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ORIGINAL PAPER

Zinc determination in samples fish by GFAAS using acid digestion in an ultrasound bath

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Abstract The aim of this paper was to determine the zinc concentrations in muscle samples of Nile tilapia (*Oreochromis niloticus*) produced in aquaculture and fed diets containing inorganic and organic zinc sources. In the sample preparation step, the samples were lyophilized and ground cryogenically to produce particles smaller than 60 μm. The zinc extraction was conducted by acid mineralization using concentrated nitric acid and hydrogen peroxide in a thermostatically controlled ultrasonic batch reactor. The zinc determinations were performed by Graphite Furnace Atomic Absorption Spectrometry at a drying temperature of 90–250 °C, pyrolysis temperature of 1400 °C, atomization temperature of 2400 °C, and cleaning temperature of 2800 °C. Niobium nitrate was used as a chemical modifier co-injected with the samples, and tungsten was employed as a permanent modifier. Among the muscle tissue samples of Nile tilapia that were analyzed, zinc concentrations were determined in the range of 3.70–19.00 mg kg^{-1} (LOD 0.012 μg L^{-1} and LOQ 0.041 μg L^{-1}). The accuracy and precision of the proposed method of zinc determination was validated using the DORM-certified standard Fish Protein 4 NRC.

Keywords Chemical modifier · GFAAS · Zinc · Fish

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Introduction

The Nile tilapia (*Oreochromis niloticus*) is quite a versatile species of fish in fish farming because it is amenable to both extensive farming without any technology employed, and high-tech production using a breeding system in cages with full rations. In addition, this fish is well-appreciated by the filleting industry, thanks to its organoleptic qualities and the absence of spines on the "Y" in your filet [1]. Due to its potential for aquaculture, the distribution of tilapia has expanded in the last 50 years. Because it is a species suitable for subsistence fish farming in developing countries and because of its importance in aquaculture many aspects of nutrition of this species have been investigated [2]. There has been a considerable increase in tilapia production in Brazil in recent years. This increase has led researchers working in aquaculture to develop their knowledge of the related behavior, physiology, genetics and nutrition of this species [1, 3, 4].

There is a consensus in the scientific community regarding the fundamental importance of metal ions to organisms. These ions are essential structural components and are active in many vital processes. The ions can act as signaling agents, such as catalysts, or alter gene expression [5]. By considering their proportions and amounts in the body tissue of animals, the metal ions are classified as macro elements (elements that the body needs in large amounts, concentration range in percent) and trace elements (elements that the body needs in smaller quantities, concentration range in mg kg^{-1} and/or μg kg^{-1}) [5–7]. Calcium, phosphorus, magnesium, potassium, sodium, chlorine and sulfur are considered to be essential macroelements, and copper, cobalt, iron, iodine, manganese, selenium and zinc are considered to be essential trace elements [5, 6].

Zinc is a trace element, the function of which is essential for many biochemical processes. Zinc is involved in the intermediate component of several proteins, and via the secretion of hormones and metabolism, zinc operates in immune defense. A number of Zn-dependent enzymes are involved in the synthesis and metabolism of proteins, lipids, carbohydrates and nucleic acids [7–9]. This element also participates in gene expression and is a component of many transcription factors. Zinc's presence in cells is processed by protein and processes storage conveyors, through metallothioneins. Changes in the concentration of this element in tissues are observed in many disease processes [7].

Thus, the development of new methodologies that enable safe quantification of trace metal ions, such as zinc and copper, which are incorporated into the organs and tissues of animals in studies of fish nutrition becomes critical. In this context, the determination of metal elements by graphite furnace atomic absorption spectrometry (GFAAS), presents itself as a fairly robust technique. This technique offers several advantages, such as high sensitivity, detection limit concentrations in ng kg^{-1}, use of small sample volumes, and determination of a wide variety of trace elements. Considering that the atomizer can act as a chemical reactor, there is a strong possibility of sampling which eliminates the step of prior complete decomposition of the sample [10–16].

Based on the above mentioned findings, this paper presents the results of the optimization of a new methodology using acid digestion with nitric acid and hydrogen peroxide assisted by a thermostated ultrasound bath reactor for the determination of zinc concentrations in the muscle tissue of Nile tilapia. Zinc was subsequently determined in the digested samples by GFAAS using a combination of niobium nitrate, used as a chemical modifier in solution, and sodium tungstate, used as permanent modifier.

