



Evaluation of Acid Digestion Procedures to Estimate Mineral Contents in Materials from Animal Trials

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ABSTRACT: Rigorously standardized laboratory protocols are essential for meaningful comparison of data from multiple sites. Considering that interactions of minerals with organic matrices may vary depending on the material nature, there could be peculiar demands for each material with respect to digestion procedure. Acid digestion procedures were evaluated using different nitric to perchloric acid ratios and one- or two-step digestion to estimate the concentration of calcium, phosphorus, magnesium, and zinc in samples of carcass, bone, excreta, concentrate, forage, and feces. Six procedures were evaluated: ratio of nitric to perchloric acid at 2:1, 3:1, and 4:1 v/v in a one- or two-step digestion. There were no direct or interaction effects ($p > 0.01$) of nitric to perchloric acid ratio or number of digestion steps on magnesium and zinc contents. Calcium and phosphorus contents presented a significant ($p < 0.01$) interaction between sample type and nitric to perchloric acid ratio. Digestion solution of 2:1 v/v provided greater ($p < 0.01$) recovery of calcium and phosphorus from bone samples than 3:1 and 4:1 v/v ratio. Different acid ratios did not affect ($p > 0.01$) calcium or phosphorus contents in carcass, excreta, concentrate, forage, and feces. Number of digestion steps did not affect mineral content ($p > 0.01$). Estimated concentration of calcium, phosphorus, magnesium, and zinc in carcass, excreta, concentrated, forage, and feces samples can be performed using digestion solution of nitric to perchloric acid 4:1 v/v in a one-step digestion. However, samples of bones demand a stronger digestion solution to analyze the mineral contents, which is represented by an increased proportion of perchloric acid, being recommended a digestion solution of nitric to perchloric acid 2:1 v/v in a one-step digestion. (**Key Words:** Ash, Feed Analysis, Chemical Analysis, Spectrophotometry)

INTRODUCTION

The ash content is a measure of the total amount of minerals present within a sample, whereas the mineral content is a measure of the amount of specific inorganic components, such as calcium (Ca), phosphorus (P), magnesium (Mg), and zinc (Zn), present within a sample. Wet ashing is primarily used in the preparation of samples for subsequent analysis of specific minerals. It breaks down and removes the organic matrix surrounding the minerals through the use of both heat and acids, so that minerals are left in an aqueous solution.

Accurate chemical analysis of feed ingredients is mandatory for precise formulation of diets. Unfortunately, some analysis results can vary among laboratories

(Cromwell et al., 2000). It has been demonstrated that analytical variability of a common diet among laboratories is large for Ca and Zn, representing twofold or more in the extremes, and considered intermediate for P (Cromwell et al., 2003). Therefore, to minimize this possible bias, an accurate and standardized procedure should be used by different laboratories.

Rigorously standardized laboratory protocols are essential for meaningful comparison of data from multiple laboratories. Mineral analyses have been performed using as a digesting solution nitric and perchloric acid in a ratio ranging from 2:1 to 4:1 v/v and with two digestion steps (AOAC, 2000; Detmann et al., 2012). Standardizing the ratio of acids used in the digestion of samples could increase the reliability of either information or comparison of results between different laboratories, while a decrease in the number of digestion steps would make the procedure simpler, faster, and less susceptible to errors.

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In minerals analyses, the digestion process is the limiting factor as to how much time the entire procedure takes and also concerns the efficiency of recovering the actual amount of mineral present in the sample (McCarthy and Ellis, 1991). Considering that the interactions of minerals with organic matrices may vary depending on the analyzed material (e.g., bones, feces, or forages), there could be peculiar demands for each material with respect to digestion procedures.

Thus, the objective of this study was to evaluate acid digestion procedures using different nitric to perchloric acid ratios and one- or two-step digestion to estimate the concentration of Ca, P, Mg, and Zn in samples of carcass, bone, excreta, concentrate, forages, and feces.

MATERIALS AND METHODS

Location and samples

This experiment was carried out at the Animal Nutrition Laboratory, Animal Science Department, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. Samples of cattle feces ($n = 10$), forages ($n = 10$), concentrates ($n = 10$), cattle bones ($n = 10$), cattle carcasses ($n = 10$), and poultry excreta ($n = 10$) were used. The fecal, carcass, and bone samples were obtained from animals involved in a feedlot cattle trial. The forage samples were fresh tropical grasses and legumes, and the feed concentrate samples were composed of grains and meals. Excreta samples were collected from a poultry trial. The range of sample types aimed to propitiate a greater representation and better comparative evaluation between the digestion procedures.

Samples with high moisture contents were freeze-dried according to the method suggested by the Brazilian Institute of Science and Technology in Animal Science (INCT-CA; method G-002/1) (Detmann et al., 2012). After that, all samples were processed in a knife mill using a 1-mm screen sieve.

