. However, more and more natural alternative sources have to be found as for example the green microalgae *H. pluvialis*, characterized by the synthesis of astaxanthin under stress conditions[10]. The concentration of astaxanthin produced by *H. pluvialis* varies depending on the synthesis conditions, it has been reported from 1 to 3% of its dry weight, mainly in esterified form (95%) like Antarctic Krill (Euphausiasuperba), while synthetic astaxanthin is unesterified[13,14]. The pigmentation with *H. pluvialis* has been reported in species as red sea bream and several ornamental fish[15].

A manufacturing process like extrusion may facilitate to crack cells to ensure maximum bioavailability. Extrusion is the main feed processing technology used in the manufacture of fish feeds. The extrusion process involves water, heat, pressure, and mechanical stress, all of which can impact on carotenoid stability.

The objective of this work was to analyze the effect of high extrusion temperatures on astaxanthin retention and antioxidant capacity provided by microalgae into a fish feed formulated with *A. platensis* and *H. pluvialis*, and replacing fish meal with vegetable protein.

Methodology

Formulation

Two formulations named C3 and C4 were made with ingredients that were chosen for their nutritional contribution and due to they have been previously reported in the literature for the elaboration of fish diets. The partial substitution of fishmeal (41 and 43%) was carried out mainly with concentrated soy flour, because it is the vegetable source with the best available amino acid profile (FAO). Sources of protein and lipids were fish meal (45-51%, PROTEÍNAS MARINAS Y AGROPECUARIAS), concentrate soy flour (19-21%, PROCESADORA DE INGREDIENTES), sunflower seed meal (13-14%, from local market). Binders used were gelatin (2-2.8%, GRENETINA DUCHE), corn flour (6-10%, MASECA) and wheat flour (6-10%, TRES ESTRELLAS). Functional additives were used algal dry biomass of *A. platensis* (2%) and *H. pluvialis* (1%). In addition, supplements were used such a mixture of vitamins (0.1%), minerals (0.1%) and ascorbic acid (0.001%).

The nutritional analysis of each ingredient was performed with methods of AOAC International 1990 (NMX-F-608, F-083, F-607, F615, F-613), in the laboratory of the Analytical and Metrological Services Unit (USAM) of CIATEJ to determine the content of protein, carbohydrates and lipids. Subsequently, the formulations were performed using the Excel program, balancing protein, lipid and carbohydrate levels. The nutritional ranges were set for a protein level of 46-48%, Lipids 11-13%, Carbohydrates 26-27%.

Two controls were prepared without microalgae replaced with corn flour, named CONC3 and CONC4.

Extrusion

1 kg of each formulated mixture (C3, C4, CONC3 and CONC4) was extruded in a laboratory prototype (CINVESTAV, Querétaro) with a temperature gradient. The first two heating zones were fixed at 80 and 90°C and the outlet temperature varying at 90 and 100°C. Compression screw used 2: 1 and a screw speed of 28 Hz (cycles per second) were used. The initial moisture of the mixture was 40%. The obtained products were named according to the extrusion profile, using the first letter to indicate the formulations C3 and C4 following the used outlet temperature. The extruded products were placed in trays in a drying oven in complete darkness where they were kept at 100°C for 1 hour to remove the greatest amount of surface moisture to reduce the time of exposure to high temperatures, as[16] recommended. Subsequently the products were kept at 40°C for 18 hours.

After drying, the samples were stored in the dark at 4°C for a period of 5 months in which they were analyzed.

Characterization of the products

Stability in water

The method recommended by[17] was followed. Extruded feeds (2 g) and a commercial feed (Malta Cleyton-Trucha-3) in triplicate were weighed, which were cut to 1 cm and placed in glass vessels with 100 ml of water. Feeds were kept for 30 minutes immersing in water with constant agitation (manually). After 30 minutes the dry matter loss was determined by draining the samples in a net, the products were placed in a drying oven at 70°C for 24 hours. The amount of loss with the difference in weight was determined.

Water Absorption Experiment

Dry feed pellets immersed in water for different time periods were examined. The experiment was carried in salt water (35 g / l) and at room temperature.1g of pellet of each type of product were randomly chosen and the dry weight determined. Pellets were left submerged for 1, 5 and 10 minutes. After the immersion period, pellets were carefully retrieved and excess water was drained with an absorbent paper[17]. Finally pellets reweighted to obtain weight increase after immersion.

