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Chromium oxide ($^{51}Cr_2O_3$) used as biological marker was not absorbed by fish

[*Óxido de crômio ($^{51}Cr_2O_3$), usado como marcador biológico, não é absorvido por peixe*]

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ABSTRACT

The aim of this study was to evaluate the rate absorption of radio-labeled chromium oxide ($^{51}Cr_2O_3$), used as biological marker in nutrition studies with Nile tilapia *Oreochromis niloticus*. An experimental diet with approximately 58 μCi of specific activity of the element was encapsulated and fed daily to 35 adult Nile tilapia; a group of 35 fish was used as control feeding on a basal diet. At the beginning of the experiment five fish from each group were randomly selected and blood samples were drawn from control (BC) and experimental fish (BE). Fish were then euthanized by anesthetic overdoses and samples of the liver tissue (LT), renal tissue (RT), stomach without content (S), intestine without content (I), gills tissue (GT), muscle tissue (fillet; MT), visceral fat (VF), content of the digestive tract (CTDE) and water aquarium were collected from the experimental fish. The procedure was repeated daily for one week. Simple linear regressions were adjusted – days of collection vs. determination coefficients, and were established for statistical comparisons of the measured activity of ^{51}Cr readings in sampled blood and tissues (logarithmic transformation) for samples of the control and experimental fish. No differences ($P>0.05$) were detected between samples from BC fish and BE, RT, VF, MT and LT of treated fish, but samples of GT, I, S, CTDE and WA from the tanks holding fish which received the experimental diet differed from control ($P<0.05$). The experimental results indicate that the trivalent chromium in the form of $^{51}Cr_2O_3$ was not significantly absorbed by the gastrointestinal tract, gills or another possible route of absorption under these experimental conditions and with Nile tilapia. Therefore, this marker was shown to be inert and can be safely used in nutrition studies.

Keywords: Nile tilapia, *Oreochromis niloticus*, absorption, digestibility, nutrition

RESUMO

O objetivo deste estudo foi avaliar a taxa de absorção de radiomarcador óxido de crômio ($^{51}Cr_2O_3$), utilizado como marcador biológico em estudos de nutrição, com tilápia-do-nilo Oreochromis niloticus. *Uma dieta experimental com cerca de 58μCi de atividade específica do elemento foi encapsulada, e 35 adultos de tilápia foram alimentados diariamente; um grupo de 35 peixes foi usado como controle e alimentado com uma dieta basal. No início do estudo, cinco peixes de cada grupo foram selecionados aleatoriamente, e amostras de sangue foram coletadas dos peixes controle (BC) e experimentais (BE). Os peixes foram sacrificados por overdose de anestésicos, e amostras do tecido do fígado (LT), rins (RT), estômago sem conteúdo (S), intestino sem conteúdo (I), brânquias (GT), tecido muscular (filé; MT), gordura visceral (VF), conteúdo do trato digestivo (CTDE) e água do aquário (WA) foram coletadas somente dos peixes experimentais. O processo foi repetido diariamente durante uma semana. As regressões lineares simples foram ajustadas – dias de coleta* versus *coeficientes de determinação – e foram estabelecidas para comparações estatísticas da leitura das atividades medidas de ^{51}Cr (transformação logarítmica) nas amostras dos peixes controle e experimentais. Não foram detectadas diferenças (P>0,05) entre as amostras BC dos peixes controle e BE, RT, VF , MT e LT dos peixes*

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experimentais, mas as amostras de GT, I, S, CTDE e WA dos peixes que receberam a dieta experimental apresentaram diferença significativa em relação aos que receberam a dieta controle (P<0,05). Os resultados experimentais indicam que o crômio trivalente na forma de $^{51}Cr_2O_3$ não foi significativamente absorvido pelo trato gastrointestinal, pelas brânquias ou por outra via possível de absorção nessas condições experimentais e com tilápia do Nilo. Portanto, esse marcador demonstrou ser suficientemente inerte, o que torna seguro seu uso em estudos de nutrição.