Digestion procedures

Six digestion procedures using nitric acid (HNO_3 ; 65%, P.A., Vetec 191; Sigma-Aldrich Ltd., São Paulo, SP, Brazil) and perchloric acid (HClO_4 ; 70%, P.A., ACS, Vetec 909; Sigma-Aldrich Ltd., Brazil) were evaluated. This six-part arrangement design included a one-step digestion process, using nitric to perchloric acid at a ratio of 2:1, 3:1, or 4:1 v/v and a two-step digestion process, using nitric to perchloric acid at a ratio of 2:1, 3:1, or 4:1 v/v.

To perform the one-step digestion procedures, approximately 250 mg of sample were poured into glass tubes. After that, 5 mL of the digestion solution (a mixture of nitric and perchloric acid at the ratio of 2:1, 3:1, or 4:1 v/v) were added. The tubes were then heated at 200°C until the solution became translucent and a brownish smoke

stopped being released, which indicated the complete digestion of the organic matter. The tubes were allowed to cool at room temperature. Then, the digested samples were quantitatively transferred to 50 mL volumetric flasks. The transfer was accomplished using ash-free quantitative filter paper (Whatman No. 41, Whatman International Ltd, Springfield, Kent, England). The volume of the solutions was made up to 50 mL using deionized water. Aliquots of the solutions were transferred to polyethylene flasks and kept cool (4°C).

The same amount of sample was used to perform the two-step digestion procedures. After pouring the samples in the tubes, the nitric acid was added at 3.3, 3.7, and 4.0 mL/tube which corresponded to the amount of acid for the 2:1, 3:1 and 4:1 ratios, respectively. The tubes were then heated at 200°C until the acid volume was the half of the initially added. After cooling at room temperature, perchloric acid was added at 1.7, 1.3, and 1.0 mL/tube, corresponding to 2:1, 3:1, and 4:1 ratios, respectively. After that, the tubes were heated again to 200°C. The endpoint of digestion, the quantitative transference, and sample storage followed the same procedures described for the one-step digestion process.

Quantification of minerals contents

The P contents were evaluated based on colorimetric reaction with sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, ACS $\geq 99\%$, Sigma-Aldrich 331058, Sigma-Aldrich Ltda, Brazil; Fiske and Subbarow, 1925). The colorimetric evaluations were carried out at 725 nm in a spectrophotometer UV/Visible BEL Photonics 2000 UV (Bel Photonics do Brasil Ltda, Osasco, SP, Brazil). Phosphorus standard solutions were previously prepared using monopotassium phosphate (KH_2PO_4 ; P.A., ACS, Vetec 1361, Sigma-Aldrich Ltda, Brazil).

The contents of Ca, Mg, and Zn were evaluated through atomic absorption spectrophotometry (Skoog et al., 2006) using a GBC Avanta Σ atomic absorption spectrophotometer (Scientific Equipment, Braeside, Victoria, Australia), hollow-cathode lamps (422.7, 285.2, and 213.9 nm for Ca, Mg, and Zn), an air-acetylene flame for Ca analysis, and a nitrous oxide-acetylene flame for Mg and Zn analysis. Different standard solutions were produced from pure stock solutions containing 1,000 ppm of the elements (Merck 1.09943 Titrisol, Merck 1.09949 Titrisol, and Merck 1.09953 Titrisol for Ca, Mg, and Zn, Merck KGaA, Darmstadt, Germany). In the Ca analysis, a strontium chloride solution (50 g/L; Merck 1.07865, Merck KGaA) was used as a releasing agent.

Statistical analysis

The statistical evaluation was performed for each mineral according to a completely randomized design

following the model:

$$Y_{ijkl} = \mu + T_i + S_{(ij)} + R_k + N_l + TR_{ik} + TN_{il} + RN_{kl} + TRN_{ikl} + \varepsilon_{ijkl}$$

where T_i is the effect of the i th type of sample (fixed effect); $S_{(ij)}$ is the effect of the j th sample within the i th type (random effect); R_k is the effect of k th nitric to perchloric acid ratio (fixed effect); N_l is the effect of the l th number of steps in digestion (fixed effect); TR_{ik} , TN_{il} , RN_{kl} , and TRN_{ikl} are the interactions (fixed effects); and ε_{ijkl} is the random error.

All statistical procedures were carried out using the MIXED procedure of SAS 9.3 (SAS Institute, Cary, NC, USA) and adopting 0.01 as the critical limit for type I error (Littell et al., 2006). When necessary, average values were compared using the SLICE statement and the Fisher's Least Significant Difference.