Materials and methods

Reagents and solutions

Ultrapure water (18.2 MΩ cm) purified in an Elga Purelab Ultra Ionic System and 14.40 mol L^{-1} nitric acid suprapur quality (Merck-Darmstadt, Germany) were used throughout the work. The stock solutions of zinc and chemical modifiers (niobium nitrate and sodium tungstate) were prepared by dilutions of standards Merck Titrisol (Darmstadt, Germany). All solutions were stored in polypropylene flasks. All of the flasks used for storing samples and standard solutions, glassware and all sampler flasks of the atomic absorption spectrometer were washed with suprapur quality nitric acid 10 % (v/v) for 24 h and later rinsed with ultrapure water and air jet-dried with pure air prior to use.

Sample collection and preparation

The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA), University of São Paulo State (UNESP), under number 04/2011. The samples of muscle tissue were provided by the Aquatic Organisms Nutrition Laboratory of the Faculty of Veterinary Medicine and Zootechny/UNESP/SP. The fish were raised using practical balanced diets (Table 1) according to Pezzato [17], Guimarães [18], National Research Council (NRC) [19], with organic and inorganic sources of zinc, the source of organic zinc being from Bioplex of Altech (zinc-methionine) and the source of inorganic was zinc sulfate monohydrate—$ZnSO_4 \cdot H_2O$ (Merck-Darmstadt, Germany) supplied to the three groups of animals with different initial weights, providing the following treatments:

- Group 1—Fish with initial weight 24 g.
  - *T1C* feed control zinc (zinc-free);
  - *T1O* feed organic zinc (Bioplex);
  - *T1I* feed inorganic zinc (zinc sulfate);

Table 1 Total zinc concentration in the muscle tissue of Nile tilapia considering the treatments with different zinc concentrations and certificate standard (DORM-4)

Treatments	Zn Concentration (mg kg^{-1})
T1C	3.65 ± 0.02
T1O	4.93 ± 0.04
T1I	6.25 ± 0.05
T2C	4.30 ± 0.04
T2O	16.80 ± 0.18
T2I	15.80 ± 0.16
T3C	4.80 ± 0.04
T3O	18.93 ± 0.20
T3I	12.35 ± 0.14
DORM-4	52.05 ± 0.80

T1C fish with initial weight 24 g and final weight 132 g with control diet (zinc-free), *T1O* Fish with initial weight 24 g and final weight 110 g fed with organic zinc (BioPlex), *T1I* fish with initial weight 24 g and final weight 129 g fed with inorganic zinc (zinc sulfate), *T2C* fish with initial weight 74 g and final weight 239 g fed with control diet (zinc-free), *T2O* fish with initial weight 74 g and final weight 293 g fed with organic zinc (BioPlex), *T2I* fish with initial weight 74 g and final weight 284 g fed with inorganic zinc (zinc sulfate), *T3C* fish with initial weight 85 g and final weight 261 g fed with control zinc (zinc-free), *T3O* fish with initial weight 85 g and final weight 201 g fed with organic zinc (BioPlex), *T3i* fish with initial weight 85 g and final weight 309 g fed with inorganic zinc (zinc sulfate), *DORM-4* standard certificate fish protein containing 52.2 ± 3.20 mg kg^{-1} of Zn

Results expressed as a mean value ± standard deviation ($n = 3$)

Confidence interval at a 99 % confidence level ($t_{student} = 5.70$)

- Group 2—Fish with 74 g.
  - *T2C* feed control zinc (zinc-free);
  - *T2O* feed organic zinc (Bioplex);
  - *T2*I feed inorganic zinc (zinc sulfate);
- Group 3—Fish with 85 g.
  - *T3C* feed control zinc (zinc-free);
  - *T3O* feed organic zinc (Bioplex);
  - *T3I* feed inorganic zinc (zinc sulfate);

After an experimental growth period of 80 days, the fish were euthanized (six fish per treatment—eighteen fish per group) with benzocaine (100 mg L^{-1}) to remove the muscle tissue. Then, to obtain the sample pool for each treatment, the samples were macerated with a turrax and stored in polypropylene flasks at −20 °C for 20 days. All samples were prepared for the zinc extraction step by initially lyophilizing for 48 h and subsequently milling in a mortar and pestle in the presence of liquid nitrogen to produce particles with a particle size smaller than 60 μm.

