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Note on the Methods for Determination of Chromic Oxide in Shrimp Feeds

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Chromic oxide is used as an inert marker to measure apparent digestibility of feeds in insects, terrestrial, and aquatic animals. Quantitative determination of chromic oxide content in the sample requires the oxidation of water insoluble trivalent chromic oxide to its water-soluble hexavalent form. The two commonly used oxidizing agents are 70% perchloric acid or a mixture of sodium molybdate, sulfuric, and perchloric acid. Chromic oxide content of the oxidized solution is then measured against known standards either directly by spectrophotometry in the visible range at 350, 370, or 440 nm or after forming a colored complex with diphenylcarbazide (DPC) by colorimetry at 540 nm. This study compared the two methods of oxidation followed by spectrophotometry at the three wavelengths and by DPC colorimetry. DPC colorimetry gave precise results than the direct measurement of dichromate ion, irrespective of the method of oxidation used. Ash from samples oxidized by perchloric acid and quantified by DPC colorimetry gave a better measure of actual chromic oxide content as compared to the other methods tested.

KEYWORDS: Method; shrimp; feed; chromic oxide

INTRODUCTION

Chromic oxide is used as an inert marker to measure apparent digestibility of feeds in terrestrial animals (*1*), in aquatic animals, in fish (*2*), and in shrimp (*3*). The primary steps required to measure chromic oxide content in a sample are oxidation of the inert and insoluble trivalent chromic oxide to its water-soluble hexavalent form before quantitative determination. Several methods have been published for oxidation of chromic oxide and its subsequent measurement in feed and in feces. In one study, the ash from fecal samples containing chromic oxide is boiled in a solution of 85% phosphoric acid containing 0.3% of manganese sulfate ($MnSO_4 \cdot 4H_2O$) and 0.18% potassium bromate ($KBrO_3$). The resulting oxidized solution is suitably diluted, and chromic oxide is measured by atomic absorption spectrometry (*1*). In another study, feed and feces of phytophagus insects were oxidized by boiling the sample without ashing in a solution of 2.5% sodium molybdate dissolved in 30% water, 30% sulfuric acid, and 40% perchloric acid (70% m/v). After oxidation, chromium content was measured in dilute solutions from the color complex formed by the interaction of hexavalent chromium with diphenylcarbazide (DPC) (*1*). Using 2% sodium molybdate instead of 2.5%, chromic oxide content in feed and fecal samples was measured after oxidation against known serial dilutions of oxidized chromic oxide at 440 nm (*5*, *6*). First, they dissolved the feed and fecal samples from fish in hot concentrated nitric acid and subsequently oxidized the chromic oxide with hot 70% perchloric acid. Chromic oxide content was then measured by the characteristic absorption of the hexavalent chromium at 350 nm. Petry and Rapp (*7*) oxidized trivalent chromic oxide with a solution of sodium molybdate described above and studied the absorption characteristics of hexavalent chromium from 350 to 440 nm. They found that pH had an influence on absorbance and recommended measuring absorbance at 370 nm after adjusting the solution to pH 11. Absorbance at 440 nm was found unsuitable for determining CrO_4^{2-} ion during digestion experiments. Pure acidic potassium dichromate solutions were used as an ultraviolet standard in the absorbance range of 250−400 nm (*8*).

The objective of this study is to evaluate and recommend an accurate and precise method that can be used as a standard for determining chromic oxide in aquaculture feeds.

MATERIALS AND METHODS

The study compares two methods of oxidation and measurement of chromic oxide after oxidation by spectrophotometry at 350, 370, and 440 nm and by DPC colorimetry. Safety, minimum use of sample, and chemicals were considered important to this study, especially minimum physical handling of perchloric acid during analysis.

Sample Preparation. A shrimp feed formulation routinely used as a control feed (**Table 1**) containing five levels of chromic oxide (Cr_2O_3) viz 1.25, 1.0, 0.75, 0.50, and 0.25% (W/W) served as samples for the study. Yttrium oxide added as an additional marker was not measured in this study. The feed samples were ground and sieved to pass through a 0.42 mm screen (40 mesh) before using them in the analysis. Shrimp feed and ash after ashing the shrimp feed at 600 °C for 6 h were used for the measurement of chromic oxide content. Feed samples were analyzed in five replicates each. All of the reagents used in this study were obtained from the Sigma Chemical Company, St. Louis, MO, and all equipment used was from Fisher Scientific, Pittsburgh, PA, unless otherwise stated.