The moisture content estimation

Three samples of each formulated feed pellets were randomly selected and weighed on an electric digital balance. Drying oven was used (Fisher Scientific Oven, Model 655F) at 105°C until a constant weight was obtained with the samples to calculate the moisture content with ASAE Standard method (Standard, 1984).

Density estimation

The density of the extruded feed from each formulation was

measured using Cruz-Suarez method[17]. 100 ml of salt water was measured in a graduated cylinder, to which 30 grams of the extruded feed was deposited, subsequently, the displacement of the water was calculated for volume calculation and the density estimated with the following formula:

Density = weight of the sample / volume of the feed
Determination of maximum strength / breaking point

The maximum force or break point (Kgf) of the developed feeds were measured, as well as the commercial feed using a texture analyzer (Stable Micro systems, model TA-XT Plus) using the procedure reported by for the characterization of physical properties in fish feed.

Of each extruded feed and commercial control, 12 samples were selected that with a cylindrical and straight shape to homogenize, which had a size of 0.5 cm. The analysis was carried out in each extrusion, by means of a double compression with a cylindrical probe with a head speed of 0.5 mm / second, until 5% of deformation of the sample was obtained.

Retention of astaxanthin

In order to determine the amount of astaxanthin in the processed feeds at the different extrusion temperatures, the extraction and quantification of the pigment in each of the extruded feeds was carried out by the modified DMSO method of[18]. To 10 mg of *H. pluvialis* biomass 2 ml of preheated DMSO to 70°C were added and in a drying oven, the DMSO samples were allowed to stand for an additional 5 minutes at 70°C, immediately the samples were placed on ice by two minutes, centrifuged at 14,000 rpm for 5 minutes and the optical density of the supernatant was measured at 491 nm. The resulting absorbance was compared to a calibration curve prepared from known concentrations with purified astaxanthin as standard (SML0982 SIGMA).

The extractions were carried out in triplicate in one gram of feed. Extraction of astaxanthin from the formulations before the extrusion process was also performed, in order to determine possible losses after processing. In addition, in order to corroborate the quantification of the formulations before the extrusion, extraction was carried out in 10 mg of the lyophilized biomass of *H. pluvialis*, equivalent to the amount of algae added per gram of feed.

At the end of the extraction, qualitative analysis of the extracts obtained by means of thin layer chromatography (TLC) was carried out. For this analysis, 10 µg of the astaxanthin standard (1 µg / ml) and 10 µl of each extract were placed in a 10 x 20 cm silica gel glass plate (Z293016 Aldrich), the separation of the compounds was developed by a mobile phase of acetone: n-hexane (30:70) for 20 minutes[19].

Quantification of β-carotene in the developed feed

For the extraction and quantification of β-carotene, the method of[20] was followed, for which 500 mg of each feed was weighed and ground, washed with distilled water, which was removed after centrifugation at 14,000 rpm for 5 minutes. 3 ml tetrahydrofuran THF was added to the samples and left overnight in the dark. Subsequently, it was centrifuged at 4000 rpm × 10 minutes and the supernatant evaluated for optical density in a spectrophotometer at 455 nm. β-carotene quantification was obtained by comparison with a standard curve previously performed, with known concentrations of β-carotene.

At the end of the extraction, the extracts were analyzed by means of thin layer chromatography (TLC). For this analysis, 10 µg of standard β-carotene (1 µg / ml) and 10 µl of each extract were placed in a 10 x 20 cm silica gel glass plate. Separation of the compounds was carried out by a mobile phase of hexane: acetone (70:30) for 20 minutes.

Evaluation of antioxidant capacity by DPPH radical inhibition

The study of antioxidant capacity was carried out using the DPPH method[21], with the purpose of comparing the antioxidant effect of the feed developed with the microalgae against the control feed made without microalgae and the commercial feed for trout.

0.5g of each feed was weighed and ground with 2 ml of cold acetone and sonicated for 10 minutes to solubilize and extract the antioxidant compounds, left for 30 minutes on ice, then centrifuged at 14000 rpm for 10 minutes, at the end the supernatant was collected. The extract was diluted 10 times in acetone, due to the intense concentration and coloration that could interfere with the reaction. From the dilution, 100 µl was taken and mixed with 900 µl of the ethanolic solution of DPPH (0.1 mM), then 30 minutes in the dark, the absorbance of the samples was measured at 517 nm in a spectrophotometer.

Inhibition of free radicals by DPPH in percent is determined with the following formula (22, 23):

$$\%Inhibition = \frac{Abs_{control} - (Abs_{sample-Absextract})}{Abs_{control}} x100$$

Where: Control Abs is the absorbance of DPPH; Abs of the sample is the DPPH in the presence of the sample and Abs of the extract is the absorbance of the sample without the DPPH.

