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Determination of chromium by GFAAS in slurries of fish feces to estimate the apparent digestibility of nutrients in feed used in pisciculture

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Abstract

This paper presents a simple, fast and sensitive method to determine chromic oxide (used as a biological marker of fish feed) in samples of fish feces by GFAAS through the direct introduction of slurries of the samples into the spectrometer's graphite tube. The standard samples of feces and of fish feed containing 0.10–1.00 mg kg^{-1} of Cr_2O_3 were pre-frozen for 1 min in liquid nitrogen and then ground a cryogenic mill for 2 min, which reduced the samples' grain size to less than 60 μm. The standard slurries were prepared by mixing 20 mg of standard samples of fish feed or feces with 1 mL of a solution containing 0.05% (v/v) of Triton X-100 and 0.50% (v/v) of suprapure HNO_3 directly in the spectrometer's automatic sampling glass. The final concentrations of Cr_2O_3 present in the standard slurries were 2, 4, 8, 16 and 20 μg L^{-1}. After sonicating the mixture for 20 s, 10 μL of standard slurries were injected into the graphite tube, whose internal wall was lined with a metallic palladium film that acted as a permanent chemical modifier. The limits of detection (LOD) and quantification (LOQ) calculated for 20 readings of the blank of the standard slurries (2%, m/v of feces or feed devoid of minerals) were 0.81 and 2.70 μg L^{-1} of Cr_2O_3 for the standard feces slurries, 0.84 and 2.83 μg L^{-1} of Cr_2O_3 for the standard feed slurries. The proposed method was applied in studies of nutrient digestibility of different fish feeds and its results proved compatible with the results obtained from samples pre-mineralized by acid digestion.

Keywords: Slurries samples; GFAAS; Metallic palladium; Chromic oxide

1. Introduction

In fish nutrition studies, determining the digestibility of the nutrients contained in the feeds given to these animals is the primary concern when evaluating their potential for inclusion in the diet [1]. Knowing the degree of digestibility of the feed allows one to ascertain the fraction of the food's nutrients or energy not excreted through the animal's feces [2]. The replacement of certain agroindustrial products and by-products used as ingredients in fish diets for other products has been done based on studies of the digestibility of these foods' nutrients and has proved to be an alternative economic practice [3].

The so-called fecal indicators are used in the determination of the digestibility of fish feeds. These indicators are divided into internal and external markers. The internal markers occur naturally in feeds, while the external markers are added to the diet or administered to the animal by some other route [4]. External markers are the ones most commonly employed in digestibility studies involving fish, and Cr_2O_3 is the most widely accepted marker because it is completely indigestible and non-absorbable, exerting no pharmacological effect on the digestive system and passing uniformly through it [5–7].

Determining the percentage of Cr_2O_3 in fish feces allows one to estimate the total or partial coefficient of digestibility of

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the metabolized nutrient, comparing it with the percentage of this oxide initially mixed with the feed [8]. To this end, the fish are kept in 250 L circular aquariums where they are given feeds marked with 0.1% of Cr_2O_3. After the feeding period, the fish are transferred to feces-collecting aquariums (also containing a volume of 250 L of water) with a conical bottom for decanting the feces. After decantation, the feces are removed from the supernatant water, then centrifuged, oven-dried, ground and stored at −20 °C [9]. The determination of the chemical–bromatological composition and of the percentage of Cr_2O_3 allows one to estimate the total or partial digestibility coefficient of the nutrients metabolized by the animal [10].

The procedures for preparing samples to determine the percentage of oxide in both feces and feed are difficult. Normally, the samples are mineralized by slow heating (60–80 °C) in digestor blocks, using a perchloric–nitric mixture (5 mL of HNO_3 conc. + 2 mL of $HClO_4$ conc.) [11]. The resulting extracts are acid-based dichromate solutions ($Cr_2O_7^{2-}$), which are usually quantified by the dyphenylcarbazide spectrophotometric method [12]. This procedure is not only lengthy but also presents the disadvantage of oxidizing chromium(III) to chromium(VI), a highly toxic species.

