**MALBER NATHAN NOBRE PALMA**

# EVALUATION OF ACID DIGESTION PROCEDURES TO ESTIMATE MINERAL CONTENTS IN MATERIALS FROM ANIMAL TRIALS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de Magister Scientiae.

**VIÇOSA**
**MINAS GERAIS – BRASIL**
**2015**

**Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa**

T

Palma, Malber Nathan Nobre, 1990-

P171e
2015

Evaluation of acid digestion procedures to estimate mineral contents in materials from animal trials / Malber Nathan Nobre Palma. – Viçosa, MG, 2015.

ix, 34f. : il. ; 29 cm.

Orientador: Edênio Detmann.

Dissertação (mestrado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. Minerais na nutrição animal. 2. Digestibilidade. 3. Espectrofotometria. I. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-graduação em Zootecnia. II. Título.

CDD 22. ed. 636.08527

**MALBER NATHAN NOBRE PALMA**

# EVALUATION OF ACID DIGESTION PROCEDURES TO ESTIMATE MINERAL CONTENTS IN MATERIALS FROM ANIMAL TRIALS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de Magister Scientiae.

APROVADA: 23 de fevereiro de 2015.

Cláudia Batista Sampaio

Kelly da Silva Coutinho Detmann

Sebastião de Campos Valadares Filho
(Coorientador)

Edenio Detmann
(Orientador)

Aos meus pais, por não medirem esforços para minha formação,

Pelo exemplo de luta e amor incondicional

Dedico

# AGRADECIMENTOS

À Universidade Federal de Viçosa e ao Departamento de Zootecnia, por tornar possível a realização deste trabalho.

Ao CNPq, pela concessão da bolsa de estudos e pelo financiamento do experimento.

À FAPEMIG e ao INCT Ciência Animal, pelo apoio para a realização deste trabalho.

Ao professor Edenio Detmann, pelo tempo e empenho dedicado à minha formação acadêmica. Exemplo de postura ética e profissional.

Aos funcionários do Laboratório de Nutrição Animal, os quais auxiliaram na execução das tarefas.

Ao Gabriel Rocha, pelas sugestões e pelo apoio técnico sem o qual teria sido impossível a realização deste trabalho.

Aos colegas do Laboratório de Nutrição Animal, William, Marcelo, Érick, Márcia, Luana e Tadeu.

A todos meus familiares e amigos, pelas palavras de incentivo.

Aos membros da banca, por aceitarem contribuir com este trabalho.

Por fim, a todos que de alguma forma ajudaram na realização deste trabalho e em minha formação profissional. Muito Obrigado!

# BIOGRAFIA

Malber Nathan Nobre Palma, filho de Antonio de Sales Palma e Zilma Maria Nobre Batista Palma, nasceu dia 25 de dezembro de 1990 em Bonfinópolis de Minas, Minas Gerais.

Em agosto de 2008 iniciou o curso de graduação em Zootecnia na Universidade Estadual de Montes de Claros, concluindo em julho de 2013. Em agosto deste mesmo ano iniciou o curso de mestrado em Zootecnia pela Universidade Federal de Viçosa, concentrando seus estudos na área de Avaliação de Alimentos para Ruminantes, submetendo-se à defesa de dissertação em 23 de fevereiro de 2015.

# ÍNDICE

**Evaluation of acid digestion techniques to estimate chromium contents in cattle feces**

**Evaluation of acid digestion procedures to estimate mineral contents in materials from animal trials**

# RESUMO

PALMA, Malber Nathan Nobre, M. Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Avaliação de procedimentos de digestão ácida para estimar o conteúdo de minerais em materiais de experimentação animal**. Orientador: Edenio Detmann. Coorientadores: Sebastião de Campos Valadares Filho e Mário Fonseca Paulino.

A presente dissertação foi elaborada com base em dois experimentos para avaliação do teor de minerais em diferentes materiais oriundos de experimentos com animais. No primeiro experimento avaliou-se a acurácia das concentrações de cromo em fezes de bovinos, utilizando-se técnicas de digestão com diferentes razões de ácido nítrico e perclórico em um ou dois passos de digestão. As técnicas avaliadas foram: razão ácido nítrico e perclórico 2:1, 3:1, e 4:1 v/v em um ou dois passos de digestão; e ácido nítrico e perclórico 3:1 v/v sem sódio molibdato em um passo de digestão. A quantificação de cromo foi realizada por espectrofotometria de absorção atômica. A acurácia das técnicas foi avaliada através de amostras-padrão com conteúdo conhecido de cromo (0, 2, 4, 6, 8 e 10 g de cromo por kg de fezes), produzidos a partir de fezes de cinco animais, pelo ajuste de um modelo de regressão linear simples dos valores estimados sobre os valores reais de cromo. A melhor recuperação de cromo foi obtida usando a razão 3:1 v/v, com uma etapa de digestão e molibdato de sódio como catalisador. O uso de molibdato de sódio como catalisador deve ser visto como obrigatório. Conclui-se que a concentração de cromo nas fezes de bovino é quantificada com acurácia pela técnica de digestão com ácidos nítrico e perclórico na razão de 3:1 v/v em um passo de digestão com molibdato de sódio como catalisador. No segundo experimento seis técnicas de digestão ácida foram avaliadas utilizando diferentes relações de ácido nítrico e perclórico (2:1, 3:1 e 4:1 v/v) em uma ou duas etapas de digestão para estimar a concentração de cálcio, fósforo, magnésio e zinco em amostras de carcaça, osso, excreta, concentrado, forragem e fezes. Não houve efeito direto ou interação da relação ácido nítrico e perclórico e número de etapas de digestão na concentração de magnésio e zinco. Houve interação significativa entre o tipo de amostra e a relação ácido nítrico e perclórico para concentração de cálcio e fósforo. Houve maior recuperação de cálcio e fósforo nas amostras de ossos utilizando-se solução de digestão de 2:1 v/v em comparação a 3:1 e 4:1 v/v. A relação entre os ácidos não influenciou a concentração de cálcio e fósforo na carcaça, excreta, concentrado, forragem e fezes. O número de etapas de digestão não afetou a concentração de mineral. A concentração de cálcio, fósforo, magnésio e zinco

em carcaça, excreta, concentrado, forragens e fezes pode ser estimada utilizando-se solução com relação entre ácido nítrico e perclórico 4:1 v/v em uma etapa de digestão. No entanto, para as amostras de ossos, solução com relação entre ácido nítrico e perclórico 2:1 v/v em uma etapa de digestão é recomendada.

