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Astaxanthin and Canthaxanthin (Xanthophyll) as Supplements in Rainbow Trout Diet: In Vivo Assessment of Residual Levels and Contributions to Human Health

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ABSTRACT: Many studies have demonstrated that xanthophylls, such as astaxanthin, have beneficial effects in human health, and their use in food supplements is thus encouraged. Moreover, such nutrients are frequently used in aquaculture to meet consumer demand for salmonoid flesh pigmentation. In this study different xanthophyll administration protocols were tested to verify pigmentation properties and safety of such mixtures of additives in trout diet. Residues of xanthophylls in muscle samples were determined by HPLC-MS/MS, reaching levels of 3.70 ± 0.04 mg/kg (astaxanthin) and 1.21 ± 0.06 mg/kg (canthaxanthin) during a 56 day administration period. On the basis of the average fish consumption in the human diet, the highest astaxanthin and canthaxanthin concentrations detected in trout fillets could result in weekly intakes of 1.63 and 0.53 mg, respectively, in humans; these values are not sufficient to achieve the positive effects described by many authors, but their residues could still represent an important source of carotenoids, alternative to the use of synthetic dietary supplements.

KEYWORDS: astaxanthin, canthaxanthin, rainbow trout, HPLC-MS/MS, human health

INTRODUCTION

Xanthophylls are part of the wider group of carotenoids, a family of tetraterpenes produced by several microorganisms (Haematococcus pluvialis, Phaffia rhodozyma), higher plants, and invertebrates.1 They are derived from a ψ,ψ-carotene precursor that is modified by a combination of different processes (hydrogenation, dehydrogenation, cyclization, and oxidation). This large class of natural and synthetic products includes carotenes (e.g., β-carotene), retinoids (e.g., retinoic acid), and, of course, xanthophylls (canthaxanthin, astaxanthin, cryptoxanthin, lutein, etc.).

The characteristic red-to-pink flesh color of salmonids, an important criterion of quality for farmers and consumers, is due to the ability of these fishes to combine xanthophylls, such as astaxanthin (Ax) and canthaxanthin (Cx), with actomyosinic complexes in muscles.2 For this reason, such substances have

been introduced as specific feed additives and, due to their wide use in aquaculture, a maximum content in complete feed has been established by European Union (EU) legislation.3,4 In fact, toxicologically, xanthophylls are considered to be safe additives for animal and human consumption,5 with the only warning referring to Cx administration (also known as food additive E161g), for which the maximum level as an additive in aquaculture feed was reduced from 100 to 25 mg/kg.6,7 Potential toxic effects of Cx are ocular lesions due to macular crystal formation, observed in monkeys, fish, and other farmed species, as well as in sporadic human cases.8 In the majority of described human cases, the Cx toxic effects were due to a large ingested quantity of food supplements for cosmetic skin coloration and/or dermal photo protection.9 Therefore, the acceptable daily intake (ADI) for Cx in humans was set to 0.03 mg/kg bw/day by the Joint FAO/WHO Expert Committee on Food Additives in 1995. Feeding Ax and Cx at the maximum admitted level, once a day, is the prevalent practice in certain cases of trout farming, to obtain the most appealing flesh color on the market. However, some studies10 have demonstrated that this practice could be counterproductive, from an economic perspective, without providing any advantage in Ax and/or Cx accumulation in salmonid flesh. Therefore, one of the aims of this study was to evaluate different in vivo experimental administrations of Ax and Cx, in terms of muscle coloration and Ax and Cx flesh concentrations, to verify the absence of any toxic effect on the animals.

