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Pigmentation of the rainbow trout (*Oncorhynchus mykiss*) with oil-extracted astaxanthin from the langostilla (*Pleuroncodes planipes*)

Gladis Coral Hinostroza [1], *Alberto Huberman W.* [2], *Guadalupe de la Lanza* [3], *José Monroy-Ruiz* [1]

Instituto Nacional de la Nutrición Salvador Zubirán

SUMMARY. The effect of oil-extracted astaxanthin from the red crab or langostilla (*Pleuroncodes planipes*) on the growth and pigmentation of forty five rainbow trouts (*Oncorhynchus mykiss*) was investigated by feeding a test diet supplemented with 75 mg/kg of astaxanthin during six weeks and compared with a control diet. The oil-extraction process of the pigment is described. Weight, flesh astaxanthin concentration, and color (L*, a*, b*) of the flesh were measured at 0, 3, and 6 weeks. No apparent effect of astaxanthin supplementation was observed on fish development. In spite of the low free astaxanthin amount in the diet (8%), an acceptable carotenoid concentration in the flesh (3,60±0,78 mg/kg, w/w), and a red hue ($H°_{ab}$=44,13±2,36) were obtained at the end of the study. The red hue was strongly correlated with the carotenoid concentration (r=0,98).

RESUMEN. Astaxantina de la langostilla, *Pleuroncodes planipes*, extraída con aceite y su uso en la pigmentación de la trucha arcoiris *Oncorhynchus mykiss*. Se investigó el efecto de la astaxantina de la langostilla (*Pleuroncodes planipes*) en el desarrollo y pigmentación de 45 ejemplares de trucha arcoiris (*Oncorhynchus mykiss*). El grupo experimental ingirió una dieta suplementada con 75 mg/kg de pigmento del crustáceo durante seis semanas y fue comparado con un grupo testigo. Se describe el proceso de extracción del pigmento en aceite. Al inicio, a las tres y a las seis semanas se hicieron mediciones de peso, contenido muscular de astaxantina y color (L*, a*, b*). No se observó ningún efecto del carotenoide sobre el desarrollo de los organismos. A pesar del bajo contenido de astaxantina libre en la dieta (8%), al finalizar el estudio los peces presentaron buena concentración de astaxantina en el músculo (3,60±0,78 mg/kg) y tonalidad roja ($H°_{ab}$=44,13±2,36). La tonalidad y la concentración del pigmento mostraron alta correlación (r=0,98).

INTRODUCTION

Astaxanthin (Ax) (3,3'-dihydroxy-b,b-carotene-4,4'-dione) is the main natural pigment responsible for the typical red coloration of salmonid muscle (1). This oxycarotenoid and mainly the free form is more efficiently utilized by these fish than other pigments (2). Since Ax can not be synthesized by salmonids (3), they depend on the natural feed intake source or it needs to be included as a feed ingredient in reared organisms. Apart from its important value as a pigmenter in imparting a desirable pink-red color to salmonid flesh, numerous other biological roles of dietary Ax have been proposed (4,5,6,7).

Most of the farmed fish is pigmented with synthetic Ax and canthaxanthin. However; attention has been concentrated during the last two decades on obtaining red-colored muscle with natural sources. As crustaceans are a rich supply of Ax (8), different species have been tested including shrimp, red crab, crayfish and krill, to induce pigmentation in reared rainbow trout and salmon (9,10,11,12,13,14).

With the purpose of obtaining a more suitable and economic pigmentation process, in some cases whole organisms have been added to the salmonid's feed, resulting in a low pigmentation (13,15,16) as a consequence of the high content of chitin and mineral salts that limit good food digestibility in fish (17). To avoid this inconvenience, the pigment extraction with oil has been efficiently tested (11,18,19,20). The patented method (20) can be considered a suitable process mainly because it avoids organic solvents residues in the extracts. The extraction process results in a pigmented oil rich in omega-3 fatty acids and sterols, enhancing its value as fish feed ingredient (19).