Statistical analysis

The software used for statistical analysis is Statgraphics Centurion, XVI (Statpoint technologies INC.), ANOVA and Tukey's multiple range test were used. Differences were considered significant if $P < 0.05$.

Results

Formulation and extrusion

Nutritional composition of the elaborated formulations are shown in table 1. The percentage of fish meal replacement with protein from plant sources is also indicated.

Table 1: Chemical and Nutritional composition of formulated feed. Numbers are expressed as a percentage of the dry weight

Nutritional composition (% (w/w DM))	C3	C4
Protein	47.6	46.2
Lipid	12.7	11.9
Carbohydrate	27	26
Fishmeal replacement Vegetal protein	41	43
Ingredients		
Fishmeal	50.5	46.5
Concentrated Soy flour	19.5	21
Sunflower seed	13.99	13.29
Gelatin	2.8	2
Cornflour	10	6
Wheat flour	0	8
A. platensis	2	2
H. pluvialis	1	1
Ascorbic acid	.0001	0.0001
Mix vitamins	0.1	0.1
Mix minerals	0.1	0.1

Protein, lipid and carbohydrate content in extruded feeds are shown in table 2. After processing, the extruded feeds showed variations due to humidity reduction caused by outlet temperatures in processing. However, the nutritional composition was maintained in desired values of high protein content and low fat are which they were formulated.

Table 2: Nutritional composition (% of dry weight) of extruded feed

% of DW	C390	C3100	C490	C4100
Humidity	3	3.3	3	3.9
Protein	49.7	51.9	49.7	48.9
Fiber	12.1	12	12.1	11.3
Fat	10.7	10.6	10.7	9.9
Carbohidrate	24.4	21.9	24.4	25.7
CrudeFiber	2.7	4.4	2.7	3.4

Food compositions after extrusion, C390, C3100: C3 composition extruded with 90 and 100 degrees, C490,C4100: C4 composition extruded with 90 and 100 degrees

The principal variables were the type of agglutinant used like gelatin and wheat flour used for better stability. For C3 formulation agglutinant used were gelatin and maize flour but for C4 formulation agglutinant used were gelatin, maize flour and wheat flour. This, because previous experiments (unpublished) where we found with only maize flour and wheat flour did not give good stability in water. Here the gelatin served as the key agglutinant for better stability. The products extrudes with C3 and C4 formulations showed a solid consistency at both the temperatures like 90°C and 100°C upon exiting from the extruder.

Stability in water

Pellet disintegration and nutrient leaching are quality factors important in aquatic feeds, due to the impacts on the water contamination problem. In our experiment, the stability in water of feeds extruded shown that C3 at 90°C and 100°C (Figure 1) presented lower losses of matter with respect to C4 after the immersion in water. In addition, both formulations made with microalgae presented greater stability than the control without microalgae. Based on the ANOVA and Tukey's multiple range test, it was found that the loss of matter after immersion in water is significantly different between each treatment. Since the P-value of the F-test is less than 0.05 ($p = 0.0000$), there is a statistically significant difference between the means of the 4 variables with a 95.0% confidence level. Moreover, the two extrusion temperatures evaluated have an effect on stability in water. Extruded products are shown retention of 82-90% of their dry weight after 30 minutes, indicating good stability in water. The highest stability found in C3 composition which was 89.6% (± 0.14) in C390 and 90% (± 0.03) in C3100, for the control without microalgae retention was 82 to 87% and in commercial control retention was 85% (± 0.8).

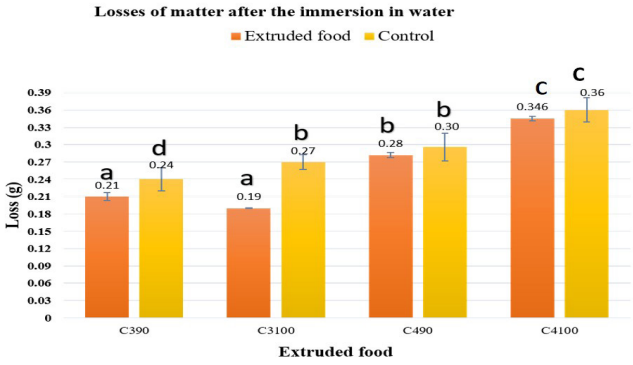

Figure 1: Loss of matter in feed developed with respect to control with same composition without microalgae, after the immersion in water 30 minutes (Water stability test).