Reagent Preparation. Two oxidizing solutions were prepared. The sodium molybdate reagent (molybdate reagent) was prepared as

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Table 1. List of Ingredients and Percentage Used in the Formulation of Shrimp Feeds[a]

LT 94 fishmeal	24.50
whole hard red winter wheat	48.84
squid meal	2.50
vital wheat gluten	4.00
brewers yeast	3.00
corn starch	2.67
protamino aqua	2.00
soybean meal	5.00
soy lecithin–CSM	2.00
fish oil	2.89
cholesterol–FG	0.23
vitamin premix	0.40
choline chloride	0.12
stay C-35	0.07
potassium phosphate, dibasic	0.56
sodium phosphate, dibasic	0.56
calcium phosphate, monobasic	0.56
chromic oxide	0.00
yttrium oxide	0.10
total	100.00

[a] The addition of chromic oxide at 0.25, 0.50, 0.75, 1.00, and 1.25% replaced an equal amount of corn starch in the feed formulation.

follows: Dissolve 5 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) in 75 mL of distilled water, and add 75 mL of concentrated sulfuric acid (H_2SO_4). Cool to room temperature and add 100 mL of 70% perchloric acid ($HClO_4$) and mix. The reagent can be stored in glass-stoppered bottles for 2 weeks. Perchloric reagent was prepared as follows: To 100 mL of distilled water, add 200 mL of concentrated nitric acid (HNO_3), cool, and add 200 mL of 70% perchloric acid ($HClO_4$). The reagent can be stored in a glass-stoppered bottle for 4−6 weeks.

Sample Oxidation with Molybdate Reagent. Oxidation of feed and/or ash with molybdate reagent was done as follows: A known weight of feed (200−250 mg) or ash (5−10 mg) was placed in a thick-walled Pyrex boiling tube (20 mm × 150 mm), and 3 mL of molybdate reagent was added. One glass bead (2 mm diameter) was added to prevent bumping during boiling. The tube was then clamped at a 45° angle in a retort stand in a fume hood and heated to a boil until fumes subsided to a minimum (2−3 min). The solution becomes clear and turns yellow due to oxidation of chromic oxide to its monochromate (CrO_4^{2-}). Care is needed to ensure that charred particles formed during boiling do not adhere to the sides of the test tube. The charred particles are usually carried down by the liquid condensing from the vapor in the upper part of the test tube if boiling is carefully controlled. After the solution is cooled to ambient temperature (10 min), 3 mL of 70% perchloric acid is added and boiling is repeated (10 min) to ensure completion of oxidation. The cooled liquid is quantitatively transferred and made up to 25 mL in a volumetric flask by rinsing repeatedly the boiling tube with distilled water. The solution is now ready for the estimation of chromic oxide content. A known weight (2−4 mg) of chromic oxide, a feed, and an ash sample without chromic oxide were similarly treated. The oxidized chromic oxide solution was used as the standard, and the feed and ash samples served as blanks.

Sample Oxidation with Perchloric Reagent. Oxidation with perchloric reagent was done as follows. A known weight of ash (5−10 mg) from the samples containing chromic oxide was placed in disposable flint glass test tubes (16 mm × 125 mm). One glass bead (2 mm diameter) is added to each tube to prevent bumping during boiling. Perchloric reagent, 4 mL, is then added along the sides of the test tube to wash down any adhering ash. The tubes were set in an aluminum heating block and then heated over a temperature-controlled hot plate kept in a fume hood. Several tubes can be heated at the same time depending on the number of holes available in the block. A thermometer was inserted somewhere in the middle of the heating block in a test tube half-filled with silica sand. The heating block is heated to a temperature range of 210−220 °C. The tubes in the heating block are maintained at that temperature for 10−12 min, by which time oxidation of chromic oxide to its monochromate (CrO_4^{2-}) was complete, and it was allowed to cool. It is not advisable to heat any longer than 15 min as this can result in drying of the reagent, thereby generating a possible explosion hazard. After the contents of the tubes were cooled to room temperature, the liquid was quantitatively transferred and made up to 25 mL in a volumetric flask by rinsing repeatedly with distilled water. The solution was now ready for the estimation of chromic oxide content. A known weight (2−4 mg) of chromic oxide (Sigma) and a sample of ashed feed containing no chromic oxide were similarly treated. The oxidized chromic oxide solution was used as a standard, and the ashed feed sample served as a blank. Only ash samples were used for perchloric oxidation. Direct oxidation of feed with perchloric acid reagent (without ashing) gave erratic results and was therefore not included in the experiments.