Moreover, different studies on xanthophylls, from both natural sources and dietary supplements, have also been performed in human diets,11 revealing, especially for Ax, several beneficial effects.12,13 Recently it has been reported that flavonoids have neuroprotective activities in cultured cortical neurons.14 Carotenoids can attenuate amyloid β-induced damage in different cell types,15 as well as interfere with the activities of ABC-transporters in cancer cells, often involved in multidrug-resistance episodes.16,17 Particularly, in vitro studies have clearly demonstrated that Ax is several-fold more active as a free radical antioxidant than β-carotene and α-tocopherol.18

The consumption of foods or dietary supplements that provide Ax, lycopene, lutein, and glabridin can ameliorate endothelial inflammation and oxidative stress, retard at herogenesis, and decrease the risk for atherogenic cardiovascular disease.19

It has been reported that xanthophylls could provide antioxidant protection for human skin20 and that they could also protect mice against UVB-induced epidermal hyperproliferation and acute inflammation.21 The same antioxidant effects were observed in humans and rodents supplemented with β-carotene,22 lycopene,23 canthaxanthin,24 and mixed carotenoid administration.25,26

Recently, Park et al.27 demonstrated that dietary astaxanthin supplements could reduce in young healthy females both DNA damage biomarkers and acute phase proteins, enhancing immune response.

In light of the interesting effects of these molecules, especially astaxanthin, efforts have been channeled into investigating sources of xanthophyll in foods and food supplements in recent years.28

However, there is a lack of studies that clearly link Ax and Cx carry-over into the food chain and their contribution to the human diet.

Therefore, the first aim of this study was to verify the entity of xanthophyll residues in farmed trout muscle when administered through different diet protocols.29

Second, we aimed to verify the human intake of such molecules through farmed trout consumption and, consequently, the achievement of Ax doses related to beneficial effects.27

MATERIALS AND METHODS

Chemicals. Feed additives CAROPHYL Pink and CAROPHYL Red were obtained from DSM Nutritional products (Basel, Switzerland). Both astanxanthin and canthaxanthin were certified as analytical standards and were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). All solvents used for analytical determinations were of high-performance liquid chromatography (HPLC) grade.

Butylated hydroxytoluene (BHT) and MS-222 (tricaine methanesulfonate) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pure water used for all experiments was prepared by using a Milli-Q system (Millipore, Bedford, MA, USA).

Animal Research and Diets. A total of 480 rainbow trout (Oncorhyncus mykiss), of both sexes, were kept at the aquaculture center of Turin University (Carmagnola, Turin, Italy) for 2 weeks to facilitate acclimation to culture conditions; during this period, fish were fed a pigment-free diet.

A total of 60 individual rainbow trouts (initial mean weight = 200 g) were then allocated to each of the eight freshwater tanks. In-house pelleted feedstuffs were prepared from the same basal composition (Table 1) and supplemented with Ax and/or Cx. Analyses of their content in prepared feedstuffs revealed final concentrations higher than those expected, probably due to natural sources of the pigments in the raw materials used (Table 2).

Trouts were hand-fed at 1% body mass, providing the same amount of feedstuffs once a day or split into two meals a day, according to the planned trial (Table 2), for 8 weeks. This protocol was approved by the scientific committee of our institution. Every 14 days, at different time points (T1 = 14 days, T2 = 28 days, T3 = 42 days, and T4 = 56 days), five specimens from each tank were collected and euthanized with a lethal dose of MS-222 (250 mg/L). Wet weight and length measurements were noted together with colorimetric determination of flesh pigmentation, performed by a bench colorimeter Chroma Meter CR-400 Konica Minolta Sensing (Minolta Sensing Inc., Osaka, Japan).

Chemical Determination

Fish tissues were extracted using the Baker et al.31 method that was slightly modified to avoid the use of salts and to obtain cleaner extracts: the skin- and bone-free fillet of each fish was minced while frozen, and 2 g of minced muscle was then accurately weighed into a polypropylene tube and homogenized with 0.5 mL of ultrapure water and 3 mL of acetone. Tubes were then firmly closed, vigorously shaken, and then centrifuged at 3500 rpm for 10 min. Supernatants were then transferred into a clean glass tube; an additional 3 mL of acetone was added to the initial sample, the extraction procedure was repeated, and the new extract was combined with the first one. Five milliliters of tert-butyl methyl ether was then added to the extract, and tubes were again vigorously shaken for 5 min.