# ABSTRACT

PALMA, Malber Nathan Nobre, M. Sc., Universidade Federal de Viçosa, February 2015. **Evaluation of acid digestion procedures for estimating the mineral content in materials from animal trials**. Adviser: Edenio Detmann. Co-Advisers: Sebastião de Campos Valadares Filho and Mário Fonseca Paulino.

This dissertation was based on two experiments which were carried out to evaluate mineral contents in different materials from animal trials. In the first one, it was evaluated the accuracy of chromium contents in cattle feces using digestion techniques with different nitric to perchloric acid ratios and one or two digestion steps. The following techniques were evaluated: ratio of nitric to perchloric acid 2:1, 3:1, and 4:1 v/ v in a one- or two-step digestion; and nitric to perchloric acid 3:1 v/v without sodium molybdate in a one-step digestion. The chromium quantifications were carried out using atomic absorption spectrophotometry. The accuracy of the estimates was evaluated through standard samples containing known chromium contents (0, 2, 4, 6, 8 and 10 g of chromium per kg of feces) which were produced from feces obtained from five animals. Accuracy evaluation was performed by adjusting a simple linear regression equation of estimated chromium concentrations on actual concentrations. The chromium recovery closest to unity was obtained using the ratio 3:1 v/v, with one-step digestion and sodium molybdate as catalyst. The utilization of sodium molybdate as catalyst was found mandatory. It could be concluded that the chromium content in cattle feces is accurately evaluated using digestion procedures based on nitric to perchloric acids ratio of 3:1 v/v in a one-step digestion with sodium molybdate as catalyst. In the second experiment, 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 of nitric to perchloric acid ratio or number of digestion steps on magnesium and zinc contents. Calcium and phosphorus contents presented a significant interaction between sample type and nitric to perchloric acid ratio. Digestion solution of 2:1 v/v provided greater recovery of calcium and phosphorus from bone samples than 3:1 and 4:1 v/v ratio. Different acid ratios do not affect calcium or phosphorus contents in carcass, excreta, concentrate, forage, and feces. Number of digestion steps did not affect

mineral content. Estimated concentration of calcium, phosphorus, magnesium, and Zn 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, for bone samples, digestion solution of nitric to perchloric acid 2:1 v/v in a one-step digestion is recommended.

# Introduction

Chromic oxide is the most commonly external marker used in digestion assays with animals. There are several techniques to estimate fecal chromium concentration, and, theoretically, they should converge to the same final result. However, disagreements between techniques in the quantification of fecal chromium have been demonstrated (Saha & Gilbreath, 1991; Kozloski et al., 1998; Souza et al., 2013). Such analytical constraints can indirectly affect the fecal recovery of this marker.

Several aspects of the procedures for chromium quantification in excreta or feces samples have been investigated, including time and temperature of heating, the acid combinations, and the quantification techniques (Kimura & Miller, 1957; Fenton & Fenton, 1979; Kozloski et al., 1998; Souza et al., 2013). The main objective of those investigations was to obtain the best combination of procedures to ensure accuracy of the estimates of fecal concentration and, as a consequence, of the estimates of fecal excretion or digesta flow. However, some constraints to the complete chromium recovery in feces have been associated with acid digestion process (Saha & Gilbreath, 1991).

On the other hand, 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 these possible biases, an accurate and standardized analytical procedure should be used by different laboratories.

In minerals analyses, the digestion process is the limiting factor as to how much time the entire procedure takes and also concerning the efficiency of recovering the actual amount of mineral present in the sample (McCarthy & Ellis, 1990). Considering that the interactions of minerals with organic matrices may vary depending on the material nature (e.g., bones, feces, forages), there could be peculiar demands for each material with respect to digestion procedures.

In this way, studies have been carried out to verify the accuracy of different sample digestion techniques in order to define the most appropriate digestion procedure and quantification technique. The digestion technique using nitric and perchloric acids has been recommended for chromium and different minerals (Kimura & Miller, 1957; AOAC, 2002; Souza et al., 2013). However, it has not been investigated either the best ratio of nitric to perchloric acid or the optimal number of steps in the digestion procedures.

Therefore, the objectives of this dissertation were:

1. to evaluate the accuracy of acid digestion techniques through atomic absorption spectrophotometry using different nitric to perchloric acid ratio and one or two steps for digestion on the accuracy of chromium content estimates in cattle feces; and
2. to evaluate acid digestion procedures using different nitric to perchloric acid ratios and one- or two-steps digestion to estimate the concentration of different minerals in samples of carcass, bone, excreta, concentrate, forage, and feces.

# References

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS – AOAC. **Official methods of analysis.** 17 ed. Gaithersburg: AOAC International, 2000. paginação descontínua.

CROMWELL, G.L.; CLINE, T.R.; CRENSHAW, J.D.; CRENSHAW, T.D.; EASTER, R.A.; EWAN, R.C.; HAMILTON, C.R.; HILL, G.M.; LEWIS A.J.; MAHAN, D.C.; NELSSEN, J.L.; PETTIGREW, J.E.; VEUM, T.L.; YEN J.T. Variability among sources and laboratories in analyses of wheat middlings. **Journal of Animal Science,** v.78, p.2653-2658, 2000.

CROMWELL, G.L.; BRENDEMUHL, J.H.; CHIBA, L.I.; CLINE, T.R.; CRENSHAW, T.D.; DOVE, C.R.; EASTER, R.A.; EWAN, R.C.; FERRELL, K.C.; HAMILTON, C.R.; HILL, G.M.; HITCHCOCK, J.D.; KNABE, D.A.; KORNEGAY, E.T.; LEWIS, A.J.; LIBAL, G.W.; LINDEMANN, M.D.; MAHAN, D.C.; MAXWELL, C.V.; MCCONNELL, J.C.; NELSSEN, J.L.; PETTIGREW, J.E.; SOUTHERN, L.L.; VEUM, T.L.; YEN J.T. Variability in mixing efficiency and laboratory analyses of a common diet mixed at 25 experiment stations. **Journal of Animal Science,** v.81, p.484-491, 2003.

FENTON, T.W.; FENTON, M. An improved procedure for the determination of chromic oxide in feed and feces. **Canadian Journal of Animal Scicence**, v.59, p.631-634, 1979.