Mineralization of muscle tissue samples

Approximately 100 mg of lyophilized muscle tissue samples were transferred to 25 mL digestion flasks. Aliquots of 3 mL of concentrated nitric acid and 1 mL of hydrogen peroxide 30 % (m/m) (Darmstadt, Germany) were transferred to each digestion flask.

The set of digestion flasks was placed in a UNIQUE ultrasonic bath at a temperature of 40 °C and a power of 135 Watts until complete mineralization of the sample occurred or until the extract became transparent. Next, the acid extracts were transferred to 10 mL volumetric flasks and brought up to volume with ultrapure water for subsequent determination of zinc by GFAAS.

Determination of zinc concentration by GFAAS

Preparation of the graphite tube coated internally with tungsten carbide

The pyrolytic graphite tubes with integrated platform used in the determination of zinc had their inner walls coated with tungsten. For this, aliquots of 25 μL of a solution containing 1000 mg L^{-1} sodium tungstate modifier were injected into the atomizer, which was then subjected to the following heating program: Drying: 150 °C for 10 s (ramp) and 25 s (heating), 250 °C for 10 s (ramp) and 25 s (heating); Pyrolysis: 800 °C for 10 s (ramp) and 20 s (heating), 1500 °C for 10 s (Ramp) and 20 s (heating); Atomization: 2500 °C for 2 s (heating) and Clean: 2600 °C for 5 s (heating). This procedure was repeated 40 times and allows a layer of tungsten carbide to be deposited on the platform of the graphite tube, which acts as a permanent chemical modifier [12, 13]. The mass of tungsten deposited on the platform graphite tube was 1 mg. Argon was used at all stages as a cleaning gas with a flow rate of 1 L min^{-1}.

Preparation of analytical curve

The analytical curve was prepared using a stock standard solution of zinc. For this, volumes of 8, 16, 24, 32 and 40 μL of the standard 25 μg L^{-1} zinc solution, 5 μL of 12 mo L^{-1} hydrochloric acid suprapure® (Merck-Darmstadt, Germany), 50 μL Triton X-100 (Merck-Darmstadt, Germany) 1 % (v/v)—used to assist in lowering of the solution viscosity, 100 μL of 1000 mg L^{-1} niobium nitrate—used as a chemical modifier in solution, and ultrapure water q.s. were transferred to the autosampler flasks of the spectrometer, so that the final volume of the standard solutions was uniformly 1000 μL and the concentration of zinc in the standard solutions was in the range of 0.20–1.00 μg L^{-1}. Solution containing all reagent (hydrochloric acid suprapure®, Triton-X 100 and niobium nitrate) at the same concentrations it was used as blank.

Preparation of acid extracts for injection into the graphite tube

500 μL acid extracts of the samples were transferred directly to the autosampler cups of the atomic absorption spectrometer to which were then added 50 μL of Triton X-100 1 % (v/v), 100 μL of 1000 mg L^{-1} niobium nitrate, and 350 μL of ultrapure water. Solution containing all reagent used in the samples mineralization (concentrated nitric acid and hydrogen peroxide) and Triton-X 100 and niobium nitrate it was used as blank.

Analytical procedures

After these steps to prepare the samples (acid extracts) and the standard solutions, which were prepared directly in the autosampler cups, a volume of 20 μL of standard and/or sample was injected into the graphite tube (internally coated with tungsten carbide) by the micropipette of the autosampler. The heating program used for the determination of zinc in the graphite furnace has been optimized and was as follows: Drying: 90 °C for 10 s (ramp), 150 °C for 10 s (ramp) and 5 s (heating), 250 °C for 10 s (ramp) and 5 s (heating); Pyrolysis: 1400 °C for 5 s (ramp) and 10 s (heating); Atomization: 2400 °C for 2 s (heating); Cleaning: 2800 °C for 5 s (heating). Three determinations were carried out per sample analyzed. (n = 3).

Statistical analyses

The experimental data were analyzed and presented in accordance with procedures established by the Statistics Analysis System Institutes [20]. The Tukey test was used to demonstrate possible significant differences between the results obtained in the determination of zinc in muscle tissue samples of Nile tilapia in the three groups of animals with different weights and to validate the results obtained by the proposed method using certified reference material. The variability of the data was expressed as the relative standard deviation, applying a significance level of 5 % ($P < 0.05$).