Water absorption

The percentage of water absorbed by the extruded feeds after the first minute of hydration was 13 to 25% on its own initial weight, 22-36% absorption at minute 5 and 28-43% at minute 10. The percentage of water absorbed by C390 in the first minute of hydration was 16.7% on its own weight, 23% absorption at minute 5 and 29.7% at minute 10, compared to a commercial control our result is very close. Figure 3 shows the tendency of the different treatments, those absorbed more water as the time of immersion in water is longer

Density

The extruded products showed an average density of 1.14 (± 0.02) g / cc, the controls without microalgae of 1.17 (± 0.05) g / cc, values very similar to those shown by the commercial control of 1.07 g /cc. There were no differences between feeds that include microalgae and those that do not.

Determination of hardness

For the developed feeds the average values required for the maximum breaking force were obtained in kilograms' force (kgf). The feed C390 with microalgae has a breaking strength of 1.49 Kgf, whereas the commercial feed for trout presented a strength of 1.13. The products extruded at 90°C showed a lower hardness

compared to extruded at 100°C, this because at higher temperature, the moisture content of the feed is less and the binder integration is better. The same compositions with included microalgae biomass have shown better hardness in comparison to their respective controls may be algal biomass contributes towards better binding behavior during the processing.

Performing the analysis of variance between the two temperatures, it was found that there is a significant difference of the extrusion temperature on the force maximum break (P-value 0.007). When comparing the maximum force presented by dry pellets against those that were submerged in salt water during different hydration times, a decrease could be observed as the hydration time increases.

Retention of astaxanthin and β -carotene

Extraction of astaxanthin was carried out in the mixtures of each formulation (C3 and C4), before and after the extrusion process to evaluate the possible losses. For initial concentration evaluation, extraction was done in 10 mg of *H. pluvialis* biomass, equivalent to the amount of astaxanthin added per gram of feed. The retentions of astaxanthin after the extrusion process and after 5 months of storage at 4°C were 85 to 93% of the original content (Figure 2a, 2b). Feeds extruded at 90°C retained a greater amount of astaxanthin (92%) than those extruded at 100°C (85%).

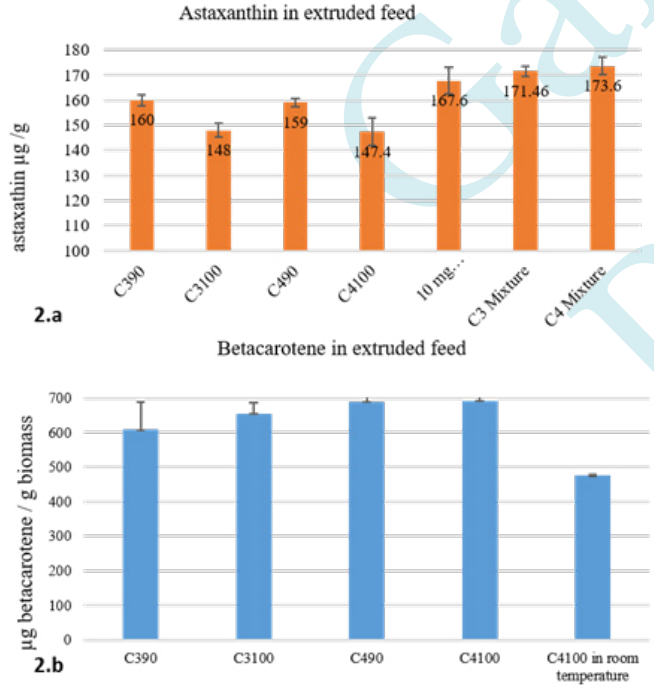

Figure 2(a): Quantification of astaxanthin from formulations C3 and C4, before and after extrusion (C390, C3100, C490, C4100) and **2(b).** Quantification of β-carotene in food: C390, C3100, C490 and C4100 stored at 4°C and C4100 at room temperature.

The thin layer chromatography (TLC) image clearly shows the bands of astaxanthin and β -carotene (figure 3a, 3b), even in the sample of feed after 5 months of storage.