KIMURA, F.T.; MILLER, V.L. Improved determination of chromic oxide in cow feed and feces. **Journal of Agricultural and Food Chemistry**, v.5, p.216-216, 1957.

KOZLOSKI, G.V.; FLORES, E.M.M.; MARTINS, A.F. Use of chromium oxide in digestibility studies: variations of the results as a function of the measurement method. **Journal of the Science of Food and Agriculture**, v.76 p.373-376, 1998.

McCARTHY, H.T.; ELLIS, P.C. Comparison of microwave digestion with conventional wet ashing and dry ashing digestion for analysis of lead, cadmium, chromium, copper, and zinc in shellfish by flame atomic absorption spectroscopy. **Journal of Association of Official Analytical Chemists**, v.74, p.566-569, 1990.

SAHA, D.C.; GILBREATH, R.L. Analytical recovery of chromium from diet and faeces determined by colorimetry and atomic absorption spectrophotometry. **Journal of the Science of Food and Agriculture,** v.55, p.433-446, 1991.

SOUZA, N.K.P.; DETMANN, E.; PINA, D.S.; VALADARES FILHO, S.C.; SAMPAIO, C.B.; QUEIROZ, A.C.; VELOSO, C.M. Evaluation of chromium concentration in cattle feces using different acid digestion and spectrophotometric quantification techniques. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.65, p.1472-1482, 2013.

# Evaluation of acid digestion techniques to estimate chromium contents in cattle feces[1]

Abstract – It was evaluated the accuracy of chromium contents in cattle feces using digestion techniques with different nitric to perchloric acid ratios and one or two digestion steps. The following techniques were evaluated: ratio of nitric to perchloric acid 2:1, 3:1, and 4:1 v $v^{-1}$ in a one- or two-step digestion; and nitric to perchloric acid 3:1 v $v^{-1}$ without sodium molybdate in a one-step digestion. The chromium quantifications were carried out using atomic absorption spectrophotometry. The accuracy of the estimates was evaluated through standard samples containing known chromium contents (0, 2, 4, 6, 8 and 10 g of chromium per kg of feces) which were produced from feces obtained from five animals. Accuracy evaluation was performed by adjusting a simple linear regression equation of estimated chromium concentrations on actual concentrations. The chromium recovery closest to unity was obtained using the ratio 3:1 v $v^{-1}$, with one-step digestion and sodium molybdate as catalyst. The utilization of sodium molybdate as catalyst was found mandatory. It could be concluded that the chromium content in cattle feces is accurately evaluated using digestion procedures based on nitric to perchloric acids ratio of 3:1 v $v^{-1}$ in a one-step digestion with sodium molybdate as catalyst.

Index terms: chromic oxide, chromium recovery, laboratory analysis.

---

[1] Artigo publicado no periódico Pesquisa Agropecuária Brasileira, v.50, p.92-95, 2015, sob o formato de Nota Técnica.

# Avaliação de técnicas de digestão ácida para estimar teores de cromo em fezes de bovinos

Resumo – Avaliou-se a acurácia das concentrações de cromo em fezes de bovinos, utilizando-se técnicas de digestão com diferentes razões de acido nítrico e perclórico em um ou dois passos de digestão. As técnicas avaliadas foram: razão ácido nítrico e perclórico 2:1, 3:1, e 4:1 v $v^{-1}$ em um ou dois passos de digestão; e ácido nítrico e perclórico 3:1 v $v^{-1}$ sem sódio molibdato em um passo de digestão. A quantificação de cromo foi realizada por espectrofotometria de absorção atômica. A acurácia das estimativas foi avaliada através de amostras-padrão com conteúdo conhecido de cromo (0, 2, 4, 6, 8 e 10 g de cromo por kg de fezes), produzidos a partir de fezes de cinco animais, pelo ajuste de um modelo de regressão linear simples dos valores estimados sobre os valores reais de cromo. A melhor recuperação de cromo foi obtida usando a razão 3:1 v $v^{-1}$, com uma etapa de digestão e molibdato de sódio como catalisador. O uso de molibdato de sódio como catalisador deve ser visto como obrigatório. Conclui-se que a concentração de cromo nas fezes de bovino é quantificada com acurácia pela técnica de digestão com ácidos nítrico e perclórico na razão de 3:1 v $v^{-1}$ em um passo de digestão com molibdato de sódio como catalisador.

Termos para indexação: análise laboratorial, quantificação de cromo, óxido de cromo.

# Introduction

The recovery of a marker used in a digestion assay can be defined as its quantity collected from the total collection of feces expressed as a proportion of its dietary dose. The recovery is the most important indicator of the efficiency of a marker. Ideally the recovery value should be 1.00 or, at least, a constant value which would allow using a correction factor in the calculation of digestibility (Jagger et al., 1992; Detmann et al., 2004).

Chromic oxide is the most commonly external marker used in digestion assays with animals. There are several techniques to estimate fecal chromium concentration, and, theoretically, they should converge to the same final result. However, disagreements between techniques in the quantification of fecal chromium have been demonstrated (Saha & Gilbreath, 1991; Kozloski et al., 1998; Souza et al., 2013). Such analytical constraints can indirectly affect the fecal recovery of this marker.

Several aspects of the procedures for chromium quantification in excreta or feces samples have been investigated, including time and temperature of heating, the acid combinations, wavelengths and the quantification techniques (Kimura & Miller, 1957; Fenton & Fenton, 1979; Kozloski et al., 1998; Souza et al., 2013). The main objective of those investigations was to obtain the best combination of procedures to ensure accuracy of the estimates of fecal concentration and, as a consequence, of the estimates of fecal excretion or digesta flow.

Some constraints to the complete chromium recovery in feces have been associated with acid digestion process (Saha & Gilbreath, 1991). In this way, studies have been carried out to verify the accuracy of different sample digestion techniques in order to define the most appropriate digestion procedure and quantification technique. The digestion solution of nitric to perchloric acid 2:1 $v\ v^{-1}$ in a two-step-digestion has been recommended (Kimura & Miller, 1957). Recently, digestion of organic matter with nitric and perchloric acid was confirmed to be better than either sulfuric and perchloric acids or phosphoric acid and the quantification of chromium using atomic absorption spectrophotometry was found to be most appropriated (Souza et al., 2013). However, those authors have not investigated the best ratio of nitric to perchloric acid or compared the possibility to work with one step for digestion instead of two.