The determination of metallic nutrients in biological samples (vegetals, blood, animal feces, foods, etc.) by graphite furnace atomic absorption spectrometry (GFAAS) offers several advantages, such as high sensitivity, detection limits at levels of $\mu g\,kg^{-1}$, the use of small sample volumes, and the determination of a wide variety of trace elements. Moreover, solid sampling is also possible because the atomizer can act as a chemical reactor, eliminating the sample's total pre-decomposition step [13]. Considering the sample's treatment stage, procedures using solid samples in the form of slurries offer advantages over conventional digestion procedures, such as shorter sample preparation times, reduction of analyte loss through excessive handling or retention on insoluble products, less possibility of sample contamination, and principally, minimization of the action of harmful acids on the analyst [14,15].

In view of the above, this paper describes the development of a method to determine chromium in slurries of fish feed and feces samples by GFAAS that eliminates the sample's mineralization step and allows for an estimate of the digestibility of nutrients in samples of fish feed.

2. Materials and methods

2.1. Reagents, standard solutions and samples

Superpure deionized water (18.2 $M\Omega\,cm^{-1}$) obtained with a Milli-Q® system (Millipore, Bedford, USA), Suprapure nitric acids (Merck), hydrogen peroxide (Merck) and Triton X-100 (Merck) were used throughout this work. The solution containing palladium, which was employed to coat the inside of the graphite tube and used as a permanent modifier, was prepared by diluting a stock solution containing 50 $g\,L^{-1}$ of palladium nitrate (Merck) with ultrapure water.

Stock solutions of the analytes and the concomitants were prepared from reagents of spectroscopic purity (Johnson & Matthey, Royston, Hertfordshire, UK). The remaining solutions used here, including the concentrated acid solutions used for mineralizing the samples, were all analytical grade. All the solutions were stored in polypropylene bottles.

All the bottles for storing samples and standard solutions, the glassware and the containers of the atomic absorption spectrometer's autosampler were washed in 10% (v/v) nitric acid for 24 h, rinsed with ultrapure water and dried by shaking before being used.

The fish feces and feed samples were dried at 50 °C in an oven with forced air circulation for 48 h and then cryogenically ground. To this end, approximately 1.0 g of the sample, together with a magnetic bar, were put into a polycarbonate flask, which was then closed and immersed in liquid nitrogen. The impact between the sample and the magnetic bar subjected to an oscillating magnetic field (20 impacts s^{-1}) pulverized the sample. The sample-grinding program consisted of an initial stage of 2 min of prefreezing, 1 min of pulverization, and again 1 min of freezing, followed by a second stage comprising two cycles of two pulverization and freezing stages, making a total of 8 min. This procedure yielded particles with a granulometry of less than 60 μm [16].

A portion of the samples was also mineralized in a microwave oven, as follows. Portions of 100 mg of cryogenically ground samples were transferred directly to the Teflon flasks of the microwave oven, and 2.5 mL of suprapure nitric acid 14 mol L^{-1} plus 0.50 mL of hydrogen peroxide 30% (m/m) were added. The heating program employed was the one proposed in the oven's user manual.

2.2. Preparation of slurry samples

After cryogenic grinding, 20 mg of samples of biological material (fish feed or feces) were transferred directly to the containers of the spectrometer's autosampler, to which were added 5 μL of suprapure nitric acid 14 mol L^{-1}, 50 μL of Triton X-100 at 1% (v/v) and 945 μL of ultrapure water. The slurry samples of biological material were then sonicated for 20 s directly in the autosampler's containers.

2.3. Apparatus

A Provecto Analítica model DGT 100 *plus* microwave oven (Campinas, SP, Brazil) was used to mineralize the samples whenever necessary.