RESULTS

The average contents of Ca, P, Mg, and Zn in samples

Table 1. Average contents of calcium (Ca), phosphorus (P), magnesium (Mg), and zinc (Zn) in carcass, bone, and excreta samples according to the different nitric to perchloric acid ratios and the number of digestion steps

Sample	Combination		Minerals			
	R	N	Ca (g/kg)	P (g/kg)	Mg (g/kg)	Zn (mg/kg)
Carcass	2:1	1	47.3	32.7	1.7	14.5
	2:1	2	49.4	31.3	1.7	14.5
	3:1	1	47.6	32.0	1.6	14.6
	3:1	2	52.7	37.5	1.8	14.4
	4:1	1	43.0	30.4	1.6	14.4
	4:1	2	54.0	36.4	1.8	15.1
Bone	2:1	1	185.6	94.3	3.9	9.9
	2:1	2	182.2	95.0	3.7	10.8
	3:1	1	172.5	85.4	3.8	9.7
	3:1	2	173.2	90.7	3.9	9.5
	4:1	1	172.6	87.5	4.3	9.9
	4:1	2	173.0	89.3	3.9	10.6
Excreta	2:1	1	9.4	9.5	6.1	25.6
	2:1	2	11.6	10.0	6.2	26.2
	3:1	1	9.3	8.3	6.4	26.4
	3:1	2	10.3	9.4	6.3	25.1
	4:1	1	9.4	8.9	6.1	24.9
	4:1	2	10.3	9.1	6.4	25.8
SEM			4.2	1.8	0.5	1.1

R, ratio of nitric to perchloric acids; N, number of digestion steps; SEM, standard error of mean.

of carcass, bone, excreta, concentrate, forage, and feces obtained by the different procedures are presented in Table 1 and 2.

There were no direct or interaction effects ($p > 0.01$) of nitric to perchloric acids ratio or number of digestion steps on Mg and Zn contents (Table 3).

On the other hand, both Ca and P contents presented a significant interaction between type of sample and nitric to perchloric acids ratio ($p < 0.01$) (Table 3). The evaluation of this effect indicated that different acid ratios do not affect either Ca or P contents in carcass, excreta, concentrate, forage, and feces samples ($p > 0.01$) (Table 4). Despite this, when bone samples were evaluated, it was observed that the digestion solution of 2:1 v/v (nitric to perchloric) provided greater recovery of Ca and P than the 3:1 and 4:1 v/v ratio ($p < 0.01$) (Table 4).

It should be highlighted that the number of digestion steps did not affect any mineral content ($p > 0.01$).

DISCUSSION

Proper handling or preparation of samples prior to the final analysis is very important for obtaining reliable

Table 2. Average contents of calcium (Ca), phosphorus (P), magnesium (Mg), and zinc (Zn) in concentrate, forage, and feces samples, according to the different nitric to perchloric acid ratios and the number of digestion steps

Sample	Combination		Mineral			
	R	N	Ca (g/kg)	P (g/kg)	Mg (g/kg)	Zn (mg/kg)
Concentrate	2:1	1	1.4	7.4	3.8	6.8
	2:1	2	1.4	6.5	3.8	7.1
	3:1	1	1.3	6.7	3.8	6.6
	3:1	2	1.4	5.9	3.8	6.1
	4:1	1	1.4	6.4	3.9	6.5
	4:1	2	1.4	6.0	3.9	6.8
Forage	2:1	1	6.4	1.3	3.7	3.2
	2:1	2	6.5	1.3	3.5	3.0
	3:1	1	5.6	1.1	3.5	2.8
	3:1	2	6.1	1.2	3.7	2.9
	4:1	1	6.4	1.2	3.6	3.1
	4:1	2	6.3	1.2	3.7	4.3
Feces	2:1	1	5.9	4.5	4.5	13.2
	2:1	2	6.0	4.4	4.7	12.5
	3:1	1	5.4	4.3	4.5	12.6
	3:1	2	5.8	4.6	4.6	13.3
	4:1	1	5.4	4.3	4.5	13.7
	4:1	2	5.3	4.2	4.5	13.0
SEM			4.2	1.8	0.5	1.1

R, ratio of nitric to perchloric acids; N, number of digestion steps; SEM, standard error of mean.