The objective of this study was to evaluate the accuracy of acid digestion techniques through atomic absorption spectrophotometry using different nitric to perchloric acid ratios and one or two steps for digestion on the accuracy of chromium content estimates in cattle feces.

## Material and Methods

Seven digestion techniques using nitric ($HNO_3$; 65% P.A.; Vetec 191) and perchloric ($HClO_4$; 70% P.A. ACS; Vetec 909) acids were evaluated: digestion using a ratio of nitric to perchloric acid 2:1 v $v^{-1}$ in a one-step digestion, nitric to perchloric acid 2:1 v $v^{-1}$ in a two-step digestion, nitric to perchloric acid 3:1 v $v^{-1}$ in a one-step digestion, nitric to perchloric acid 3:1 v $v^{-1}$ in a two-step digestion, nitric to perchloric acid 4:1 v $v^{-1}$ in a one-step digestion and nitric to perchloric acid 4:1 v $v^{-1}$ in a two-step digestion. All those cited procedures were done using sodium molybdate ($Na_2MoO_4.2H_2O$; ≥99% ACS; Sigma-Aldrich 331058) as a catalyst. The seventh technique was a digestion using nitric and perchloric acid at the ratio of 3:1 v $v^{-1}$ without sodium molybdate in a one-step digestion. The spectrophotometric quantifications were carried out using atomic absorption spectrophotometry.

Several fecal standards containing known chromium contents were produced using cattle feces (organic matrix) obtained from five different animals (one calf, one growing heifer, one bull, one non-lactating dairy cow, and one lactating dairy cow) to evaluate the accuracy of the different techniques. The animals were fed with corn silage-based diets containing different forage:concentrate ratios and none of them had received chromium neither as mineral supplement nor as external marker. The fecal samples were collected on the same day, oven-dried (60°C) and processed in a knife mill (1-mm). From each organic matrix, six different standards were produced containing 0, 2, 4, 6, 8 and 10 g of chromium per kg of feces, totalizing 30 fecal standards. The standard concentrations were produced on as-is basis in order to avoid the accumulation of error from the estimation of the total dry matter content (Mertens, 2003). Pure chromic oxide ($Cr_2O_3$; 99.9% trace metals basis; Sigma-Aldrich 203068) was employed to produce the standards.

To perform the one-step digestion procedures, approximately 250 mg of the fecal standards 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 $v^{-1}$) were added. When necessary, sodium molybdate was added at 1 g $L^{-1}$ of digestion solution. The use of sodium molybdate serves as catalyst for helping the acid digestion to convert $Cr^{+3}$ into $Cr^{+6}$, the quantifiable form of chromium (Kimura & Miller, 1957). The tubes were then heated at 200°C until the appearance of a yellowish/orangish color and a brownish smoke stops to be released, which indicated the complete digestion of the organic matter and the change of chromium valence from $Cr^{+3}$ (sesquioxide) to $Cr^{+6}$ (dichromate). The tubes were allowed to cool at room temperature. After that, the digested samples were quantitatively transferred to 50 mL volumetric flasks. The transfer was done using ash-free quantitative filter paper **(**Whatman #41). The volume of the solutions was made up to 50 mL using de-ionized water. Aliquot of the solutions were transferred to polyethylene flasks and kept cooled (4°C).

The same amount of sample was used to perform the two-step digestions procedures. After pouring the samples in the tubes, the nitric acid was added at 3.3, 3.7 and 4.0 mL/tube which correspond to the amount of acid for the 2:1, 3:1 and 4:1 ratios, respectively. Sodium molybdate was added to the nitric acid in order to provide 1 g $L^{-1}$ of final digestion solution. The tubes were then heated at 200°C until acid was half gone. After cool 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 at 200°C. The end of digestion point, the quantitative transference and sample storage followed the same procedures described for the one-step digestion.

To performed atomic absorption spectrophotometry procedures, standard solutions containing 0, 2, 4, 6, 8, and 10 ppm of chromium were used to produce the standard curve. Those solutions were produced from a stock solution containing 1000 ppm of chromium (Merk 1.09948 Tritisol®). The samples were evaluated in the spectrophotometer GBC Avanta Σ, using a hollow-cathode lamp (357.9 nm) and a nitrous oxide-acetylene flame.

The accuracy of techniques was evaluated by adjusting a simple linear regression equation of chromium concentrations estimated by each technique (dependent variable) on the actual concentrations of chromium in the fecal standards (independent variable). The statistical analysis was performed under the hypotheses:

$H_0 : \beta_0 = 0$ vs. $Ha : \beta_0 \neq 0$ (1),

$H_0 : \beta_1 = 1$ vs. $Ha : \beta_1 \neq 1$ (2).

The slope of the adjusted function must be interpreted as the recovery of chromium added in the fecal standards. Additionally, the intercept should represent some kind of interference in the medium which could be originated from chemical interferences, reagents impurity, as well as incomplete digestion. Accordingly, the estimated concentrations of chromium were considered to be accurate when both null hypotheses were not rejected. The different organic matrixes were included as a random effect during the adjustment of the models and also for estimating the standard errors of the parameter estimates.

The acid digestion technique that was found accurate and presented the greatest recovery was then compared with the digestion technique adopted by National Institute of Science and Technology in Animal Science (INCT-CA; method M-005/1; Detmann et al., 2012) by evaluating the chromium concentration in 104 feces samples obtained from grazing calves (n = 20) and lactating dairy cows (n = 84) which received 10 to 20 g of $Cr_2O_3$ per day.

The feces samples were oven-dried (60°C) and processed in a knife mill (1-mm). After that, all samples were evaluated to chromium content.

The estimates of chromium content obtained by the accurate technique which presented the best recovery were compared to those values obtained by INCT-CA method by adjusting a simple linear regression equation, considering both null hypotheses previously presented. The techniques were considered to be similar when both null hypotheses were not rejected. The effect of animal category (calves or cows) was included as a random effect in the model.

All statistical procedures were carried out using the MIXED procedure of SAS (Statistical Analysis System; version 9.2) and adopting $\alpha = 0.05$.