In order to find a suitable utilization of the Mexican red crab or langostilla, *Pleuroncodes planipes* (Stimpson) for aquaculture purposes, the present study was carried out with the aim of evaluating the effect of the mainly esterified oil-extracted Ax from this crustacean, on rainbow trout *Oncorhynchus mykiss* (Walbaum) pigmentation and growth.

The langostilla, *Pleuroncodes planipes*, is an anomuran (Crustacea: Decapoda: Galatheidae) which occurs mostly along the western coast of Baja California Peninsula, Mexico. Its greatest standing stock (460.000 MT/km2) has been calculated during winter-spring (21). At these seasons, they occupy all depth strata of the inner continental shelf including the shallow waters of Magdalena Bay (22) and they can even be beached by wave action, allowing an easier collection. At present, this species represents the focus of important studies, mainly on applied technology (23,24,25).

MATERIALS AND METHODS

Organisms: The langostilla, *Pleuroncodes planipes*, with a carapace length ranging from 19 to 26 mm, was collected live in the beach of Magdalena Bay, Baja California, Mexico. They were light-

1. Ph. D. candidate, Faculty of Science, Universidad Nacional Autónoma de México.
2. Head, Department of Biochemistry. Instituto Nacional de la Nutrición Salvador Zubirán
3. Research scientist, Department of Zoology, Instituto de Biología, Universidad Nacional Autónoma de México.
4. Research scientist, Department of Nutrition, Universidad Iberoamericana, México.

protected with black plastic bags and transported frozen at -15 °C to the laboratory. Rainbow trout, *Oncorhynchus mykiss*, «kamloops» variety, were purchased from the El Pedregal fish farm, State of Mexico, Mexico.

Experimental design and diets: Ninety fishes were alloted to two groups: pigmented (I) and control (II), of three tanks each, with 15 organisms per tank. Fishes were previously acclimatized for four weeks with astaxanthin-free feed. They were individually weighed at the beginning (average weight 218±7 g), at the middle and at the end of the experiment. The animals were reared indoors in 600 L fibreglass tanks supplied with fresh running water at an average temperature of 9.5 °C and 8 ppm of oxygen.

The experimental diets were prepared by pelleting (4 mm diameter in a California pelletizer) the unpellitized rainbow trout feed purchased from Alimentos El Pedregal (Toluca, State of Mexico) as a standard feed (38% protein, 10% fat) under license from Silver Cup (Salt Lake, Utah, USA). Diet for group I, was made by adding the pigmented oil (6% w/w) to the pellets (mixing in a bowl by hand rotation). Diet for group II, had the same preparation but coated with cod oil (6%, w/w) instead of Ax pigmented oil.

Feeding and sampling: Before starting the experiment ten fishes were sampled, sacrificed and filleted for initial analysis (flesh pigment content and color). Fishes were fed twice a day at a feeding rate of 1,5% body weight/day during 6 weeks. The daily ration was corrected on the third week. At this time, three fishes per tank were randomly sampled for analysis. At the end of the study ten organisms from each tank were weighed, sacrificed and filleted. Two fillets were removed from each organism (approximatly 30 cm2 each, under the dorsal fin), one for pigment analysis which was protected against light with black plastic bags and frozen at -20 °C and another for color measuring that was promptly done in fresh.

Pigment extraction: All extractions and analyses were performed, as far as possible, at reduced ligth and under a nitrogen stream, with analytical grade solvents. To extract total pigment from the fresh langostilla they were ground in a blender. Ten g of the «puree» sample were mixed with the same amount of anhydrous sodium sulfate in order to reduce humidity. The pigments were extracted with acetone until remove all pigments.

Oil pigment extraction was performed with cod oil. Previously, the normal langostilla humidity (80%) was reduced to 46% in an air-circulating oven (APEX type 48 BE) at 60 °C for 4 h in order to facilitate grinding. Organisms were then ground in a mill (Hobart-Dayton, 84181-DG) to pass through a 2 mm mesh screen. After restoring the humidity to 80%, cod oil (10%, w/w) was added as extractant and ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) (0,05%, w/w) as antioxidant (11). A single-stage heat extraction was made at 90 °C/30 min with regular stirring (19) in darkness and then a coarse separation by hand pressing was done before centrifugation at 11.000 g/10 min/0 °C (18). The pigmented oil extracts were analyzed, sealed and frozen at -20 °C until utilization.