Palavras-chave: tilápia-do-nilo, Oreochromis niloticus*, absorção, digestibilidade, nutrição*

INTRODUCTION

The feedstuffs nutritional value can be measured by the apparent digestibility coefficient (ADC) of ingredients, that is, the percent of ingested nutrients not recovered in fecal excretions. The indirect method uses a dietary inert marker, not requiring, therefore, total feces recovery. Among the ideal characteristics of a marker is the capacity of the marker to be completely recovered in feces and not be absorbed (Souza *et al.*, 2013), and among other criteria, an external marker has to be inert, with no toxic or physiologic effects (Ng and Wilson, 1997).

Currently, Cr_2O_3 can be added to experimental diets at rates between 0.01% and 3% (Bremer Neto *et al.*, 2005), but even in such small amounts it has been shown that the chromium oxide may not be totally inert, affecting the assessment of diet digestibility with implications in the nutrition and feeding practices of the species (Shiau and Liang, 1995), which seems to indicate that it is being absorbed and retained in the fish body (Shiau and Liang, 1995; Shiau and Shy, 1998). They speculated that the poorer growth performance of tilapia fed the higher level of chromic oxide may be due to a toxic effect of dietary chromium (Ng and Wilson, 1997).

The chromium mineral, when absorbed by fish, mainly accumulates in metabolically active organs such as the liver and kidneys at high concentrations (Svecevicius, 2009) and previous studies reported a fast passage rate of ^{51}Cr, through the gastrointestinal tract (Oberleas and Stoecker, 1987) and fast peaks of ^{51}Cr in the blood (Hopkins Junior, 1965) of animals.

This study aimed to determine if there is actually absorption of radio-labeled chromic oxide III – $^{51}Cr_2O_3$ – used as inert marker in experimental diets for Nile tilapia, *Oreochromis niloticus*.

MATERIAL AND METHODS

A total of 400 adult Nile tilapia (*Oreochromis niloticus*), sexually reverted to male were acclimatized in the laboratory at the Center for Nuclear Energy in Agriculture - CENA / USP, Piracicaba, Brazil for one month at 28±0.2°C, which was the temperature for the experimental conditions and feeding once a day to near satiety with a basal diet included into 0.5g jelly capsules (Tab. 1), containing 3,200 kcal kg^{-1} of digestible energy (DE) and 32% of crude protein (CP) (Association of Official Analytical Chemists – AOAC, 1990; National Research Council – NRC, 1993). Cornstarch (Amilogill 2100; Cargill Agr. S.A., Uberlândia, MG, Brazil) was added to the diet (40%) as a source of carbohydrates.

The fish were distributed in 400 110L aquariums, with external measurements of 80x35x45cm, with 1 fish per tank. The tanks were fitted with constant aeration stones through micropores connected to a central blower, an individual biological filtering system. The water was heated with individual 100W heaters. A 12h dark:12h light photoperiod was maintained throughout the trial. The ration level, feeding regimen, temperature and light-dark cycle were the same as during the acclimation period and during this period the stabilization of the biological filter occured.

Routinely, 15 minutes after feeding all uneaten capsules were removed from tanks and the water was replenished. Every other day tanks were siphoned to reduce residues and avoid accumulation of feed waste and feces. This study was approved by the Institutional Ethics and Animal Welfare Committee.

Water quality parameters – temperature, dissolved oxygen (DO), pH, total ammonia nitrogen (TAN), un-ionized ammonia (NH_3-N), nitrite-nitrogen (NO_2-N), nitrate-nitrogen (NO_3-N) – were monitored routinely using a Shimadzu

UV 1601 PC spectrophotometer (Kyoto, Japan) and were determined following standard methods (APHA, 1995).