## Results

There were no interferences from the medium for any technique combinations. This can be affirmed because the contents obtained for the standard 0 g kg$^{-1}$ were very close or equal to zero (Table 1) and none of the intercept estimates were found to be different from zero ($P>0.05$; Table 2). The chromium recovery was found complete ($P>0.05$) when the ratio 3:1 v v$^{-1}$ (nitric to perchloric acids), using sodium molybdate and one-step digestion was used as well as 4:1 v v$^{-1}$ regardless the number of digestion steps. However, the chromium recovery was found incomplete when other techniques were used ($P<0.05$).

Considering the acid digestion ratio of 3:1 v v$^{-1}$ in a one-step digestion, the use of sodium molybdate as catalyst increased the chromium recovery and influence the accuracy of the estimates (Tables 1 and 2). Working with two digestions steps did not improved the chromium recovery of none of technique used. Moreover, recovery was found incomplete ($P<0.05$) when the 2:1 and 3:1 v v$^{-1}$ ratios and two-step digestion were used.

Considering that a one-step digestion should be used to decrease the labor of the analytical procedures, both 3:1 v v$^{-1}$ and 4:1 v v$^{-1}$ ratios produced accurate results (Table 1). However, the nitric to perchloric ratio of 3:1 v v$^{-1}$ with sodium molybdate in a one-step digestion presented the recovery closest to unity (Table 2) and the lower mean prediction error compared to 4:1 v v$^{-1}$ ratio (-0.195 vs. -0.282 g kg$^{-1}$, respectively). From these results, the fecal samples obtained from the digestion trials were compared using the digestion techniques based on nitric and perchloric acids ratio of 2:1 and 3:1 v v$^{-1}$ both with sodium molybdate in a one-step digestion. The first one corresponds to the official method adopted by INCT-CA (Detmann et al., 2012; Souza et al., 2013).

Considering this, it was verified that both technique combinations provided similar results ($P>0.05$) and were strongly correlated ($r = 0.996$; $P<0.05$; Figure 1).

## Discussion

According to Kozloski et al. (1998), the atomic absorption spectrophotometry is the more precise and sensible method for chromium analysis. Recently the accuracy of atomic absorption spectrophotometry was also confirmed by Souza et al. (2013), who compared this method to the colorimetric evaluation using different acid combinations in the digestion procedure. These authors also suggest that the use of nitric and perchloric acid instead of sulfuric and perchloric or phosphoric acid is the most appropriated due to the greater accuracy, lower time and labor consuming. Currently, the 2:1 v $v^{-1}$ nitric to perchloric acid digestion has been suggested in Brazil by the INCT-CA (Detmann et al., 2012).

In the present study, the estimation of chromium was considered to be accurate when the nitric to perchloric ratio of 3:1 v $v^{-1}$ with sodium molybdate in a one-step digestion and 4:1 v $v^{-1}$ ratio regardless the number of step digestions were used. The greatest chromium recovery of the nitric to perchloric ratio of 3:1 v $v^{-1}$ with sodium molybdate in a one-step digestion suggested that it was more accurate to estimate the chromium contents.

The use of predigesting step with nitric acid showed to be unnecessary once none of the digestion techniques were improved by the use of two digestion steps. Moreover, the use of a predigesting step makes the technique more labor and time consuming. Long time analyses make the technique tedious and more steps make it more susceptible to errors due to greater number of analytical steps and sample manipulation. In view of these, a unique step-digestion with a mixture of nitric acid and perchloric acid is recommended.

According to Kimura & Miller (1957), the use of sodium molybdate serves as catalyst for the acid digestion to convert $Cr^{+3}$ into $Cr^{+6}$, the quantifiable form of chromium. In the present study the importance of this reagent was confirmed because the digestion technique based on 3:1 v $v^{-1}$ ratio without sodium molybdate provided incomplete recovery of the fecal chromium presenting the lowest slope while the same procedure with sodium molybdate provided the best recovery amongst all techniques (Table 2). From this, the utilization of sodium molybdate must be assumed as mandatory to correctly perform chromium analysis.

Interestingly, in the present study independent of the step numbers the acid ratio of 2:1 v $v^{-1}$ did not present accurate results. This is not in agreement with Kimura & Miller (1957) and Souza et al. (2013) who worked with the nitric to perchloric ratio of 2:1 v $v^{-1}$ in two- or one-step digestion, respectively. Perhaps the biggest problem is the balance in the action of different acids on the conversion of the chromium valence. The difference between the estimates of concentration and the actual concentration values of the standards increases as the chromium concentration increases when the 2:1 ratio was used (Table 1). This indicate that the function of nitric acid is most important in the conversion of $Cr^{+3}$ to $Cr^{+6}$, which could explains the low recovery when using the 2:1 v $v^{-1}$ ratio.

The digestion techniques based on nitric and perchloric acids ratio of 2:1 and 3:1 v $v^{-1}$ both with sodium molybdate in a one-step digestion were found similar each other (Figure 1) although just the last one was found accurate (Table 2). Besides the accurate results of the 3:1 v $v^{-1}$ with sodium molybdate in a one-step digestion this technique has the advantage over the 2:1 v $v^{-1}$ due to lower cost of the nitric acid compared to perchloric acid.

Frequent testing of large numbers of samples requires a technique that additionally to be accurate should be rapid and cost effective (McCarthy & Ellis, 1990). In accordance, the findings of the present study indicate that chromium estimation should be done using a nitric to perchloric acids ratio of 3:1 v $v^{-1}$ with sodium molybdate in a one-step digestion by atomic absorption spectrophotometry.

## Conclusion

The chromium contents in cattle feces are accurately evaluated through atomic absorption spectrophotometry using digestion procedures based on nitric to perchloric acids ratio of 3:1 v $v^{-1}$ in a one-step digestion with sodium molybdate as catalyst.

## References

DETMANN, E.; VALADARES FILHO, S.C.; PAULINO, M.F.; ZERVOUDAKIS, J.T.; CABRAL, L.S. Avaliação da técnica dos indicadores na estimação do consumo por ruminantes em pastejo. **Cadernos Técnicos de Veterinária e Zootecnia**, v.46, p.40-57, 2004.

DETMANN, E.; SOUZA, M.A.; VALADARES FILHO, S.C.; QUEIROZ, A.C.; BERCHIELLI, T.T.; SALIBA, E.O.S.; CABRAL, L.S.; PINA, D.S.; LADEIRA, M.M.; AZEVEDO, J.A.G. (Eds.) **Métodos para análise de alimentos - Instituto Nacional de Ciência e Tecnologia em Ciência Animal**, Visconde do Rio Branco: Suprema, 2012. 214p.