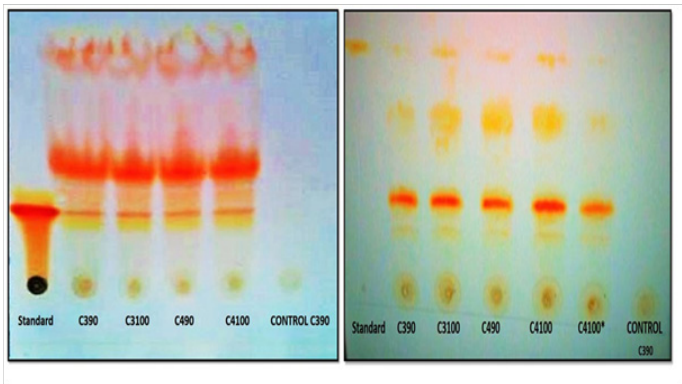

Figure 3: Thin layer chromatography performed with extractions of the extruded products a) Astaxanthin, b) β-carotene.

A simple correlation analysis was performed between the temperature and the amount of astaxanthin. The results of fitting an X-inverse Y-square model to describe the correlation indicate that the P-value is less than 0.05 (P = 0.0006), it can be stated that there is a statistically significant correlation between the amount of astaxanthin and the final extrusion temperature. This indicates that a higher extrusion temperature decreases the retention of astaxanthin.

The extraction of β-carotene in the feed (stored at 4°C) yielded from 600 - 687 µg / g of β-carotene after processing. The presence of astaxanthin and β-carotene was demonstrated by means of thin layer chromatography (Figure 2 a, b), in that image the absence of astaxanthin or β-carotene in the control feed was clear. It is observed that the feed stored at room temperature has 37% less β-carotene than that stored at 4°C.

There is a statistically significant relationship between the amount of β -carotene and the final extrusion temperature at 95% confidence level (P = 0.0000).

Evaluation of antioxidant capacity by DPPH radical inhibition

Figure 6 shows the values obtained for the antioxidant capacity of the extruded products, the commercial control and the negative control without the microalgae. This graph highlights the higher antioxidant activity of fish feed with microalgae compared to controls that were without microalgae.

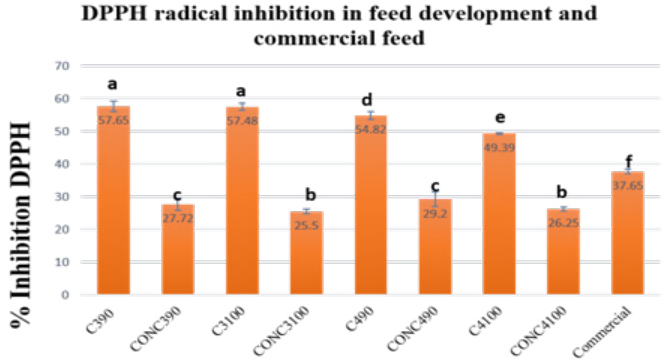

Figure 4: Antioxidant capacity by DPPH radical inhibition in developed feed and commercial feed.

In addition, a higher percentage of inhibition of the

DPPH radical was corroborated in feeds developed with the microalgae, ranging from 49 to 57% compared to commercial feed for trout that was 37% inhibition.

Discussion

The extrusion process improves characteristics such as the digestibility of proteins by inactivation of enzyme inhibitors, in addition to the partial denaturation of the protein that exposes diverse sites for the enzymatic attack.

There are very few reports on physical quality of the feeds and its impact. Production of extruded fish feed pellets demands a strict process to obtain acceptable physical product quality specifications. The physical properties of pellets should be suitable for handling and storage, in order to withstand handling and transportation without generation of an excessive amount of dust and fine particles. No standards are established in terms of hardness or durability[24]. reported the impact of physical pellet quality on the biological response of the fish but not in detail. Studies regarding the physical characteristics of feed pellets involved in seawater[25] and freshwater[26] fish farming systems have been previously published but with lack of information regarding the characteristics of the feed employed. Therefore, here we tried to characterize an antioxidant functional product with microalgae by analyzing its physical quality like density, hardness, water absorbance and stability in water comparing with another commercial product.

Pellet stability in water is an important quality parameter in the manufacture of aquaculture diets. Stability in water is the retention of pellet physical integrity with minimal disintegration and nutrient leaching. Pellets with less stability will quickly leach nutrients, contaminating water in the culture environment and lead to low growth and survival in fish.

Water stability depends on several factors like the method of diet preparation and processing[27], types of ingredients, binding agents, diet composition and coating materials such as microcapsules[28]. Semi-crystalline structure of starch can be modified during extrusion process which could favor the interactions among the components and can help for stability.

Hence in this work, we used ingredients with microalgae, plant based ingredients and protein binding agents such gelatin. Furthermore, microalgae may be contributing towards the binding capability among ingredients with adding functional properties such as antioxidant value and vitamins contained.