The experimental diet was prepared with a pre-mixture of 20 g basal diet plus 0.0074g $^{51}Cr_2O_3$ [gamma emission; half-life 27.8 days (Anderson, 1981)], specific activity 16 μCi (592 MBq)[$^{51}Cr_2O_3$ 99,99% purity (Nuclear and Energies Research Institute – IPEN, São Paulo, Brazil)], and 0.00635g Cr_2O_3 (99.99 % purity; Aldrich Chemical Company, Inc.). It was done in a porcelain bowl. The remaining basal diet (117.5g) was then added and carefully homogenized to the pre-mixture and included into 0.5g jelly capsules. The measured average radioactivity of each capsule was 58.2μCi, a marker's total concentration of 100 $\mu g.g^{-1}$ (0.01% in diet) (Comar, 1955; Bremer Neto *et al.*, 2005).

Table 1. Formulation and approximate composition (%) of the experimental diet

	Diet
Formulation	
Premix [a.b]	58.50
Corn starch	40.00
Carboximethyl cellulose	1.49
Cr_2O_3 ($\mu g.g^{-1}$)	53.80
$^{51}Cr_2O_3$ ($\mu g.g^{-1}$)	46.20
Concentration of analyzed Cr (%)	0.01
Approximated composition	
Water (W)	6.75
Crude protein (CP)	31.98
Ether extract (EE)	11.11
Mineral matter (MM)	9.64
Crude fiber (CF)	4.76
Free nitrogen extract (FNE)[c]	35.74

[a]Premix containing (%): fish flour (Agribands of Brasil Ltda, Brasil), 45.00; cod-liver oil, 2.0; wheat germ oil, 6.0; lisina, 1.0; cellulose, 4.0 and mineral and vitamin premix, 0.5;
[b]Supplement vitamin + mineral (Supremais): Composition for kilogram of the product: Vit. A = 1 200,000 UI; vit. D3 = 200,000 UI; vit. E = 12,000 mg; vit. K3 = 2,400 mg; vit. B1 = 4,800 mg; vit. B2 = 4,800 mg; vit. B6 = 4,000 mg; vit. B12 = 4,800 mg; folic acid = 1,200 mg; pantothenate of calcium = 12,000 mg; vit. C = 48,000 mg; biotin = 48 mg; hill = 65,000 mg; nicotinic acid = 24,000 mg; Fe = 10,000 mg; Cu= 600 mg; Mn = 400 mg; Zn = 6,000 mg; I = 20 mg; Co = 2 mg and Se = 20 mg;
[c]Obained through subtraction (100 – W - CP – EE – MM – CF).

On the first day of the experimental period seventy fish, randomly selected from an original group of 400 fish, were weighed (673±3.6g) and fed one capsule of the marked diet plus capsules containing the basal diet until apparent satiation; control fish were fed capsules containing basal diet alone. After 24 hours, five fish from each group were randomly selected, anesthetized [Ethylene glycol monophenyl ether - $0.2mL.L^{-1}$ (96%, Merck Chemicals)], and blood samples were drawn from control (BC) and experimental fish (BE). Fish were then euthanized by anesthetic overdoses and samples of the liver tissue (LT), renal tissue (RT), stomach without content (S), intestine without content (I), gills tissue (GT), muscle tissue (fillet; MT), visceral fat (VF), content of the digestive tract (CTDE) and water tanks were collected from the experimental fish. The procedure was repeated daily for one week. The specific activity was determined in all samples (Comar, 1955). Radioactive wastes were discarded according to normalizations CNEN-NE-6.05, November 1985.

Samples readings of ^{51}Cr activity [countings per minute (cpm)] from control and experimental fish were logarithmically transformed [$\log_{10}$ ($cpm.g^{-1}$ or $cpm.mL^{-1}$ + 10)], fitted to regression curves and compared through the angular and linear coefficients, in function of exposure time (days) (Ostle and Mensing, 1975). The differences between the regression coefficients were significant at 5% level.