After this step, 2 mL of ultrapure water was added to the tubes to allow separation of solvents. After centrifugation for 5 min at 3000 rpm, supernatants were evaporated under a flow of nitrogen. Residues were then dissolved with 0.2 mL of acetonitrile containing 500 mg L−1 BHT.

The HPLC system consisted of an Agilent 1100 series (G1311A quaternary pump), a SecurityGuard C18 (4 × 3.0 mm i.d.), and a Synergi 4 μm Polar-RP 150 × 2 mm column, both supplied by Phenomenex, Torrance, CA, USA. As detector, an API 4000 triplequadrupole mass spectrometer (ABSciex, Framingham, MA, USA) was chosen.

Chromatographic separation was performed using acetonitrile (eluent A) and ultrapure water (eluent B) for the mobile phases, at a flow rate of 0.5 mL/min: 50% eluent A at time 0, and 100% eluent A at time 6 min until 10 min. Re-equilibration of the column was carried out for 4 min prior to the next run. A 10 μL injection volume was applied in each run.

Triple-quadrupole parameters and precursor−product ion transitions are listed in Tables 3 and 4. The atmospheric pressure chemical ionization (APCI) source, in positive ion mode, was chosen, and nitrogen was used as ionization and dissociation gas (Figures 1 and 2). Astaxanthin and canthaxanthin were quantified following the integration and comparison of peak areas to those of the matrixmatched calibration curve.

Statistical Methods. To test the differences in growth between animals from treated and untreated tanks and to verify differences in Ax and Cx concentrations between treated groups, one-way ANOVA was performed. P values of <0.01 were considered statistically significant ($p < 0.01$).

RESULTS AND DISCUSSION

Morphological and colorimetric characteristics, along with astaxanthin and canthaxanthin determinations, were performed at established time points (five specimens for each tank).

Although of lesser concern, morphometric parameters such as weight and length were collected during the investigation; mean values and relative standard deviations are reported in Figures 3 and 4. No significant differences ($p > 0.01$) in growth can be observed between animals from treated tanks (tanks B, C, D, G, H, and L) and untreated ones (tanks A and E).

All fillets from treated groups acquired the expected pink/red flesh color at the end of applied exposure protocols.

Colorimetric determination of muscle pigmentation at different time points (14, 28, 42, and 56 days) highlighted no significant differences among treated groups (data not shown).

Analytical determinations were characterized by standard deviations ranging from 0.03 to 0.23 mg/kg for Cx and from 0.01 to 0.37 mg/kg for Ax: concentrations in muscle samples versus sampling times are shown in Figures 5 and 6. Observed levels of both carotenoids increase in a nonlinear way in all groups until 42 days.

After 56 days (T4), slight increases of both Ax concentration in tank L and Cx concentration (Figures 5 and 6) in tank D were recorded, but without any statistical significance ($p > 0.01$). In all other tanks, the concentration decreased after 42 days of exposure. For this reason, we considered T3 sampling (42 days of exposure) for the reference time point for xanthophyll accumulation in trout flesh.

In fact, the highest concentration of Cx (1.21 ± 0.06 mg/kg) was recorded after 42 days of treatment in tank H (two meals/ die of Cx alone). This concentration was, however, below the maximum residue level established by European legislation (5.00 mg/kg).

In addition, for Ax, the highest accumulation level in muscle samples was reached after 42 days (3.70 ± 0.04 mg/kg). No differences were observed in accumulation rates of Ax between one administration/die groups (tanks B and D) and two administration/die groups (tanks G and L), contrary to the results by Dixon et al.25

The Italian National Institute of Statistics estimates a weekly fish intake of 441 g in the Italian population.32 As previously demonstrated in this study, after 42 days of treatment, the Ax concentration in trout flesh can reach 3.70 ± 0.04 mg/kg wet tissue, resulting in an Ax weekly intake of 1.63 mg of Ax.