It has been indicated in the literature that when *A. platensis* is added 2% or equivalent inclusion in the fish feed, the microalga exerts some functional effect. In the Pagrus major (red sea bream) fed a diet with 2% of microalgae raised protein synthesis in fish;[29] reported that 0.5 to 2% showed improvement in fish coloration. reported better growth performance with *A. platensis* biomass inclusion which improves feed efficiency[30,31]. suggested that *A. platensis* improves the intestinal microbiota in fish by break down of indigestible feed components to extract more nutrients; this also stimulates fat metabolism by stimulating the production of enzymes.

The main contribution of *H. pluvialis* is the presence of astaxanthin, a potent antioxidant. However, there are few reports on the effective dose that a diet for fish should carry. established minimum doses of 25 to 50 mg / kg for an effect on meat pigmentation, higher than that reported by[32] of 100 mg astaxanthin per kg. In this work, the biomass of *H. pluvialis* was included as an astaxanthin source, with an inclusion of 172 ± 1.4 mg / Kg of feed, above the recommended dose in the literature. The advantage of including *H. pluvialis* biomass is that the cell wall provides stability to astaxanthin when to be included before the extrusion process. During the extrusion process, the high temperature, moisture and pressure it may serve to partial breakdown the cell walls to make it more available.

In several reports, it has been pointed out that natural astaxanthin was found to be equally effective or even better than synthetic astaxanthin for fish pigmentation as trout, red sea brem and various ornamental fish[33,15]. The above has been corroborated in several studies that report that astaxanthin incorporated in biomass, presents greater stability in comparison with the extract.

The most susceptible form to oxidation degradation is the non-esterified form of astaxanthin. *H. pluvialis* presents 70% as a monoester of the isomer (3S, 3'S), 10-15% in diester form and in free form it is 4 to 5% with the unesterified hydroxyl groups.

Astaxanthin resistance in *H. pluvialis* is reflected in the feeds developed, which presented high retention levels of 85 to 92.4% on the original astaxanthin content after the extrusion. The fish feed was prepared with 172 ± 1.4 mg of astaxanthin total per kg of feed. Of which, after the extrusion process and 5 months storage at 4° C retained from 147 to 160 mg / kg. The extrusion temperature exerts a significant effect on the retention of astaxanthin in feeds. Nevertheless, astaxanthin was stable to the extrusion process, similar results were reported by Anderson and Sunderland they report 86% retention in final product[34]. On the other hand, Haaland et al. (1993) indicated values of 86 to 94% of astaxanthin retention in extruded feeds.

Storage, extrusion and drying temperatures have an effect on the stability of astaxanthin as Niamnuy et al. (2008) point out, they report that the loss of astaxanthin is lower at drying temperatures of 100 and 120°C due to the shorter time of exposure. In this work feeds were analyzed after 5 months of storage to evaluate the losses. The products extruded at 100°C retained 147 to 148 mg of astaxanthin per kg, compared to feeds extruded at 90°C that retained 159 to 160 mg / kg[35].

report that astaxanthin (20 ppm) exerts an antioxidant activity of 50% (inhibition of the DPPH radical), moreover indicate an activity of 25 to 70% in a complex with cyclodextrin and astaxanthin in carotenoid concentrations ranging from 1.14 to 4.55 µmol / Lt. The added ascorbic acid has a protective effect of astaxanthin, retaining up to 87% of astaxanthin after one year stored at -18° C as reported by[36].

Conclusions

It was possible to verify that the microalgae contributed antioxidant activity in the extruded feed ranging from 49 to 57% inhibition of the DPPH radical, compared to the controls without microalgae with only 25-27%. When the comparison was made with the commercial feed for trout that presented 37% inhibition of the radical, it was corroborated that it presents a lower antioxidant capacity than feed (Figure 6) developed in our experiment. It was found that the antioxidant activity of microalgae included

feeds is significantly higher.

When astaxanthin was added to the formulation by adding *H. pluvialis* biomass, it remained stable to the extrusion process, retaining more than 90% of the initial amount (92.7%), even after being stored for 5 months at 4°C. The best extrusion conditions were with 40% initial moisture of the mixture and an exit temperature of 90°C, obtaining a product with high levels in retention of astaxanthin, antioxidant capacity and absorption of water that allowed low levels of hardness for better digestion. The storage conditions at 4°C and in the total absence of light favors the retention of the functional properties of carotenoids, as evidenced by the quantification of β-carotene and astaxanthin.

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