For the chromium determinations, a Shimadzu model AA-6800 atomic absorption spectrometer was used, equipped with a background absorption corrector with a deuterium lamp and self-reverse (SR) system, and a pyrolytic graphite tube with integrated platform and automatic ASC-6100 sampler. A Shimadzu hollow cathode chromium lamp operated with a 10 mA current was also used. The wavelength applied was 357.9 nm and the spectral resolution was 0.5 nm. Argon was used as inert gas at a constant flow of 1 L min^{-1} throughout the heating program, except during the atomization step, when the gas flow was interrupted. The absorbance signals were measured in the peak area. In the absorbance readings by the flame module (FAAS),

Table 1
Heating program used for coating the inner wall of the graphite tube with metallic palladium (Pd^0)

Stages	Temperature (°C)	Ramp (s)	Hold (s)	Argon flow ($L\,min^{-1}$)
Drying	95	40	0	0.30
Drying	120	10	0	0.30
Reduction	500	10	5	0.30
Reduction	550	5	1	0.30

acetylene was used as combustible gas under a constant flow of $2\,L\,min^{-1}$, and air was used as oxidizing gas at a constant flow of $0.80\,L\,min^{-1}$.

The samples were cryogenically ground in a SPEX—Freezer model Mill 6750 cryogenic mill.

The slurries of fish feces and feeds were shaken in a UNIQUE ultrasonic cell disruptor.

2.4. Preparation of the graphite tube coated internally with metallic palladium

The inner walls of the pyrolytic graphite tubes with integrated platform used for determining chromium were coated with metallic palladium. This was done by injecting aliquots of 10 μL of a solution containing $10\,g\,L^{-1}$ of the $Pd(NO_3)_2$ modifier into the atomizer, which was then subjected to the stages of the heating program described in Table 1. This procedure was repeated eleven times. When heated to 500 °C, the Pd(II) deposited on the graphite tube's inner wall forms a layer of Pd^0 that acts as a chemical modifier [17]. In this case, the mass of Pd^0 deposited was 506 mg. With the treatment it was possible the use of the graphite tubes for up to 260 firings.

2.5. Preparation of the standard slurries

Analytical curves were prepared using fish feed and feces slurries containing 2, 4, 8, 16 and 20 $\mu g\,L^{-1}$ of Cr_2O_3 (Merck), with the absorbance readings done by GFAAS. These standard slurries were prepared under the same conditions as those used for preparing the slurries of feed and feces samples using 20 mg of standard samples of fish feces or fish feed containing 0.10–1.00 $mg\,kg^{-1}$ of Cr_2O_3. Analytical curves were also prepared by dissolving 100 mg of each of the aforementioned standard mixtures in a 1 + 3 nitric–perchloric solution under heating in a microwave oven so as to obtain standard solutions containing 1, 2, 4, 8 and $10\,mg\,L^{-1}$ of Cr_2O_3, with the absorbance readings of these solutions done by FAAS.

2.6. Analytical procedures

After the sonication step of the sample in slurries and/or standard slurries directly in the autosampler's containers, a volume of 10 μL of standard or sample was injected into the graphite tube (coated internally with metallic palladium), using the autosampler's micropipette. Each measurement was repeated five times.

Table 2
Graphite tube heating program optimized for the determination of Cr_2O_3 in slurries of fish feces and feed samples

Stages	Temperature (°C)	Ramp (s)	Hold (s)	Argon flow ($L\,min^{-1}$)
Drying	120	10	0	1
Drying	250	10	5	1
Pyrolysis	1400	10	20	1
Pyrolysis	1400	5	10	1
Atomization	2500	1	5	0
Cleanup	2800	5	0	1

Table 2 describes the heating program of the graphite tube, which was optimized to determine chromium.