FENTON, T.W.; FENTON, M. An improved procedure for the determination of chromic oxide in feed and feces. **Canadian Journal of Animal Scicence**, v.59, p.631-634, 1979.

JAGGER, S.; WISEMAN, J.; COLE, D.J.A.; CRAIGON, J. Evaluation of inert markers for the determination of ileal and faecal apparent digestibility values in the pig. **British Journal of Nutrition**, v.68, p.729-739, 1992.

KIMURA, F.T.; MILLER, V.L. Improved determination of chromic oxide in cow feed and feces. **Journal of Agricultural and Food Chemistry**, v.5, p.216-216, 1957.

KOZLOSKI, G.V.; FLORES, E.M.M.; MARTINS, A.F. Use of chromium oxide in digestibility studies: variations of the results as a function of the measurement method. **Journal of the Science of Food and Agriculture**, v.76 p.373-376, 1998.

McCARTHY, H.T.; ELLIS, P.C. Comparison of microwave digestion with conventional wet ashing and dry ashing digestion for analysis of lead, cadmium, chromium, copper, and zinc in shellfish by flame atomic absorption spectroscopy. **Journal ofAssociation of Official Analytical Chemists**, v.74, p.566-569, 1990.

MERTENS, D. R. Challenges in measuring insoluble dietary fiber. **Jounal of Animal Science**, v.81, p.3233-3249, 2003.

SAHA, D.C.; GILBREATH, R.L. Analytical recovery of chromium from diet and faeces determined by colorimetry and atomic absorption spectrophotometry. **Journal of the Science of Food and Agriculture,** v.55, p.433-446, 1991.

SOUZA, N.K.P.; DETMANN, E.; PINA, D.S.; VALADARES FILHO, S.C.; SAMPAIO, C.B.; QUEIROZ, A.C.; VELOSO, C.M. Evaluation of chromium concentration in cattle feces using different acid digestion and spectrophotometric quantification techniques. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.65, p.1472-1482, 2013.

**Table 1.** Means and standard errors for the chromium contents in the standard samples obtained by different combinations of techniques.

| Combinations | | | Standards (g chromium kg$^{-1}$ sample) | | | | | |
|---|---|---|---|---|---|---|---|---|
| R[1] | N[2] | Mo[3] | 0 | 2 | 4 | 6 | 8 | 10 |
| 2:1 | 1 | + | 0.01±0.00 | 1.98±0.21 | 3.77±0.39 | 5.94±0.34 | 7.72±0.49 | 8.93±0.34 |
| 2:1 | 2 | + | 0.01±0.00 | 1.81±0.05 | 3.94±0.54 | 5.59±0.11 | 6.56±0.36 | 8.61±0.38 |
| 3:1 | 1 | + | 0.00±0.00 | 1.96±0.26 | 3.40±0.62 | 5.80±0.44 | 7.60±0.47 | 10.35±0.82 |
| 3:1 | 1 | - | 0.01±0.00 | 1.71±0.05 | 3.45±0.08 | 5.11±0.15 | 7.37±0.29 | 8.04±0.38 |
| 3:1 | 2 | + | 0.01±0.00 | 1.57±0.13 | 3.65±0.35 | 6.33±0.53 | 6.40±0.29 | 8.37±0.93 |
| 4:1 | 1 | + | 0.00±0.00 | 1.67±0.24 | 4.06±0.29 | 5.87±0.27 | 7.60±0.62 | 9.10±0.53 |
| 4:1 | 2 | + | 0.01±0.00 | 1.82±0.12 | 3.94±0.44 | 5.26±0.10 | 7.44±0.30 | 9.51±0.36 |

[1]R, ratio of nitric to perchloric acids. [2]N, number of digestion steps. [3]Mo, sodium molybdate as catalyst.

**Table 2.** Estimates of linear regression parameters for the chromium concentration in the fecal standards obtained by different technique combinations.

| Combination | | | Regression Parameters | | | | P-value | |
|---|---|---|---|---|---|---|---|---|
| R[1] | N[2] | Mo[3] | Intercept | Slope | $s_{xy}$ | r | Ho: $\beta_0 = 0$ | Ho: $\beta_1 = 1$ |
| 2:1 | 1 | + | 0.154±0.249 | 0.914±0.037 | 0.69 | 0.981 | 0.571 | 0.029 |
| 2:1 | 2 | + | 0.214±0.235 | 0.841±0.036 | 0.67 | 0.979 | 0.414 | <0.001 |
| 3:1 | 1 | + | -0.197±0.422 | 1.000±0.069 | 1.30 | 0.944 | 0.665 | 0.955 |
| 3:1 | 1 | - | 0.082±0.178 | 0.840±0.027 | 0.51 | 0.985 | 0.460 | <0.001 |
| 3:1 | 2 | + | 0.178±0.372 | 0.842±0.059 | 1.12 | 0.945 | 0.657 | 0.013 |
| 4:1 | 1 | + | 0.071±0.281 | 0.930±0.042 | 0.79 | 0.976 | 0.813 | 0.108 |
| 4:1 | 2 | + | -0.023±0.189 | 0.938±0.031 | 0.59 | 0.986 | 0.910 | 0.060 |

[1] R, ratio of nitric to perchloric acids. [2] N, number of digestion steps. [3] Mo, sodium molybdate as catalyst.