Measurement of Chromic Oxide. Measurement of chromic oxide content in feed and ash samples oxidized by molybdate reagent and ash samples oxidized by perchloric reagent was done by measuring absorbance of the oxidized solution at 350, 370, and 440 nm. The oxidized solutions that were made up to 25 mL were directly measured at these wavelengths without any further dilution. The standard oxidized chromic oxide solution was serially diluted with distilled water in the range of 20, 16, 12, 8, 4, and 2 μg/mL. Absorbance of known serial dilutions and of the unknown sample concentrations was measured using a 1 cm path length, 4.5 mL quartz cuvette with a Beckman DU 70 spectrophotometer (Beckman Coulter, Fullerton, CA). Absorbance of a known serial dilution was used to generate a regression equation to calculate unknown concentrations in the samples.

Chromic oxide content in feed and ash samples oxidized by molybdate reagent and ash samples oxidized by perchloric reagent was also measured by DPC colorimetry. A 0.25% solution of 1,5-DPC was prepared by first dissolving 0.25 g in 50 mL of acetone and then diluting up to 100 mL with distilled water. The reagent solution is stable for 1 week if stored in a dark bottle under a nitrogen atmosphere below 10 °C. Sulfuric acid solution, 3 N, was prepared by adding 42 mL of 95% sulfuric acid to 458 mL of distilled water. The standard oxidized chromic oxide solution was serially diluted with distilled water in the range of 10, 8, 6, 4, and 2 μg/mL. All of the samples were then further diluted with distilled water to fall within the range of 10−2 μg chromic oxide/mL. To 1 mL of sample placed in a test tube (16 mm × 125 mm) were added 1.5 mL of 3 N sulfuric acid and 3.5 mL of distilled water, and the mixture was mixed well in a vortex shaker. Then, 0.5 mL of DPC was added to each tube and immediately mixed in a vortex shaker. An additional 3.5 mL of distilled water was then added to make the final volume to 10 mL. Absorbance of the colored complex of known serial dilutions and of the unknown sample concentrations was measured using a 1 cm path length, 4.5 mL disposable polystyrene cuvette with a Beckman DU 70 spectrophotometer at 540 nm. The absorbance for the known serial dilution was used to generate a regression equation to calculate unknown concentrations of chromic oxide in the samples.

Statistical Analysis. Regression analyses were performed using SigmaStat (version 2.0) on the actual and predicted chromic oxide values. A correlation coefficient was used to describe the fit of the data on the regression line. The intercept and slope of the regression line were evaluated and compared among the four methods using *t*-test and analysis of variance.

RESULTS AND DISCUSSION

Results for the percent chromic oxide content of feed and ash samples, oxidized by molybdate and perchloric reagents and measured at 350, 370, and 440 nm and by DPC colorimetry, are summarized in **Table 2**. Regression analyses performed on these data are presented in **Figures 1−3**. The figures show relatively high correlation coefficients by all methods but differ in slope and intercept values. The ideal prediction is given by a correlation coefficient value of 1, which is indicated by a 1:1 agreement between the actual and predicted chromic oxide values, with an intercept value of zero and a slope value of 1.0.

The regression results show that the DPC colorimetry method gave the best prediction of chromic oxide values for feed and ash samples. The regression equation for chromic oxide on feed

Table 2. Percent Chromic Oxide Content of Feed and Ash Samples Determined by Molybdate and Perchloric Oxidation and Measured at 350, 370, and 440 nm and by DPC Colorimetry at 540 nm[a]