3. Results and discussion

3.1. Optimization of the instrumental conditions

In the determination of metals by GFAAS using the sample in slurry technique, obtaining exact and reproducible analytical results depends on the optimization of the temperatures of pyrolysis and atomization of the analyte present in the slurry samples. Therefore, pyrolysis and atomization curves were drawn to determine the optimal pyrolysis and atomization temperatures of the Cr in standard slurries of fish feed and feces containing 16 $\mu g\,L^{-1}$ of Cr_2O_3, using the graphite tube coated internally with Pd^0, which acted as a permanent modifier, and the sample preparation conditions described under Section 2.2. Figs. 1 and 2 illustrate the influence of the pyrolysis and atomization temperatures on the absorbance signal obtained for the Cr in the standard slurries of the biological materials. The pyrolysis temperature of 1400 °C was selected because, as Fig. 1 indicates, the absorbance signals obtained for the Cr remained constant from 800 °C up, declining rapidly after reaching 1400 °C. As for the atomization temperature (Fig. 2), the absorbance signals obtained for the Cr were constant from 2300 °C up for both standard slurries, so the atomization temperature of 2500 °C was selected for

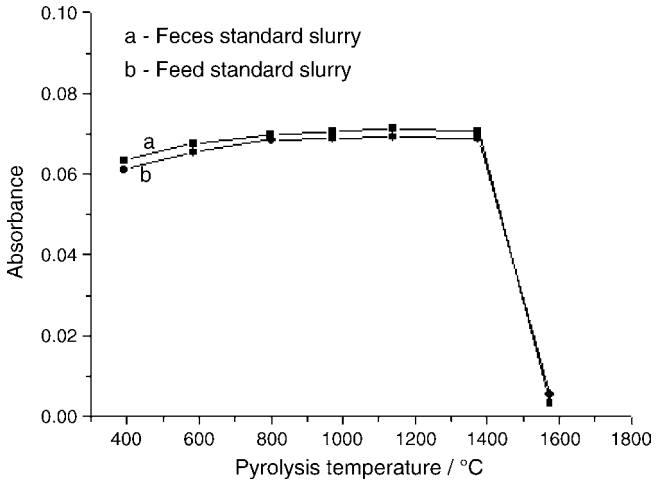

Fig. 1. Pyrolysis temperature curves of the slurries of feces and feed samples containing 16 μL^{-1} of Cr_2O_3.

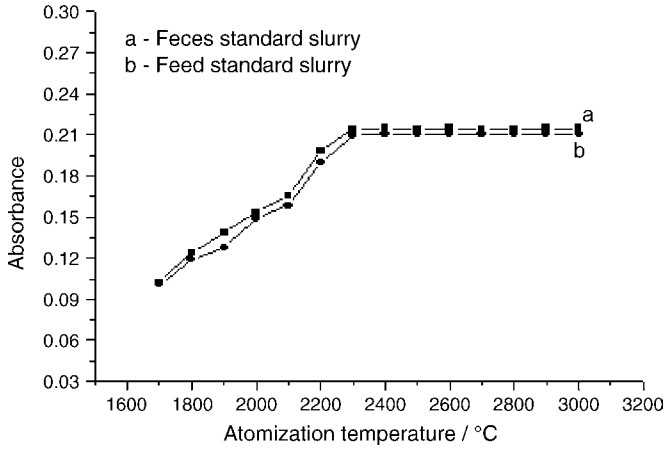

Fig. 2. Cr atomization temperature curves of the slurries of feces and feed samples containing 16 μL^{-1} of Cr_2O_3.

all the remaining experiments. Figs. 3 and 4 illustrate the analyte absorbance (AA) and background absorbance (BG) signals for the standard slurries of the biological materials. Both figures show a relatively low background absorbance, indicating the efficiency of Pd^0 as a chemical modifier. Because the biological materials studied here displayed magnesium content of about 0.12%, this element in the matrix may help in the thermal stabilization of chromium [16,18].

3.2. Determination of the optimal sonication time of the slurry samples

Ultrasonic shaking to analyze slurries of solid materials ensures good homogenization of the sample, allowing for better reproducibility between measurements [19]. Thus, the samples sonication time was evaluated in the interval of 5–60 s of agitation. Fig. 5 depicts the influence of the sonication time of samples on the absorbance signals obtained for Cr. An analysis of this figure indicates that the absorbance signals remain constant starting from 20 s of sonication. This sonication time was considered optimal, for not only was a good absorbance signal obtained but also the R.S.D. among the measurements was relatively low (3.4%).