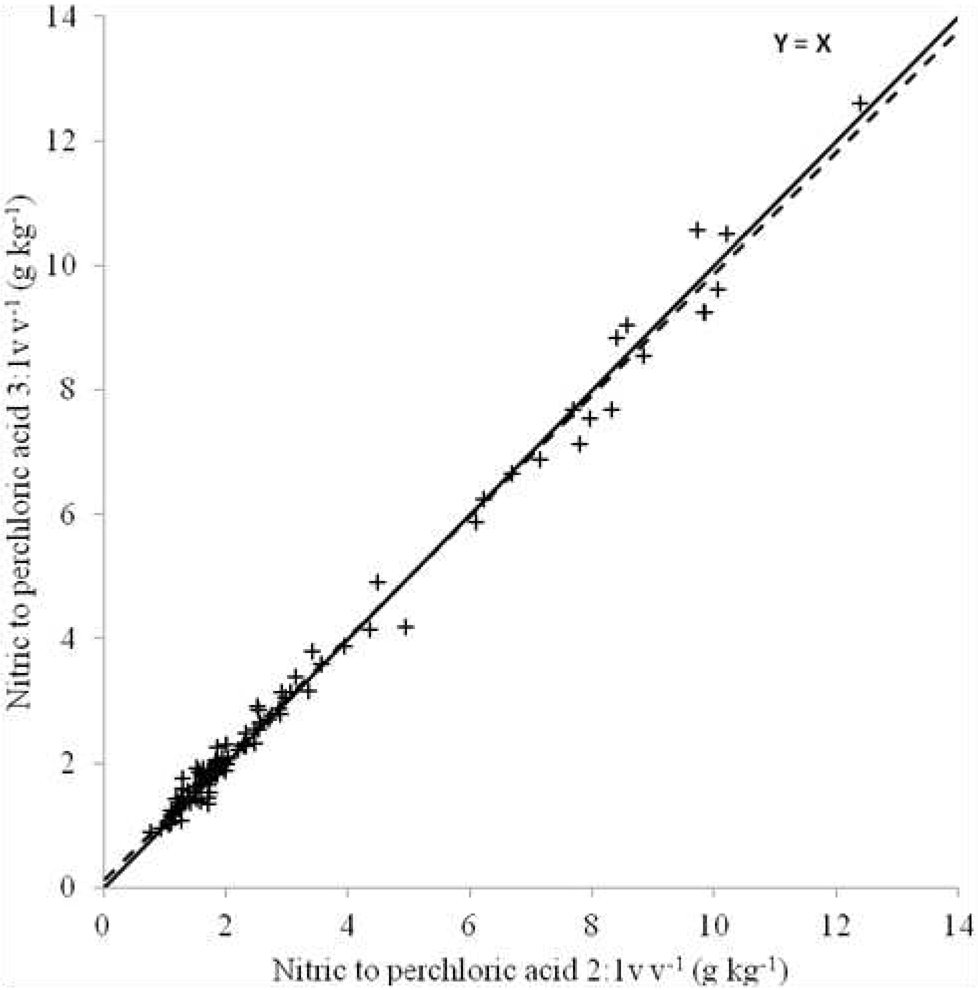

**Figure 1.** Relationship between the fecal chromium concentrations obtained with the digestion technique using nitric to perchloric acids at the ratios of 2:1 and 3:1v $v^{-1}$, both using sodium molybdate as catalyst ($\hat{Y} = -0.1527 + 1.0367 \times X$; $r = 0.996$; $n = 104$; the dashed line corresponds to the least squares straight line).

# EVALUATION OF ACID DIGESTION PROCEDURES TO ESTIMATE MINERAL CONTENTS IN MATERIALS FROM ANIMAL TRIALS

# Avaliação de técnicas de digestão ácida para estimar a concentração de minerais em materiais de experimentação animal

## ABSTRACT

Rigorously standardized laboratory protocols are essential for meaningful comparison of data from multiple laboratories. Considering that interactions of minerals with organic matrices may vary depending on the analyzed material, there could be peculiar demands for each material with respect to digestion procedures. 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 of nitric to perchloric acid ratio or number of digestion steps on magnesium and zinc contents. Calcium and phosphorus contents presented a significant interaction between sample type and nitric to perchloric acid ratio. Digestion solution of 2:1 v/v provided greater recovery of calcium and phosphorus from bone samples than 3:1 and 4:1 v/v ratio. Different acid ratios did not affect calcium or phosphorus contents in carcass, excreta, concentrate, forage, and feces. Number of digestion steps did not affect mineral contents. Evaluation of calcium, phosphorus, magnesium, and Zn contents 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, for bone samples, digestion solution of nitric to perchloric acid 2:1 v/v in a one-step digestion is recommended.

**Index terms:** ash, chemical analysis, feed analysis, spectrophotometry.

# RESUMO

Protocolos rigorosos são essenciais para comparação de dados entre diferentes laboratórios. Considerando-se que as interações de minerais com as matrizes orgânicas podem variar dependendo da natureza do material, poderia haver especificidades para cada um dos materiais em relação aos procedimentos de digestão ácida. Seis técnicas de digestão ácida foram avaliadas utilizando diferentes relações de ácido nítrico e perclórico (2:1, 3:1 e 4:1 v/v) em uma ou duas etapas de digestão para estimar a concentração de cálcio, fósforo, magnésio e zinco em amostras de carcaça, osso, excreta, concentrado, forragem e fezes. Não houve efeito direto ou interação da relação entre ácido nítrico e perclórico e número de etapas de digestão na concentração de magnésio e zinco. Houve interação significativa entre o tipo de amostra e a relação entre ácido nítrico e perclórico para concentração de cálcio e fósforo. Houve maior recuperação de cálcio e fósforo nas amostras de ossos utilizando-se solução de digestão de 2:1 v/v em comparação a 3:1 e 4:1 v/v. A relação entre os ácidos não influenciou a concentração de cálcio e fósforo em amostra de carcaça, excreta, concentrado, forragem e fezes. O número de etapas de digestão não afetou as concentrações de nenhum mineral. A concentração de cálcio, fósforo, magnésio e zinco em amostra de carcaça, excreta, concentrado, forragens e fezes pode ser estimada utilizando-se solução com relação entre ácido nítrico e perclórico 4:1 v/v em uma etapa de digestão. No entanto, para as amostras de ossos, solução com relação entre ácido nítrico e perclórico 2:1 v/v em uma etapa de digestão é recomendada.

**Termos para indexação:** análise de alimentos, análise química, cinzas, espectrofotometria.

# 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. Currently, mineral analyses have been performed using as a digesting solution based on nitric and perchloric acid in a ratio ranging from 2:1 to 4:1 v/v and with two digestion steps (AOAC, 2002; 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 more simpler, faster, and less susceptible to errors.

In minerals analyses, the digestion process is the limiting factor as to how much time the entire procedure takes and also concerning the efficiency of recovering the actual amount of mineral present in the sample (McCarthy & Ellis, 1990). Considering that the interactions of minerals with organic matrices may vary depending on the analyzed material (e.g., bones, feces, 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.