Moreover, the highest Cx concentration detected was 1.21 ± 0.06 mg/kg, corresponding to a weekly intake of 0.53 mg; this value, for a person of 75 kg, corresponds to a daily intake level of 0.001 mg, still well below the ADI set for this substance (0.003 mg).

These values obtained from data on fresh muscle should be further rearranged, taking into account the average carotenoid depletion due to domestic cooking procedures.33,34 The results of this study assessed that canthaxanthin and astaxanthin levels, in all muscle samples from rainbow trout fed different diet protocols, were compliant with legislation.

In the concentration ranges of both supplements used in the exposure protocols, no adverse effects on animals were observed, according to the concentration established for feed safety assurance. Furthermore, accumulation rates of canthaxanthin and astaxanthin in muscle samples reached the highest values after 42 days, allowing reduction of the treatment time and, consequently, the production costs in aquaculture. Variations in muscle Ax and Cx concentrations are due to different factors, such as the carotenoid nature and concentration, fish size, or physiological state.10 In agreement with Wathne et al.35 and in contrast with Choubert et al.,29 the alternate Ax feeding, compared to continuous feeding, did not influence Ax deposition in fish muscle. In light of several studies on carotenoid effects, the integration of "xanthophylls-enriched" foods in the diet represents a good opportunity for increasing human health.

Xanthophylls, such as zeaxanthin, lutein, and Ax, can protect against DNA damage. Furthermore, carotenoid addiction in neuroblastoma cells, after UVA ray exposure, has been demonstrated to positively influence DNA repair kinetics. In vitro studies have also highlighted the beneficial effects of Ax on human neutrophil functions.36 Moreover, Kanazashi et al.37 have recently demonstrated that Ax can up-regulate angiogenic factors and reduce capillary regression in rats with hindlimb unloading.

Miyazawa et al.38 suggested that even with a low dose of astaxanthin (1 mg/die), the concentration in the plasma could reach levels suitable for antioxidant effects.

Even if many studies have demonstrated that Ax is more efficiently deposited than Cx in rainbow trout,39,40 concentrations detected in the flesh are not sufficient to reach the beneficial effects described by many authors; nevertheless, it could represent an additional source of carotenoids, limiting the use of dietary supplements. Moreover, Barros et al.41 have recently suggested that habitual consumption of fish containing astaxanthin (such as salmon or, in our case, trout) can improve the immune response and reduce vascular and infectious diseases. These effects can be due to the combined cumulative effects of Ax and fish oil consumption, resulting in hypolipidemic/hypocholesterolemic actions, improvement of phagocytic activity in activated neutrophils, and an antioxidant effect in PMA-activated neutrophils.

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Notes: The authors declare no competing financial interest.

ABBREVIATIONS USED

HPLC-MS/MS, high-performance liquid chromatography– tandem triple-quadrupole mass spectrometry; Ax, astaxanthin; Cx, canthaxanthin; APCI, atmospheric pressure chemical ionization; EFSA, European Food Safety Agency; ADI, acceptable daily intake; FAO, Food and Agricultural Organization; WHO, World Health Organization; MS-222, tricaine methanesulfonate; BHT, butylated hydroxytoluene; ANOVA, analysis of variance; IZS PLV, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta

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Table 1. Ingredients and Proximate Composition of Experimental Diets (on Weight Basis)

ingredient	%
fish meal	50
corn gluten meal	10
dehulled barley meal	18
corn meal	9
cod liver oil	7
binder (Merigel)	3
mineral mixture[a]	1.5
vitamin mixture[b]	1.5
proximate composition	**%**
crude protein	44.6
ether extract	11.88
ash	7.92
gross energy[c] (MJ/kg)	20.60