RESULTS AND DISCUSSION

Water quality parameters – temperature (28.2 ± 0.2°C), dissolved oxygen % (107.4 ± 0.8), pH (7.9 ± 0.1), total ammonia nitrogen (2.8 ± $0.0mg.L^{-1}$), un-ionized ammonia (0.004 ± $0.001mg.L^{-1}$) nitrite-nitrogen (0.08±$0.01mg.L^{-1}$) and nitrate-nitrogen (1.19±$0.06mg.L^{-1}$) – were considered appropriate for fish welfare and natural development (Boyd, 1990).

Chromium oxide is the most extensively used inert marker in nutrition studies with animals, and feedstuffs enable its evaluation and precise formulation of balanced diets. Complete recovery of Cr^{3+} in oxide form was confirmed through the radioactive isotope technique (Kane

et al., 1959) and for this reason it was used in this study, as $^{51}Cr_2O_3$ (99.99% purity).

The ^{51}Cr was used in this study to prevent possible errors due to recovery of the chromium in feed, water of aquarium, faeces and tissue samples in fish, since it allows direct, simple, sensitive and more accurate, elemental reading in sample material, reducing bias associated to chemical analysis, especially in digestibility and nutrition studies. All these factors were carefully monitored and controlled in this study so results could be credited exclusively to treatment effects. Table 2 demonstrates the results, standard deviation and the regression parameters established after logarithmic transformation [log10 (cpm.g^{-1} or cpm.mL^{-1} + 10)] of the specific activities of ^{51}Cr detected in samples collected from the control and experimental fish, versus time (days) collection. The regressions were compared through the angular and linear coefficients (Ostle and Mensing, 1975).

To investigate if there is chromium III absorption in fish, such as chromium oxide, simple linear regressions were adjusted – days of collection vs. determination coefficients for samples of the control and experimental fish (Figure 1). Background values measured for fish tissue samples which did not receive the experimental diet, 10,000 counts in five intervals, averaged 98.02±3.61cpm, and were subtracted from all reading values from the control and experimental fish samples.

Intestinal absorption of trivalent chromium (e.g. chlorides, fluorides, phosphates, nitrates, and hydroxides), is low in both humans and animals, varying between approximately 0.5 and 2.0% depending on dietary intake. Some data indicates that chromium absorption is inversely related to its dietary intake (Anderson and Kozlovsky, 1985). In this experiment, the level of chromic oxide added to the diet was only 0.01% and according to the authors mentioned above, at this small rate the chromium would lead to increased absorption by the digestive tract and therefore would be more readily detected in experimental fish samples.