To extract the Ax from rainbow trout muscle, 10 g of tissue were ground in a mortar and extracted with acetone until colorless. Four parts of water and one part of petroleum ether (PE) were added to the acetone solution in order to remove all pigments to PE. The aqueous phase was drained from the upper pigmented ether phase in a separatory funnel. The pigmented solution was then filtered through a medium porosity fritted-glass funnel containing a 1,5 cm bed of anhydrous sodium sulfate rinsing with PE until colorless.

Chemical analyses: Proximate analyses were performed after preparation of diets. Crude protein was calculated by the method 976.05, crude fat by 920.39, ash by 942.05, moisture by 934.01 and crude fiber by (962.09), as described by AOAC (26). Gross energy content was determined directly by bomb calorimetry using a Parr adiabatic oxygen bomb according to the manufacturer's instructions (Parr Instrument Co., Moline, IL).

Carotenoid analyses: Identification of the Ax and its esters were performed by: 1) spectral analysis in the visible range, 2) thin layer chromatography (TLC) and 3) calculating Rf values. Each analysis was made in parallel with reference Ax. To this end, synthetic Ax (Carophyll Pink, 8% formulation, F. Hoffmann-La Roche, Ltd, Basle, Switzerland) was re-purified by TLC.

Total Ax in whole langostilla organisms was determined with a UV-VIS Beckman DU-64 spectrophotometer set at lmax=475 nm, the maximum absorption spectrum of Ax in acetone (27). Calculation was made by utilizing 1900 as extinction coefficient (E1 %,1cm) of Ax in acetone (3). Total Ax in the oil extract was estimated by measuring its concentration at the maximum absorbance lmax=472 nm in hexane (27) and 1930 as extinction coefficient (E1 %,1cm). The Ax content in PE solutions from fish flesh was measured at lmax=469 nm (28) and 1910 as extinction coefficient (E1 %,1cm) (5).

For thin layer chromatography (TLC), solvent extracts were cooled to -20 °C to precipitate lipids, 5 ml of the upper layer of acetone and PE solution were submitted to determination of relative amounts of Ax and its esters. To this end, the extracts were concentrated under a stream of N2 and redissolved in hexane for chromatographic separation. TLC was made on 20x20 activated (in a vacuum drier at 120 °C/1 h) aluminium silica gel sheets (60 F254, E. Merck). The pigment was chromatographed with acetone-hexane (30:70, v/v). Each fraction was scraped off and redissolved in acetone for spectrophotometric analysis.

The carotenoid concentration of diets were calculated by the method of No and Storebakken (29) and chromatographic separation was done as above.

Relative flesh astaxanthin retention: Relative flesh astaxanthin retention was obtained by the formula: Ax in flesh (mg/kg)/Ax in diet (mg/kg) according to Torrissen (16) expressed as percentage.

Specific growth rate: Specific growth rates (SGR) were obtained according to the formula:

$$SGR = [(\ln W_1 - \ln W_0)/(\#d)] \times 100,$$

where ln = natural logarithm, W_0 = initial weight, W1 = final weight and #d = days of feeding (30).

Flesh color measurement: Flesh color was measured with a Hunter Lab (D25-PC2) colorimeter using the tristimuli CIE (1976) L*a*b*. From a* and b* values, the hue ($H°ab=\tan^{-1}b*/a*$; H°ab=0° for red and 90° for yellow) and chroma [C*ab=(a*2+b*2)1/2] were measured according to Choubert et al., (31).