[a]Mineral mixture (g or mg/kg diet): bicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt, 40 g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulfate, 20 g; zinc sulfate, 4 g; copper sulfate, 3 g; potassium iodide, 4 mg; cobalt sulfate, 20 mg; manganese sulfate, 3 g; sodium fluoride, 1 g (Granda Zootecnica, Cuneo, Italy). [b]Vitamin mixture (IU or mg/kg diet): DL-α-tocopherol acetate, 60 IU; sodium menadione bisulfate, 5 mg; retinyl acetate, 15000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnica). [c]Calculated following Cho and Kaushik.[30]

Table 2. Experimental Design

			concentration (mg/kg feed)	
tank	feed additive	meal/die	expected	experimental
A		1	0	0
B	Ax	1	75	116.2
C	Cx	1	25	19.9
D	Ax	1	75	132.9
	Cx		25	27.5
E		2	0	0
G	Ax	2	75	116.2
H	Cx	2	25	19.9
L	Ax	2	75	132.9
	Cx		25	27.5

Table 3. Triple-Quadrupole Parameters

curtain gas	15
temperature (°C)	400
ion source gas 1	30
nebulizer current (μA)	3
interface heater status	ON
collision-activated dissociation gas	8
ion spray voltage (V)	5000
entrance potential (V)	10
collision energy exit potential (V)	15

Table 4. Precursor–Product Ion Transitions

analyte	M (uma)	$(M + H)^+$	declustering potential (V)	transition	collision energy (V)
Cx	564.8	565.5	83	565.5–203.1	27
				565.5–547.3	22
Ax	596.8	597.6	78	597.6–579.3	17
				597.6–285.1	24
				597.6–299.3	24

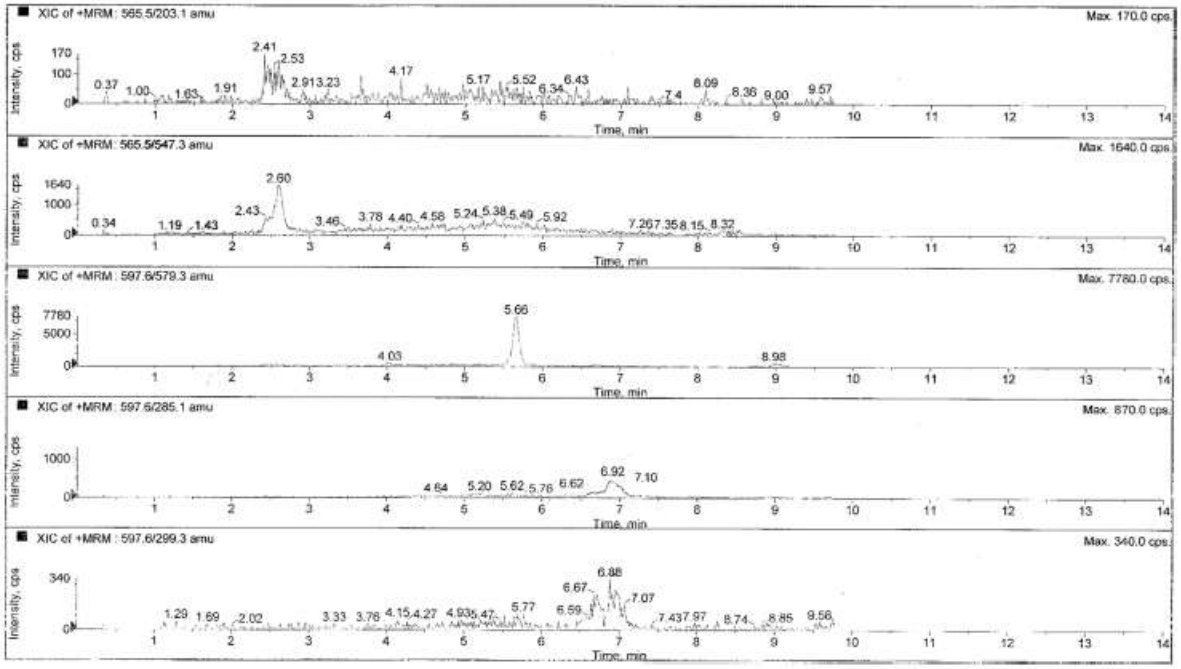

Figure 1. Selected ion chromatograms recorded from trout muscle nonspiked.