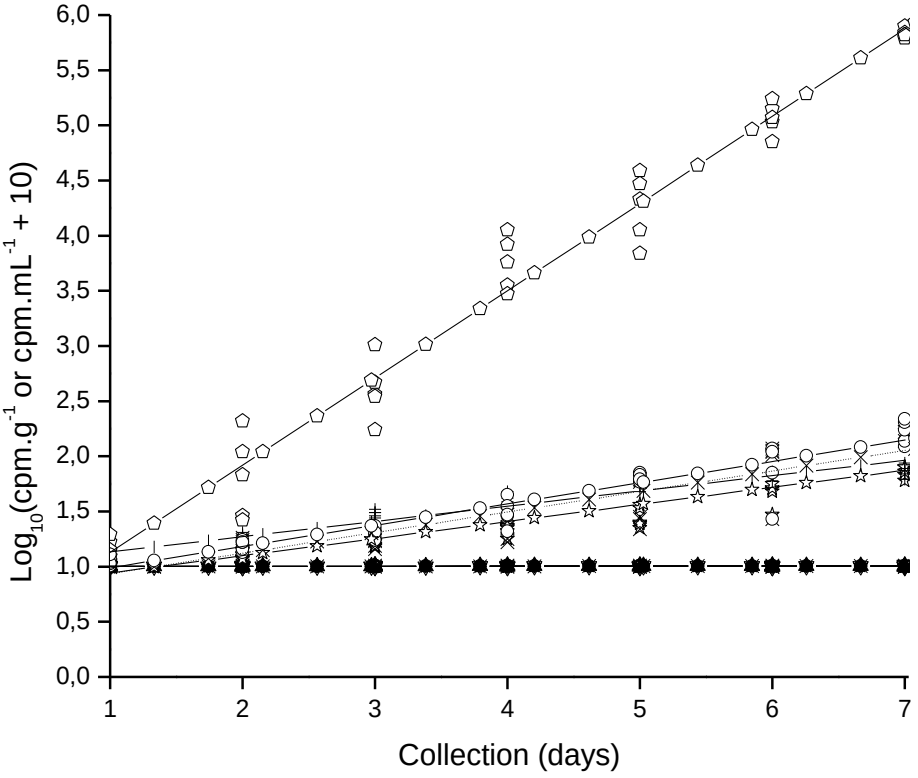
The adjusted regressions for blood samples of the fish fed with the control diet for seven days, control animals, when compared with regression adjusted with samples of fish fed with the experimental diet did not differ among themselves ($P>0.05$). These results agree with those obtained by Utley *et al*. (1970), who also used radioactive chromium oxide (III) orally, but administered to bovines, and did not detect radiation in heifers' blood. Other studies reported fast passage rate of $^{51}CrCl_3$ through the gastrointestinal tract (Oberleas and Stoecker, 1987) and fast peaks of ^{51}Cr in the blood (Hopkins Junior, 1965) of rats and levels in blood reflect chromium (III) intake. Therefore, if the chromium oxide suffers absorption by the gastrointestinal tract, their presence would have to be detected quickly in the blood of fish in this study.

In blood, absorbed chromium (III) is bound mostly to transferrin and to other proteins, which are responsible for its transport in the body. Long-term storage occurs particularly in the liver, spleen, bones and other organs (Lim *et al.*, 1983). The accumulation patterns of chromium are in the following order: kidney > liver > gill ≈ muscle, for lower concentrations (Palaniappan and Karthikeyan, 2009).

If the chromium in the form of inert marker was absorbed by the Nile tilapia, this mineral is accumulated in the fish tissues. Therefore, to determine if there was absorption of chromium as a marker, through the digestive tract of Nile tilapia, blood samples were compared to adjusted regression from control fish, with regression adjusted samples of visceral fat, liver tissue, muscle tissue and kidney tissue of fish that received the experimental diet and those did not differ among themselves ($P>0.05$), and so are characterized as belonging to a single linear regression. These results suggest that there was no radioactivity detected in samples of experimental fish, suggesting no significant uptake of the marker.

The mechanism responsible for the intestinal absorption of chromium is not well understood. It is unclear whether Cr is absorbed passively or with the aid of carrier proteins located in the intestinal mucosa. Mertz *et al.* (1965) reposted that the absorption of trivalent Cr does not appear to be a saturable process, which suggests that it is absorbed by passive diffusion. Mertz and Roginski (1971) reported contrary evidence. They found that the percentage of trivalent chromium absorbed by everted gut sacs

decreased as the Cr concentration increased in the incubation medium. This observed saturation effect suggests that carrier proteins are involved in Cr absorption. However, in the experiment by Dowling *et al*. (1989) it was concluded that inorganic, trivalent chromium is absorbed by the nonmediated process of passive diffusion in the small intestine of rats fed a Cr adequate diet.