Results

Optimization of experimental conditions for zinc determination by GFAAS

The pyrolysis and atomization curves were made to determine the optimum temperatures of pyrolysis and atomization of zinc in standard solutions and in acid extracts of muscle tissue samples of Nile tilapia. The influence of the temperatures of pyrolysis and atomization on the absorbance signal obtained for the standard of zinc containing 0.50 μg L^{-1} and the acid extracts of samples of biological material are presented in Figs. 1 and 2. The pyrolysis temperature of 1400 °C was chosen, because as shown in Fig. 1, the absorbance signals obtained for zinc remain constant from 1000 to 1400 °C, after which a rapid decrease occurs. This result indicates that up to 1400 °C zinc is thermally stable.

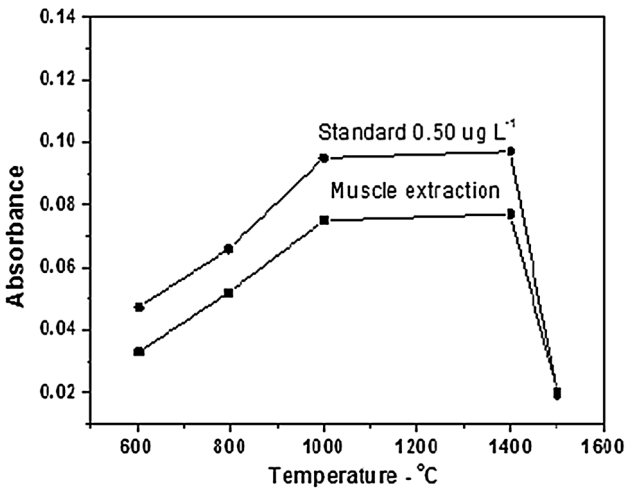

Fig. 1 Pyrolysis temperature curves of acid extracts of tissue muscle samples of Nile tilapia and standard containing 0.50 μg L^{-1} of zinc

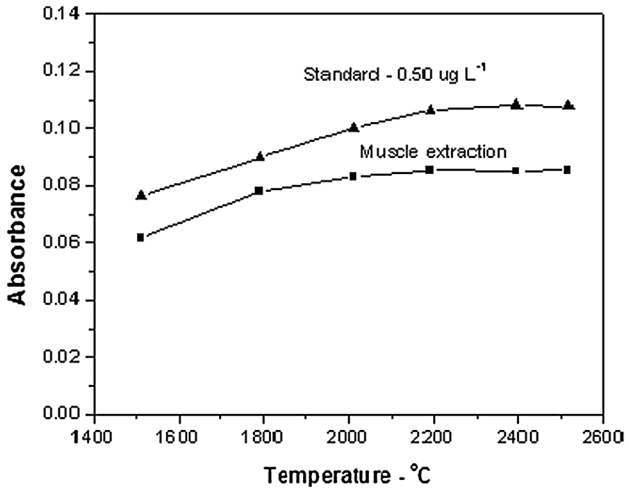

Fig. 2 Atomization temperature curves of acid extracts of tissue muscle samples of Nile tilapia and standard containing 0.50 μg L^{-1} of zinc

Regarding the atomization temperature (Fig. 2), we observed that the absorbance signals obtained for zinc remain constant from 2200 °C upward. Considering this behavior and variables such as reproducibility and repeatability of readings, an atomization temperature of 2400 °C was chosen. The thermal stability of zinc obtained in these experiments demonstrates the effectiveness of the modifier niobium nitrate injected together with the sample and the tungsten carbide that served as permanent modifier [21–24].

The physicochemical parameters that were optimized (pyrolysis and atomization) established the heating program shown in "Preparation of analytical curve" section. The calibration curve for zinc was prepared in the concentration range from 0.20 to 1.00 μg L^{-1}. Figure 3 shows the analytical curve obtained with its corresponding equation of the line. The values obtained from the absorbance readings of the zinc standard ($n = 3$) exhibited good repeatability (relative standard deviations of less than 1 %). This result supports the efficiency of the chemical modifiers used, niobium nitrate and tungsten carbide and, also, the thermal stabilization of zinc during the pyrolysis and atomization steps in from the heating program used.