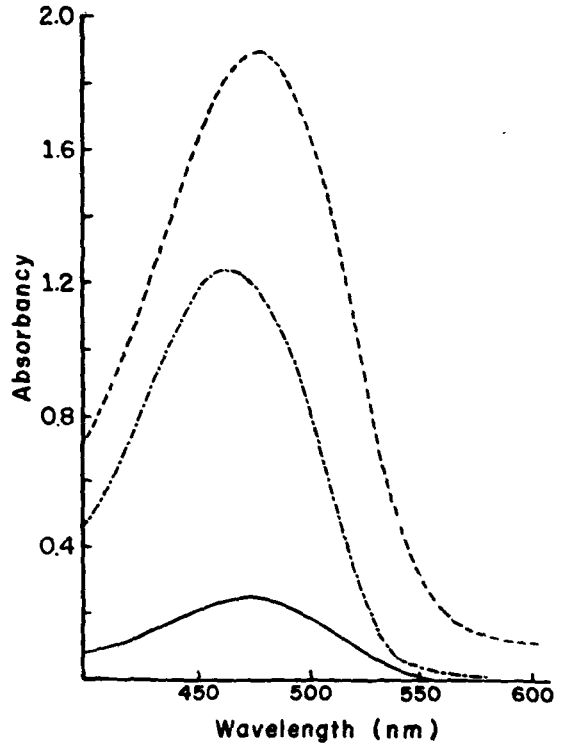
Statistical analysis: Groups I and II were compared by t-Student tests using as response variables fish weight, flesh hue, chroma and Ax concentration. At 3 and 6 weeks of experimentation a regression

analysis was made where the independent variable was the Ax concentration and as a dependent variables the hue and chroma values. The Statgraphics and Statistical graphics software version 7.0 was used in every case.

RESULTS AND DISCUSSION

The proximate chemical composition and energy content of the two diets were about the same along the experiment (Table 1). According to the identification criteria, the only carotenoid identified in the langostilla *Pleuroncodes planipes* was Ax (Fig.1) mainly esterified (Table 2). The total concentration of the carotenoid in the entire organism was 145 mg/kg (wet weight), higher than that reported by Wilkie (32) who besides found b-carotene as an other pigment constituent which was not the case in our findings.

FIG. 1.
Light absorption spectra of the astaxanthin from *Pleuroncodes* planipes (—) in acetone, of reference Ax (Carophyll pink) (_._._) in petroleum ether and of rainbow trout flesh extracts (____) in acetone.



The utilized oil extraction process yielded an Ax oil extract of 61 mg/kg, equivalent to 42% of total pigment amount, which means that an Ax rich solid fraction remains after extraction that can have other applications in aquaculture. Some of the pigmented oil could have been retained with the solids during hand pressing separation or it might be influenced by the type of oil. Meyers et al. (33) have shown that different oils have different Ax extraction efficiencies whereas Omara-Alwala et al. (19), demonstrated that the Ax content in the recovered oil is highly enhanced through the extraction process.

TABLE 1
Chemical analysis and carotenoids content of diets after preparation[1]

Component g/100g	Test Diet	Control
Moisture	12,30±0,18	9,56±0,17
Crude protein (Nx6.25)	36,24±0,21	36,70±0,16
Crude fat	11,20±0,06	10,90±0,09
Ash	6,50±0,05	6,46±0,03
Crude fibre	2,66±0,23	2,81±0,20
Gross energy (Kcal/100)	397,4±0,22	415,5±0,25
Ax (mg/kg)	75,00±0,21	
Other carotenoids (mg/kg)	0,90±0,17	0,90±0,20

1 Data presented as mean of duplicate analyses ±s.d.

TABLE 2
Astaxanthin composition after thin layer chromatography analysis[1]

Source	Fraction of Ax	R_f value	max (nm)	Amount (%)
Langostilla	free	0,35-0,38	472	24
	monoester	0,64	470	40
	diester	0,83	476	36
Pigmented oil	free	0,36	470	8
	monoester	0,65	470-480	20
	diester	0,80-0,82	470-475	72
Test diet	free	0,36	475	8
	monoester	0,60	475-480	22
	diester	0,82-0,84	475-480	70
Trout muscle[2]	free	0,40	475	100
ReferenceAx	free	0,37	475	100

1 All spectrophotometric evaluations were made in acetone
2 Mean of five fish.

The mean carotenoid content in the test diet during the experiment was 72 mg/kg (dry weight). On the basis of the Ax fractions content in the whole langostilla, these were clearly modified in the oil-pigmented diet (Table 2). This predominance of esterified forms in the oil are in accordance with previous results (19) suggesting that the esters are preferentially extracted by the oils. Also, it can be suspected that Ax could undergo esterification during heat oil extraction by chemical process.