## MATERIAL AND METHODS

### Location and Samples

This experiment was carried out at the Animal Nutrition Laboratory, Animal Science Department, Universidade Federal de Viçosa, Viçosa, MG, 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) and perchloric acid ($HClO_4$; 70%, P.A., ACS, Vetec 909) were evaluated. This six-part experimental 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. After that, the digested samples were quantitatively transferred to 50 mL volumetric flasks. The transfer was accomplished using ash-free quantitative filter paper **(**Whatman #41). The volume of the solutions was made up to 50 mL using de-ionized water. Aliquots of the solutions were transferred to polyethylene flasks and kept cooled (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 was half gone. 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 end 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 (Fiske & Subbarow, 1925). The colorimetric evaluations were carried out at 725 nm in a spectrophotometer UV/Visible BEL Photonics 2000 UV. Phosphorus standard solutions were previously prepared using monopotassium phosphate ($KH_2PO_4$, P.A., ACS, Vetec 1361).

The contents of Ca, Mg and Zn were evaluated in an atomic absorption spectrophotometer (GBC Avanta Σ), using 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 1000 ppm of the elements (Merk 1.09943 Tritisol®, Merk 1.09949 Tritisol®, and Merk 1.09953 Tritisol® for Ca, Mg, and Zn). In the Ca analysis, a strontium chloride solution (Merk 1.07865, 50 g/L) 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:

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

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 $\varepsilon_{ijkl}$ is the random error.

All statistical procedures were carried out using the MIXED procedure of SAS 9.2 and adopting 0.01 as the critical limit for type I error. When necessary, average values were compared using Fisher's Least Significant Difference.

# RESULTS AND DISCUSSION

The average contents of Ca, P, Mg, and Zn in samples of carcass, bone, excreta, concentrate, forage, and feces obtained by the different procedures are presented in Tables 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 brought into evidence that using different acid ratios does 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 ($P>0.01$).

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 more reliable and 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 (Jones Jr. & Case, 1990). 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, 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, 2002) 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, due to less manipulation of the samples. 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. The difference 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).

## CONCLUSION

The quantification of mineral 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.

## REFERENCES

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS – AOAC. **Official methods of analysis.** 17 ed. Gaithersburg: AOAC International, 2000. paginação descontínua.

CROMWELL, G.L.; CLINE, T.R.; CRENSHAW, J.D.; CRENSHAW, T.D.; EASTER, R.A.; EWAN, R.C.; HAMILTON, C.R.; HILL, G.M.; LEWIS A.J.; MAHAN, D.C.; NELSSEN, J.L.; PETTIGREW, J.E.; VEUM, T.L.; YEN J.T. Variability among sources and laboratories in analyses of wheat middlings. **Journal of Animal Science,** v.78, p.2653–2658, 2000.

CROMWELL, G.L.; BRENDEMUHL, J.H.; CHIBA, L.I.; CLINE, T.R.; CRENSHAW, T.D.; DOVE, C.R.; EASTER, R.A.; EWAN, R.C.; FERRELL, K.C.; HAMILTON, C.R.; HILL, G.M.; HITCHCOCK, J.D.; KNABE, D.A.; KORNEGAY, E.T.; LEWIS, A.J.; LIBAL, G.W.; LINDEMANN, M.D.; MAHAN, D.C.; MAXWELL, C.V.; MCCONNELL, J.C.; NELSSEN, J.L.; PETTIGREW, J.E.; SOUTHERN, L.L.; VEUM, T.L.; YEN J.T. Variability in mixing efficiency and laboratory analyses of a common diet mixed at 25 experiment stations. **Journal of Animal Science,** v.81, p.484-491, 2003.

DETMANN, E.; SOUZA, M.A.; VALADARES FILHO, S.C.; QUEIROZ, A.C.; BERCHIELLI, T.T.; SALIBA, E.O.S; CABRAL, L.S.; PINA, D.S.; LADEIRA, M.M.; AZEVEDO, J.A.G. **Métodos para análise de alimentos - INCT - Ciência Animal**. Suprema Editions, Visconde do Rio Branco, 2012. 214p.

FISKE, C.H.; SUBBAROW, Y. The colorimetric determination of phosphorus. **The Journal of Biological Chemistry,** v.66, p.375–400, 1925.

JONES Jr., J.B.; CASE, V.W. **Soil testing and plant analysis**. In Westerman, R. L. (Ed).  ampling, handling, and analyzing plant tissue samples. SSSA, Inc., Madison, WI, 1990. p.389-427.

McCARTHY, H.T.; ELLIS, P.C. Comparison of microwave digestion with conventional wet ashing and dry ashing digestion for analysis of lead, cadmium, chromium, copper, and zinc in shellfish by flame atomic absorption spectroscopy. **Journal of Association of Official Analytical Chemists**, v.74, p.566-569, 1990.

ROCHA, G.C.; PALMA, M.N.N.; DETMANN, E.; VALADARES FILHO, S.C. Evaluation of acid digestion techniques to estimate chromium contents in cattle feces. **Pesquisa Agropecuária Brasileira**, v.50, p.92-95, 2015.

Table 1 - Average contents of Ca, P, Mg, and Zn in carcass, bone, and excreta samples according to the different nitric to perchloric acid ratios and the number of digestion steps

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

a R, ratio of nitric to perchloric acids.

b N, number of digestion steps.

Table 2 – Average contents of Ca, P, Mg, and Zn in concentrate, forage, and feces samples, according to the different nitric to perchloric acid ratios and the number of digestion steps

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

[a] R, ratio of nitric to perchloric acids.

[b] N, number of digestion steps.

Table 3 - Descriptive levels of probability for type I error taken from analyses of variance of the Ca, P, Mg, and Zn contents

| Effect | Mineral | | | |
|---|---|---|---|---|
| | Ca | P | Mg | Zn |
| Type (T)[a] | <0.001 | <0.001 | <0.001 | <0.001 |
| Ratio (R)[b] | 0.046 | 0.064 | 0.429 | 0.032 |
| Steps (N)[c] | 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[d] | 11.2 | 3.92 | 0.31 | 0.14 |

[a] Type of sample.

[b] R, ratio of nitric to perchloric acids.

[c] N, number of digestion steps.

[d] RSD, residual standard deviation.

Table 4 - Least squares means of Ca and P contents in bone samples (g/kg) according to the nitric to perchloric acids ratio used in digestion procedures

| 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 |
| Forage | 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 |
| Forage | 1.3 | 1.2 | 1.2 | 0.993 |
| Feces | 4.5 | 4.4 | 4.3 | 0.981 |

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