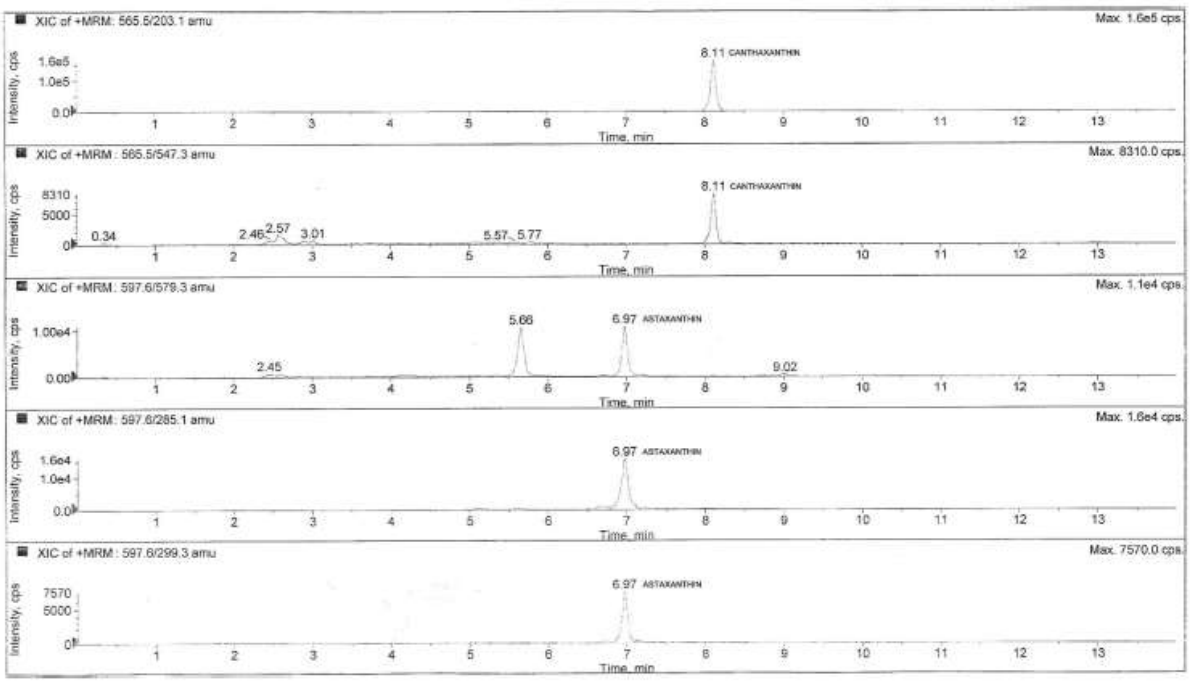

Figure 2. Selected ion chromatograms recorded from trout muscle spiked with Ax and Cx at a concentration of 1 $\mu g/mL$.