Samples control fish	Parameters of regressions - Control fish
▽ Blood (BC)	—▽— y=1.00+4.52E-4x R=0.16 SD=0.01[d]
Samples experimental fish	**Parameters of regressions - Experimental fish**
▲ Blood (BE)	—▲— y=1.00+4.59E-4x R=0.19 SD=0.01[d]
● Renal tissue (RT)	—●— y=1.00+3E-4x R=0.12 SD=0.01[d]
★ Muscle tissue - fillet (MT)	—★— y=1.00+3.43E-4x R=0.12 SD=0.01[d]
✳ Liver tissue (LT)	—✳— y=1.00+4.91E-4x R=0.20 SD=0.00[d]
ı Visceral fat (VF)	—+— y=1.00+4.26E-4x R=0.18 SD=0.00[d]
⬠ Digestive tract content (CTDE)	—⬠— y=0.34+0.79x R=0.99 SD=0.25[a]
☆ Gill tissue (GT)	—☆— y=0.84+0.13x R=0.96 SD=0.08[c]
+ Water from the aquarium (WA)	—+— y=0.99+0,14x R=0.96 SD=0.08[c]
× Stomach without content (S)	···×··· y=0.75+0.19x R=0.91 SD=0.17[b]
○ Intestine without content (I)	—○— y=0.79+0.19x R=0.95 SD=0.13[b]

Note: [a]The regression adjusted samples of CTDE differed significantly from the others (P<0.05); [b]The regression adjusted samples of S and I did not differ significantly from each other (P>0.05) and differed from the others (P<0.05); [c]The regression adjusted samples of GT and WA did not differ significantly from each other (P>0.05) and differed from the others (P<0.05);[d]The regression adjusted samples of BC, BE, RT, MT, VF and LT did not differ significantly from each other (P>0.05) and differed from the others (P<0.05).

Figure 1. Adjusted linear regressions after logarithmic transformation of the estimates of radioactive activity (cpm) of sample collections of the control and experimental fish in function of time (days) of exposure.

According to the results obtained by Febel *et al*. (2001), 2.5% of chromium oxide was absorbed during an hour and the absorbed chromium was transferred to the liver where the liver tissue retained 10.9% of chromic oxide. These results differ from the results of this study, we believe it is because there was no significant increase of chromium ^{51}Cr in the fish tissues analyzed: blood, visceral fat, liver, kidneys and file of fish, which have not occurred to suggest absorption and consequent bioaccumulation of marker. What differed between the experiments was the system employed to maintain the right conditions for fish life in the aquarium, concentration of the marker added to the food and fish species.

Table 2. Logarithmic transformation [$\log_{10}$ (cpm.g^{-1} or cpm.mL^{-1} + 10)] of the detected specific activities of the ^{51}Cr in the sample collections of the control and experimental fish and those which were used to adjust the regression curves.

Collected sample	Day of the collection						
	1	2	3	4	5	6	7
	Control fishes						
Blood (BC)	1.00±0.01[A]	1.00±0,01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.01±0.01
	Experimental fishes						
Blood (BE)	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.01±0.01
Renal tissue (RT)	1.00±0.01	1.00±0.01	1.00±0.01	1.01±0.01	1.00±0.01	1.00±0.01	1.01±0.01
Muscle tissue - Fillet (MT)	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.01±0.01
Liver tissue (LT)	1.00±0.01	1.00±0.00	1.00±0.00	1.00±0.01	1.00±0.01	1.00±0.00	1.01±0.01
Visceral fat (VF)	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01
Intestine without content (I)	1,06±0.04	1.19±0.05	1.30±0.05	1.49±0.12	1.82±0.03	1.89±0.0.27	2.22±0.11
Stomach without content (S)	1.05±0.03	1.20±0.06	1.22±0.04	1.33±0.10	1.56±0.21	1.92±0.27	2.19±0.03
Gills tissue (GT)	1.06±0.03	1.10±0.03	1.20±0.02	1.33±0.05	1.45±0.09	1.66±0.11	1.85±0.05
Water from the aquarium (WA)	1.04±0.06	1.27±0.04	1.48±0.03	1.62±0.04	1.74±0.03	1.83±0.03	1.87±0.06
Content of the digestive tract (faeces) (CTDE)	1.18±0.08	1.81±0.38	2.60±0.28	3,75±0.24	4,26±0.31	5.07±0.15	5.83±0.04

[A]Valeus are mean (standard deviation) of five repetitions.