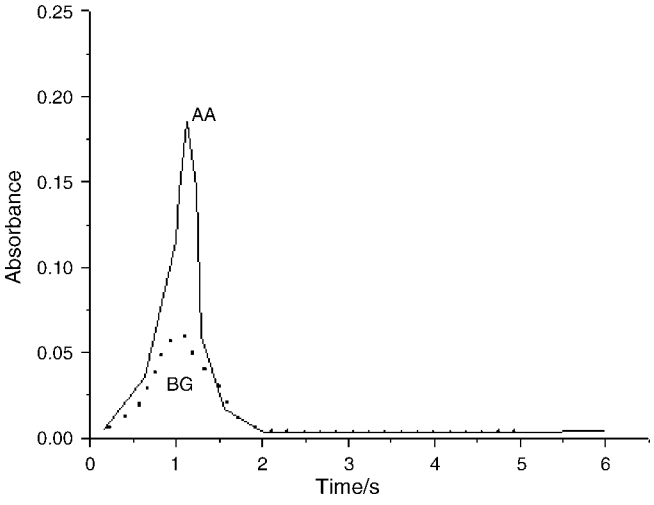

Fig. 4. Transient atomic absorption (AA) and background (BG) signals in the atomization of Cr in slurries of feed samples containing 16 μL^{-1} of Cr_2O_3.

3.3. Obtaining analytical curves

Based on the parameters of pyrolysis and atomization temperature and the profile of the optimized atomic absorption signal, analytical curves were plotted using standard slurries of fish feces and feed containing Cr_2O_3 in the range concentration of 2–20 $\mu g\,L^{-1}$ (as described before in Section 2.5). The Fig. 6 depicts the analytical curves obtained and their respective straight-line equations. A comparison of the analytical curve prepared with fish feces (curve a) and the one prepared with fish feed (curve b) indicates that their slopes do not show significant differences ($k = 0.0145$ for curve a and $k = 0.0135$ for curve b). Both analytical curves show absorbance values approximately 10–15% lower than the values of the analytical curve obtained from the standard solutions prepared in the range of 1–10 $mg\,L^{-1}$ of Cr_2O_3, whose absorbance read-

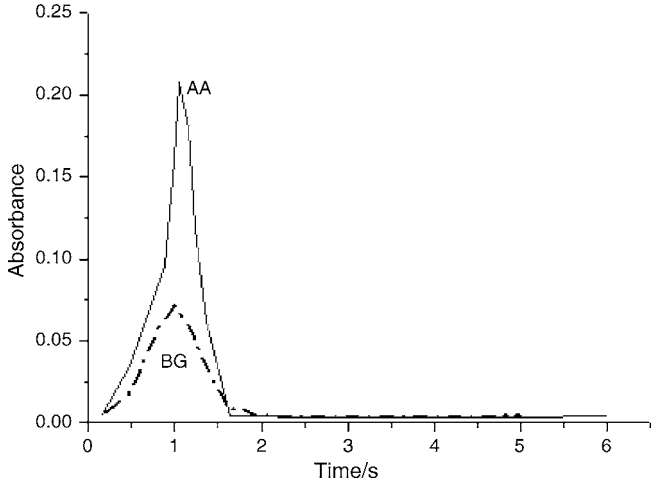

Fig. 3. Transient atomic absorption (AA) and background (BG) signals in the atomization of Cr in slurries of feces samples containing 16 μL^{-1} of Cr_2O_3.