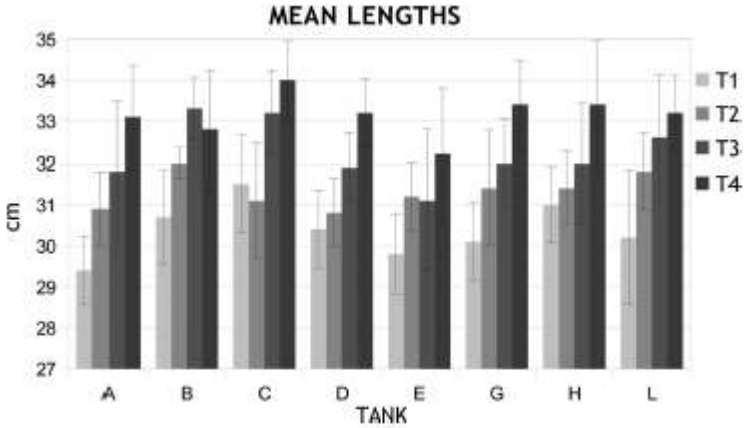

Figure 3. Mean lengths (cm) measured during the experiment.

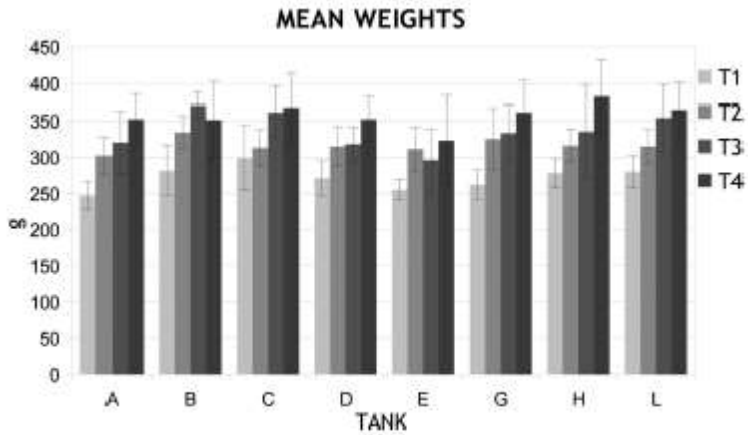

Figure 4. Mean weights (g) measured during the experiment.

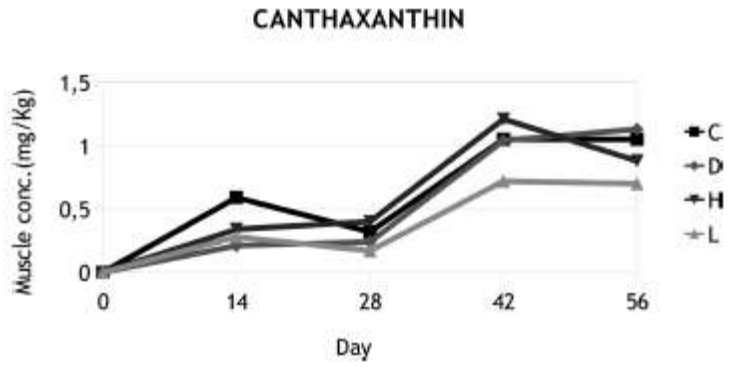

Figure 5. Ax concentrations in muscle samples versus sampling times.

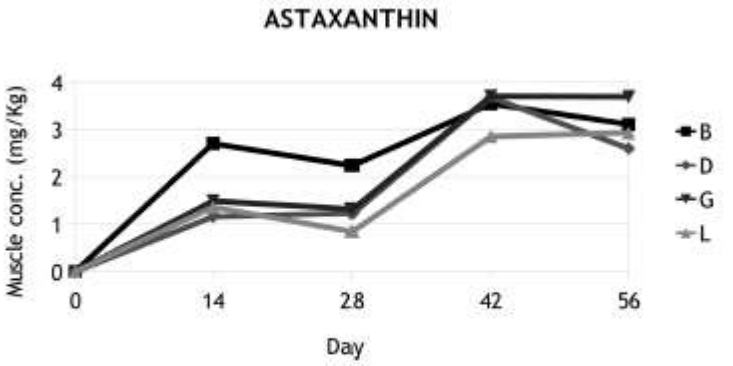

Figure 6. Cx concentrations in muscle samples versus sampling times.