Zinc concentration in the muscle tissue samples of Nile tilapia

After optimization of the physicochemical parameters in the ultrasonic extraction process and the heating program for the determination of zinc by GFAAS, the optimized method was applied to determine this analyte in samples of muscle tissue of Nile tilapia. The precision and accuracy of the method were evaluated by the determination of zinc in

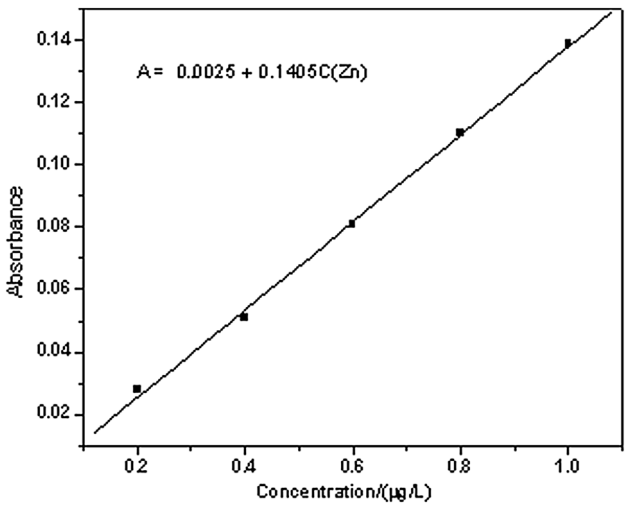

Fig. 3 Zinc analytical curve prepared in the concentration range of 0.20–1.00 µg L^{-1}

the certified standard fish protein DORM-4 NRC. The results obtained are summarized in Table 1.

Discussion

Analyzing each group separately (Table 1), there was also no difference between treatments T1O × T1I and T2O × T2I ($P < 0.05$), but the T3O x T3I concentration was different ($P < 0.05$), being higher for T3O (18.93 ± 0.20 mg kg^{-1}) than for T3I (12.45 ± 0.14 mg kg^{-1}). This result indicates that there was greater incorporation of zinc into the muscle tissue with fish feed as the organic source of zinc than in those fed with inorganic zinc. Evaluating zinc absorption due sizes within each source, it is observed that the concentrations were higher for animals fed with 85 g organic source of zinc (T3O). The zinc absorption is related to the use of dietary zinc by the intestinal symbiotic bacterial microflora. Thus, with the increase of available zinc in the intestinal microflora, that is multiplied by promoting positive action on the morphology and function of the duodenal absorptive epithelium, increasing the thickness of the intestinal mucosa and thus the absorptive area. Therefore, it can be inferred that the organic source provided higher zinc concentration in the tissue due to the size of animal favoring increased mineral absorption in the intestine by the diet. [25, 26].

In relation to the results obtained using the method proposed for the determination of zinc, the data presented can be considered quite robust because they were validated comparing the results from samples with the results obtained using the certified reference DORM 4 fish muscle protein. Table 1 shows that the relative standard deviation value against the standard experimental certificate is less than 1 %, and the absolute experimental value (52.05 ± 0.80 mg kg^{-1}) is approximately 0.30 % lower than the absolute certified value (52.20 ± 3.20 mg kg^{-1}), confirming that these results were not significantly different by Tukey test ($P < 0.05$), which demonstrates the accuracy and precision of the proposed method for zinc determination. Moreover, the characteristic mass calculated relative to the standard 0.50 µg L^{-1} was 12 pg zinc. The limits of detection (LOD) and quantification (LOQ), as calculated based on the standard deviation of 20 readings taken in relation to the blank of the standard solutions and the slope of the calibration curve (LOD = 3σ/slope and LOQ = 10σ/slope), were 0.012 and 0.041 µg L^{-1} zinc, respectively [27]. The lifetime of the graphite tube was equivalent to 800 firings. Compared to other methods described in the literature, and considering the complexity of biological matrices, this lifetime of the tube is even higher. [10–16, 24, 28–30].

Conclusions

The results obtained using the proposed method for the mineralization of muscle tissue samples of Nile tilapia and subsequent zinc determination by GFAAS exhibited excellent precision and accuracy, low limits of detection and quantification, and higher sample throughput compared with the process of acid mineralization using block digestion. Zinc concentrations in the muscle tissue of the animals exhibited no difference between organic and inorganic treatments in groups 1 and 2, but in group 3, the concentration was higher in the organic treatment.

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Compliance with ethical standards

Conflict of Interest The authors have no conflict of interest.

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