The basal formula contained 0,9 mg/kg of carotenoids other than Ax, probably derived from the component feedstuffs. Lutein, zeaxanthin and b-carotene can be present in commercial formula (10).

Organisms of group I did not accept easily the test diet during the first four days, after which they took it well. The reluctance might have been caused by natural soluble crustacean compounds with a characteristic odor. At the end of the experiment, specific growth rates (SGR) were 0,89 and 0,91 for the experimental and control group, respectively. Both trials showed no significant difference ($P>0,05$) in mean weights (Table 3) and SGR. Thus, no apparent effect of Ax oil supplementation was observed on fish development throughout the experiment. Water temperature might have affected

pigment metabolism and growth as referred by No and Storebakken (29).

On the tenth day, a bright pink color was observed along the lateral line of fish that were provided with pigmented diet. This color was intensified from the second week on and spread to the fins. The Ax was deposited in flesh at a rate of 0,06 mg/kg/day during the first three weeks, with a resultant pink color of fillets. This rate increased towards the end of the experiment to 0,10 mg/kg/day. This increase and the even flesh coloration of the experimented group obtained at the end of the study, suggests that there was a good utilization of pigmented oil by rainbow trout, *Oncorhynchus mykiss*, and could probably reach greater levels of pigmentation extending the feeding period to 8 weeks. In spite of the low free Ax amount in the diet, a final flesh Ax concentration of 3,60 mg/kg was achieved which is significantly higher than the minimum required for satisfactory pink-red color in rainbow trout (2-3 mg/kg) (14) and in the range stablished by Torrissen et al. (34) for commercial salmonids. The astaxanthin taken up from the feed might have followed two metabolic pathways in the trout: 1) it was either deposited directly as the free form, with an estimated deposition of 62%, or 2) the two fractions of Ax (free and esterified) were used prior to hydrolysis of the esterified compound as supported by Kamata and Simpson (10) and Storebakken and No (35) with an estimated deposition of 5% as free Ax in muscle.

Control group did not present any particular external pigmentation and the flesh color after six weeks was pale-gray with 0,4 mg/kg of carotenoids which were not distinguished as Ax by TLC.

Astaxanthin content and hue of rainbow muscle (Table 3) showed a significant correlation where r=0,988. According to hue values, chroma and Ax content as response variable at 3 and 6 weeks, pigmented and control group were significantly different (P<0,001). This obtained hue is comparable with that reported by Seurman et al., (12) and corresponds to 8 (according to a scale ranging from 0 to 8, 0 = means no visible pigmentation and 8 = maximum red) (3).

TABLE 3
Body weight increase and effect of dietary astaxanthin on flesh astaxanthin content and color of rainbow trout[1]

Trial	Weeks	Weight (g)	Ax content (mg/kg)	Hue angle ($tang^{-1}b^*/a^*$)	Chroma ($a^{*2}+b^{*2}$)
Experimental group (I)	0	218±7	0,20±0,05[2]	82,50±3,21[2]	9,36±1,00[2]
	3	261±8	1,55±0,25	50,27±2,95	17,52±2,73
	6	316±19	3,60±0,78	44,13±2,36	25,90±2,51
Control(II)	0	220±7	0,20±0,05[2]	82,50±3,21[2]	9,36±1,00[2]
	3	254±11	0,27±0,05	70,69±2,22	10,42±2,85
	6	325±23	0,40±0,10[2]	77,76±5,45	16,17±4,86

1 Mean ± s.d. of replicate groups.
2 Values of ten fishes for both groups at time zero.
3 Pigment not identified as Ax.

In view of our results, we conclude that although the oil extraction method was not an advantageous procedure (42% efficiency), the oil-pigmented feed did not have any deleterious effect on fish grouth and was palatable enough for the rainbow trout which obtained a good flesh Ax content (3,60±0,78 mg/kg ww), a red hue ($H°_{ab}$=44,13±2,36) and chroma (C^*_{ab}=25,90±2,51) to be considered as a marketable item.

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