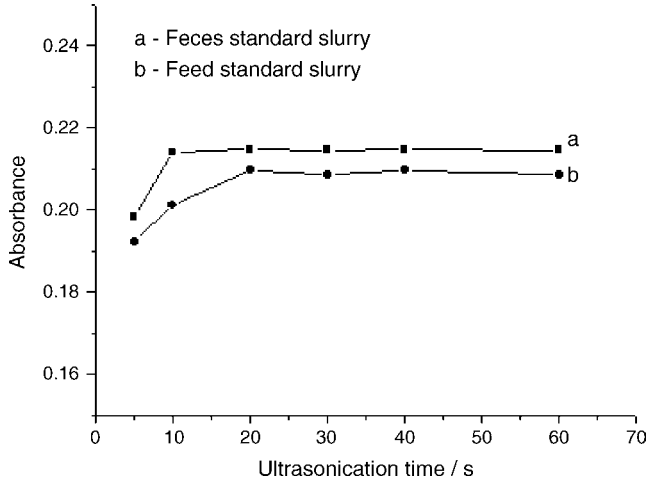

Fig. 5. Influence of ultrasonication time on the Cr absorbance signal in standard slurries of fish feces and feed containing 16 μL^{-1} of Cr_2O_3.

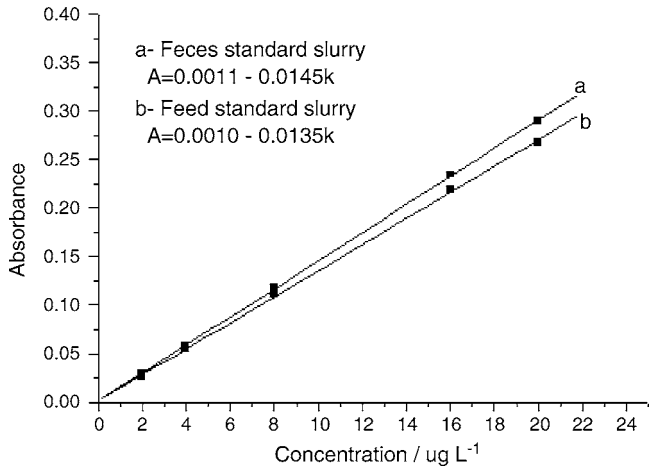

Fig. 6. Analytical curves obtained from standard slurry of fish feces and feed containing 2, 4, 8, 16 and 20 μg L^{-1} of Cr_2O_3.

ings were taken in the FAAS module (straight-line equation: $A = 0.0038 + 0.0029k$). However, the slopes of these straight lines obtained for the standard slurries were about 5-fold greater, which indicates that the sensitivity of the proposed method is also greater, attesting to the efficiency of the pyrolysis and atomization temperature stage of the heating program employed. An accumulation of carbonaceous residues inside the graphite tube, causing partial obstruction of the radiation from the hollow cathode lamp, can impair absorbance measurements [18]. Nevertheless, the low background (BG) signals obtained in the chromium optimization stage of the proposed procedure indicate that the carbon residue left behind by the pyrolysis state did not impair the absorbance measurements. The characteristic masses calculated in relation the standard slurry of 8 μg L^{-1} Cr_2O_3 were 30 and 32 pg for the standard slurry feces and feed, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of 20 readings obtained for the blanks of the standard slurries and the slopes of the analytical curves (LOD = 3σ/slope and LOQ = 10σ/slope). Their values were 0.80 and 2.70 μg L^{-1} of Cr_2O_3, respectively, that correspond to 40 and 135 μg kg^{-1}, for the standard slurry feces and 0.85 and 2.80 μg L^{-1} of Cr_2O_3, according to 42.50 and 140 μg kg^{-1}, for the standard slurry feeds [16]. The method's precision was tested by measuring the percentage of recovery of Cr_2O_3 of standard slurry of feces and feeds containing 10 μg L^{-1} of Cr_2O_3 ($n = 12$) [16,18]. A comparison between the value of the limit of detection obtained by the proposed method with that got by the FAAS method (76 μg L^{-1} of Cr_2O_3), shows that it is possible to detect concentrations about 95-fold smaller using the proposed method. The percentage of recovery achieved was 98 ± 3 for the standard slurry of feces and 97 ± 2 for the standard slurry of feeds. These results demonstrate that the proposed method offers an acceptable level of precision. The lifetime of the graphite tube was equivalent to 260 firings. Considering the complexity of the biological matrices, the tube's service life with the proposed method is acceptable when compared with other methods described in the literature [16,18].