Table 3. Descriptive levels of probability for type I error taken from analyses of variance of the calcium (Ca), phosphorus (P), magnesium (Mg), and zinc (Zn) contents

Effect	Mineral			
	Ca	P	Mg	Zn
Type (T)	<0.001	<0.001	<0.001	<0.001
Ratio (R)	0.046	0.064	0.429	0.032
Steps (N)	0.064	0.019	0.666	0.910
T×R	0.003	<0.001	0.132	0.033
T×N	0.461	0.028	0.355	0.599
R×N	0.201	0.113	0.539	0.754
T×R×N	0.467	0.402	0.217	0.335
RSD	11.2	3.92	0.31	0.14

T, type of sample; R, ratio of nitric to perchloric acids; N, number of digestion steps; RSD, residual standard deviation.

analytical results for minerals contents (Hendricks, 1998). However, a multitude of procedures exist to remove the organic matrix of the sample surrounding the minerals for mineral analysis. The standardization of analytical procedures among laboratories allows obtaining comparable estimates of mineral contents. For the analyses of specific minerals, wet ashing digestion is preferred to dry ashing. The main constraints of the dry ashing procedure include low micro-minerals recovery and the possibility of volatilization losses due to high temperature ashing (Pomeranz and Meloan, 1978; Jones Jr. and Case, 1990; Faithfull, 2002). In the present study, different procedures using wet ashing digestion were evaluated and a wide range of sample types were used aiming to identify a method that could be applicable to the differing materials evaluated in animal trials.

The estimated contents of Ca, P, Mg, and Zn in carcass,

Table 4. Study of the interaction effect between the nitric to perchloric acids ratio and type of the samples on calcium (Ca) and phosphorus (P) contents

Type	Ratio			p-value
	2:1	3:1	4:1	
Ca (g/kg)				
Carcass	48.3	50.2	48.5	0.853
Bone	183.9 ^a	172.8 ^b	163.4 ^c	<0.001
Excreta	10.5	9.82	9.85	0.977
Concentrate	1.40	1.32	1.37	>0.999
Grass	6.47	5.81	6.34	0.980
Feces	5.97	5.58	53.5	0.984
P (g/kg)				
Carcass	32.0	34.7	33.4	0.088
Bone	94.6 ^a	88.0 ^b	88.4 ^b	<0.001
Excreta	9.8	8.9	9.0	0.732
Concentrate	6.9	6.3	6.2	0.804
Grass	1.3	1.2	1.2	0.993
Feces	4.5	4.4	4.3	0.981

^{ab} Means with different superscripts differ (p<0.01).

excreta, concentrate, forage, and feces samples were not affected either by acid ratios or number of digestion steps. The AOAC recommendation for mineral analyses (method 935.13; AOAC, 2000) is based on a nitric to perchloric acid ratio ranging from 2 to 3:1 v/v, whereas the official Brazilian method of INCT-CA (method M-003/1; Detmann et al., 2012) recommends a ratio of 4:1 v/v. Both methods are performed using two digestion steps. First, a predigestion with nitric acid and then a digestion with perchloric acid. The results presented here are in agreement with those methods (except for bone samples), but they further demonstrated that working with one- or two-step digestion leads to similar results.

Clearly, working with one-step digestion could be a preferable procedure because it is simpler, faster and less susceptible to errors. Generally, the possibility of errors through sample alteration, contamination or loss is increased by handling, so that variation is proportional to the number of steps or operations (Van Soest and Robertson, 1985). On the other hand, using a 4:1 v/v nitric to perchloric acid ratio, rather than 2 or 3:1 v/v, could be more advantageous due to the lower cost of nitric acid when compared to perchloric acid. Therefore, the digestion procedure based on 4:1 v/v, in a one-step digestion process seems to be the most appropriate procedure to quantify minerals in samples of carcass, excreta, concentrate, forage, and feces. These findings are useful once the same procedure can be made applicable to samples of a wide range of materials, including animal and vegetable.

Despite this, for Ca and P in bone samples, the 2:1 v/v nitric to perchloric acid ratio provided greater recovery regardless the number of steps. The lower recovery obtained by using the 3:1 and 4:1 v/v ratios indicated an incomplete liberation of minerals during digestion. Mixed acids are the usual reagents for the decomposition of organic material; however, the quantities of each acid, order, and rate of addition may vary with different biological materials (Pomeranz and Meloan, 1978). Accordingly, the difference observed for bones when compared to other types of samples could be associated either with the greater contents of Ca and P in bones or the different and stronger linkages of minerals to the organic matrix. Thus, a stronger digestion solution would be necessary to analyze minerals in bones, in this case represented by an increased proportion of perchloric acid in the digestion solution.

On the other hand, the estimation of Mg and Zn in bones samples provided the same result regardless of the digestion procedure. However, in this case, it is recommended that one use the digestion procedure based on 2:1 v/v in a one-step digestion process, following the same procedure used for Ca and P.

It is significant that regardless of sample or solution ratio, one-step digestion can be used for all the minerals.

Previous work in our laboratory showed a similar pattern for chromium analysis (Rocha et al., 2015).

In conclusion, the quantification of Ca, P, Mg, and Zn contents in samples of carcass, excreta, concentrated, forage, and feces can be performed using a digestion solution of nitric to perchloric acid 4:1 v/v in a one-step digestion. However, for bone samples, a digestion solution of nitric to perchloric acid 2:1 v/v in a one-step digestion is recommended.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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