One possible explanation is that chromic oxide is not an inert marker, and that there is substantial absorption of chromium, in the form of chromic oxide or other chromium derivatives produced by digestion, through the intestinal wall (Fernandez *et al.*, 1999). Another explanation, suggested by Ng and Wilson (1997) to explain the results of Shiau and Liang (1995), is the possible incorporation through the fish's gills of the chromium present in the aquarium water resulting from the fish voiding their chromium-containing feces in the aquarium water. Even with an efficient turnover of the aquarium water, the concentration of chromium increases, resulting in the mineral absorption (Fernandez *et al.*, 1999).

In this experiment, with a static system, everyday aquaria were siphoned to reduce residues and avoid accumulation of feed and feces in the water and only the water lost by evaporation and management was restored. This procedure caused the concentration of chromium and conduction to a linear increase of the mineral in the aquarium water and in the gills samples. The accumulation varies with exposure period and environmental concentrations. At low concentration, the accumulation was in accordance with exposure time (Palaniappan and Karthikeyan, 2009).

Heavy metals (e.g. cobalt, copper, manganese, molybdenum, zinc and chromium) in aquatic environments are of critical concern because of their accumulation in aquatic organisms (Dimari

et al., 2008). Fish, being major components of most aquatic habitats, have also been recognized as good bioaccumulators of inorganic minerals (King and Jonathan, 2003). The gill has also been reported as an important site for the entry of heavy metals, which provokes gills lesions and damage (Bols *et al.*, 2001). To further test this hypothesis, in this experiment, the dietary concentration of $^{51}Cr_2O_3$ supplied in the diet of experimental fish was 100 µg.g^{-1}, with a specific activity of 58.2 µCi. Therefore, if the element had been absorbed by the digestive tract of fish, possibly through the incorporation through gills present in the aquarium water or by any other route, at least the gama radiation, originated from the decline of the ^{51}Cr, should have been detected in Nile tilapia.

When the regressions set, the blood samples were taken from control fish were contrasted with those representing results of the collected samples of the intestine without content, stomach without content, digestive tract content of fish that received the experimental diet, and there was a linear increase and significant differences were detected between established regressions ($P<0.05$). These results obtained suggest that the gut without content, without stomach contents and contents of the digestive tract had a saturation effect during the seven day experimental period.

The results obtained by Clawson *et al*. (1955) indicate that the chromium oxide concentration in the feces comes into equilibrium with that of the feed consumed between three and four days after the initial feeding of this compound. However, in this study, until the seventh day there was an increased concentration of the marker in the faeces, this difference may be due to low concentration of the marker used in this experiment and the necessary saturation of the digestive tract.

Considering the results obtained in this study, we agree with Fernandez *et al*. (1999), that another possible explanation for the results obtained by Shiau and Liang (1995) and Shiau and Shy (1998), would be that the chromium content of the fish follows the same pattern that they found for other inorganic nutrients (calcium, phosphate, ashes), increasing its concentration in the fish fed with the diets supplemented with chromic oxide, with a maximum at a chromic oxide level around 5–10g.kg^{-1}. This increase could have more to do with a higher retention of the natural chromium present in the diet than with the absorption of the supplemented chromic oxide. It has been reported (Evtushenko *et al.*, 1986) that the level of accumulated metals in tissues remained invariably at a plateau even when the organisms were exposed to them continuously for a sufficiently long period.

Furthermore, the levels of the marker (5 to 10% of chromic oxide incorporated in the diet) used in these studies, even with a high degree of purity, can provide other forms of chromium complexation that can be absorbed by the digestive tract or other routes by the fish.

CONCLUSIONS

The experimental results indicate that the trivalent chromium in the form of Cr_2O_3 was not significantly absorbed by the gastrointestinal tract, gills or another possible route of absorption under these experimental conditions with Nile tilapia. Therefore, this marker was shown to be inert and can be safely used in studies of nutrition and digestibility.

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