3.4. Application of the proposed method

After the procedures of optimization and the LOD and LOQ determination, the applicability of the newly developed method was tested in the determination of Cr_2O_3 in four samples of feed containing different food supplements used in the diet of Nile tilapia juveniles and in samples of feces from these fish. Then, based on the values of the percentage of Cr_2O_3 and the bromatological analysis of the feeds, a calculation was made to estimate the coefficient of apparent digestibility of the fractions of dry matter (DM), gross protein (GP), total lipids (TL) and gross energy (GE), using Eq. (1) [10]:

$$\mathrm{Da} = 100 - \left[100 \left[\frac{\%\mathrm{Cr_2O_{3r}}}{\%\mathrm{Cr_2O_{3f}}}\right] \left[\frac{\%N_\mathrm{f}}{\%N_\mathrm{r}}\right]\right] \quad (1)$$

where Da(n) is the apparent digestibility; $\%Cr_2O_{3r}$ the percentage of chromic oxide in the feed; $\%Cr_2O_{3f}$ the percentage of chromic oxide in the feces; $\%N_r$ the percentage of nutrients in the feed; $\%N_f$ is the percentage of nutrients in the feces.

Table 3 lists the values of the coefficients of apparent digestibility (CAD) calculated based on the percentages of Cr_2O_3 determined by the proposed method and by FAAS after mineralization of the feed samples in a microwave oven.

A comparison of the values of the coefficients of apparent digestibility (CAD) of the nutrients present in the four types of feed used in the diet of Nile tilapia juveniles (see Table 3) reveals that the values found based on the determinations of the percentage of Cr_2O_3 by the proposed method are congruent with those obtained by the FAAS method after mineralization of the feed and feces samples. The FAAS method is normally used in fish nutrition digestibility studies [20–22]; hence, our results attest to the applicability of the proposed method in such studies.

Table 3
Coefficient of apparent digestibility of dry matter (DM), gross protein (GP), total lipids (TL) and gross energy (GE) of Nile tilapia juveniles fed with feed containing different food supplements

Feeds	Coefficient of digestibility apparent							
	DM (%)		GP (%)		TL (%)		GE (%)	
Yeast	67 ± 2[a]	65 ± 2[b]	89 ± 4[a]	87 ± 3[b]	84 ± 3[a]	83 ± 3[b]	69 ± 2[a]	68 ± 3[b]
Corn	69 ± 2[a]	67 ± 2[b]	91 ± 4[a]	89 ± 3[b]	85 ± 2[a]	86 ± 2[b]	73 ± 3[a]	74 ± 3[b]
Soybean bran	68 ± 2[a]	69 ± 2[b]	91 ± 3[a]	90 ± 2[b]	87 ± 3[a]	89 ± 4[b]	71 ± 3[a]	70 ± 3[b]
Rice bran	69 ± 2[a]	68 ± 2[b]	77 ± 2[a]	78 ± 2[b]	78 ± 2[a]	76 ± 2[b]	73 ± 2[a]	72 ± 2[b]

[a] Calculation based on the % Cr_2O_3 determined by proposed method.
[b] Calculation based on the % Cr_2O_3 determined by FAAS after mineralization of the feed samples in a microwave oven.

4. Conclusions

The proposed method for quantifying Cr_2O_3 using samples of fish feed and feces in the form of suspensions to estimate the apparent availability of nutrients in feeds used in fish nutrition yielded results equivalent to those obtained with the FAAS quantification method, whose initial step involves the mineralization of samples. The main advantage of the proposed method is that it does not generate toxic dichromate residues, which can be harmful to the analyst's health and contaminate the environment. Moreover, since this new method does not require mineralizing the samples, it considerably reduces the time spent on analytical determinations in fish nutrition analyses. In addition it offers limits of detection (LOD) and of quantification (LOQ) in the order of 0.80 and 2.80 $\mu g\,L^{-1}$, respectively, that correspond to 40 and 140 $\mu g\,kg^{-1}$, using only 10 μL of slurry samples for each analytical determination. The values of the limits of detection calculated by the proposed method were about 95-fold smaller than those obtained by the FAAS technique.

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