	actual percent chromic oxide of sample				
method	0.25	0.50	0.75	1.00	1.25
			Molybdate Oxidation (Feed Samples)		
350 nm	0.139 ± 0.033	0.376 ± 0.014	0.593 ± 0.025	0.818 ± 0.047	1.055 ± 0.041
370 nm	0.323 ± 0.043	0.647 ± 0.015	0.955 ± 0.024	1.256 ± 0.075	1.576 ± 0.051
440 nm	0.185 ± 0.031	0.461 ± 0.021	0.753 ± 0.028	1.039 ± 0.074	1.314 ± 0.019
DPC	0.279 ± 0.029	0.564 ± 0.027	0.813 ± 0.028	1.083 ± 0.035	1.284 ± 0.124
			Molybdate Oxidation (Ash Samples)		
350 nm	0.161 ± 0.007	0.327 ± 0.041	0.257 ± 0.019	0.403 ± 0.033	0.544 ± 0.022
370 nm	0.148 ± 0.007	0.337 ± 0.048	0.478 ± 0.018	0.666 ± 0.031	0.847 ± 0.020
440 nm	0.242 ± 0.006	0.477 ± 0.064	0.697 ± 0.009	0.926 ± 0.012	1.149 ± 0.011
DPC	0.223 ± 0.004	0.518 ± 0.013	0.782 ± 0.008	1.057 ± 0.009	1.294 ± 0.012
			Perchloric Oxidation (Ash Samples)		
350 nm	0.203 ± 0.004	0.430 ± 0.003	0.647 ± 0.006	0.887 ± 0.078	1.060 ± 0.014
370 nm	0.191 ± 0.004	0.408 ± 0.002	0.632 ± 0.005	0.816 ± 0.010	1.038 ± 0.013
440 nm	0.067 ± 0.012	0.120 ± 0.032	0.282 ± 0.009	0.722 ± 0.013	0.928 ± 0.017
DPC	0.254 ± 0.004	0.507 ± 0.002	0.744 ± 0.009	1.018 ± 0.001	1.262 ± 0.023

[a] Values represent the mean ± standard deviation of five replicate samples.

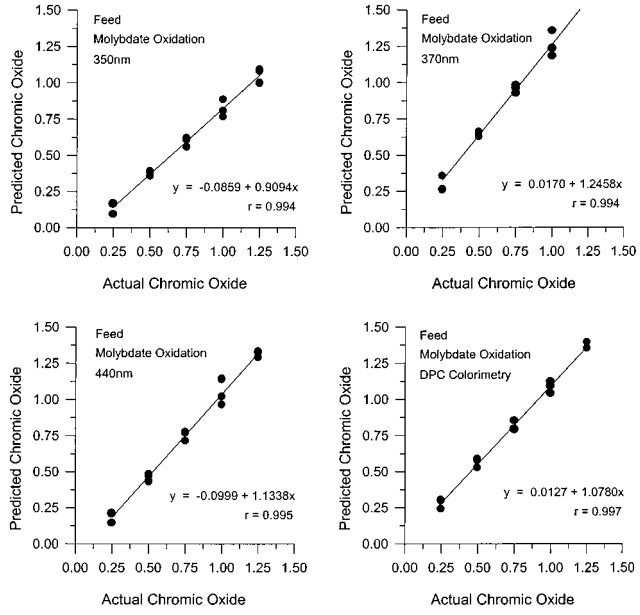

Figure 1. Predicted percent chromic oxide values for shrimp feed samples determined by four methods (molybdate oxidation and measured at 350, 370, and 440 nm absorbance and DPC colorimetry) as a function of the actual percent chromic oxide values.

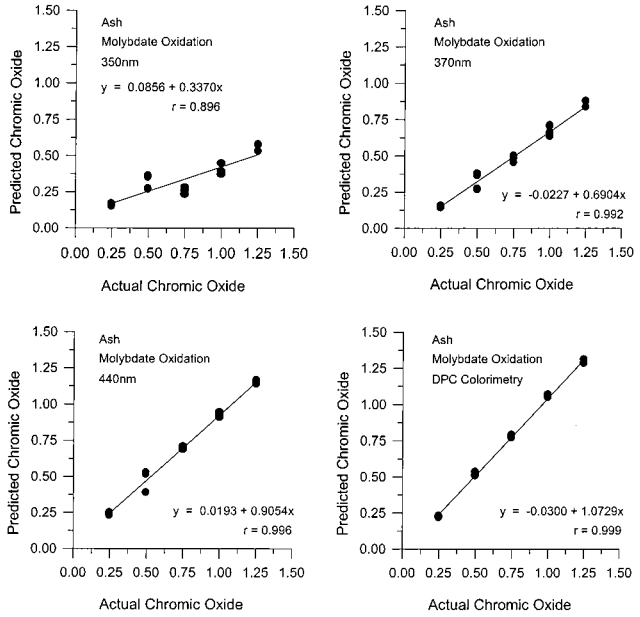

Figure 2. Predicted percent chromic oxide values for ash samples determined by four methods (molybdate oxidation measured at 350, 370, and 440 nm absorbance and DPC colorimetry) as a function of the actual chromic oxide values.

samples determined by molybdate oxidation and the DPC colorimetry method had an intercept value of 0.0127, which is not significantly different from zero ($P = 0.543$); a slope value (1.078) very close to 1.0 ($P < 0.001$); and a correlation coefficient of 0.997. This coefficient indicates a 99.7% agreement between the actual and the predicted chromic oxide values (**Figure 1**). On the other hand, the regression equation for chromic oxide on ash samples determined by perchloric oxidation and the DPC colorimetry method had an intercept value of −0.0013 ($P = 0.880$), a slope value of 1.011 ($P < 0.001$), and a correlation coefficient of 0.999 (**Figure 3**). This method appeared to provide a better prediction of actual chromic oxide values of ash samples tested than the molybdate oxidation and DPC colorimetry method as shown in **Figure 2** (intercept = −0.03 with $P = 0.015$; slope = 1.073 with $P < 0.001$; and correlation coefficient = 0.999). The method employing molybdate oxidation and 350 nm absorbance gave the worst prediction equation for chromic oxide on all samples tested (**Figures 1**−**3**).

It is seen that DPC colorimetry results are more precise than the results obtained by the other methods. This can be attributed to the fact that DPC colorimetry does not directly measure absorbance of the chromate ion but measures a chromatophore resulting from the interaction between chromate ion and DPC. The imprecise readings obtained when chromate concentrations in samples are measured directly as absorbance at 350, 370, and 440 nm were caused by interference from the impurities other than the chromate ion present in sample solutions. Furthermore, the absorbance of sample solutions was compared with pure dichromate ion solutions as standards. Acidic potassium dichromate solution is routinely used as a standard to calibrate spectrophotometers (*8*), but it should be remembered that purity of the hexavalent ion is important for such measurements.

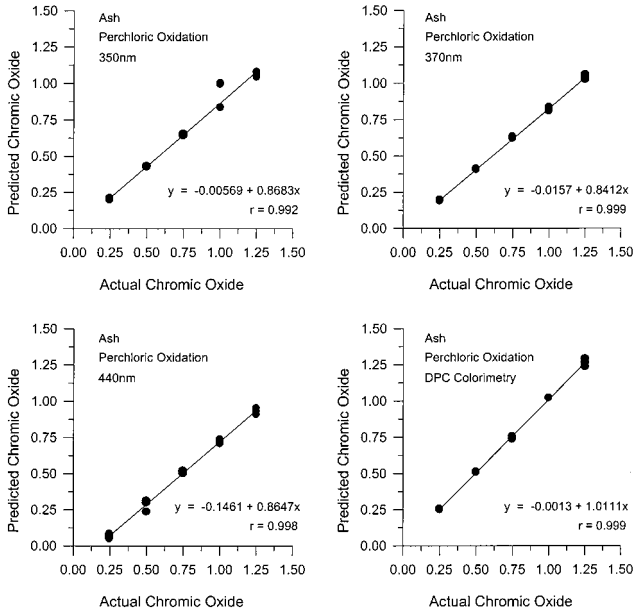

Figure 3. Predicted percent chromic oxide values for ash samples determined by four methods (perchloric oxidation measured at 350, 370, and 440 nm absorbance and DPC colorimetry) as compared to the actual chromic oxide values.

In recent years, attempts have been made to replace chromic oxide with other trivalent metal oxides such as yttrium. Fifteen trivalent metal oxides were evaluated (*9*) as inert markers to estimate apparent digestibility in salmonids, and it was concluded that trivalent metal oxides of lanthanum, yttrium, and ytterbium can substitute chromic oxide in digestibility studies in salmonids and can be used in lower concentrations without affecting accuracy. However, accurate determination of these metal oxide contents in feed and fecal samples requires either atomic absorption or inductively coupled plasma emission spectrometry, and these instruments can be expensive. It is reasonable to assume that the use of chromic oxide as a marker in any animal feed for the determination of apparent digestibility will remain the method of choice as a simple and economic method. Although the present study was restricted to feed samples, the method without any modification is equally applicable to ash from feces.

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