DAIANA FRANCISCA QUIRINO

STUDIES ON METHODS FOR DRY MATTER, CRUDE ASH, AND NITROGEN ANALYSIS IN ANIMAL FEEDS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Edenio Detmann

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ABSTRACT

QUIRINO, Daiana Francisca, D.Sc., Universidade Federal de Viçosa, February, 2023. **Studies on methods for dry matter, crude ash, and nitrogen analysis in animal feed**. Adviser: Edenio Detmann.

This study encompassed three experiments to evaluate different methods for quantifying the dry matter, crude ash, and nitrogen contents in animal feeds. In the first experiment, our objective was to evaluate the accuracy, repeatability, and physicochemical impacts of oven-drying times on laboratory dry matter estimates by loss on drying at 105°C. We evaluated eighteen different feeds, which were clustered into high- and low-moisture materials. The tested methods were based on loss on drying at 105°C and the Karl Fischer titration was adopted as the reference method. Test portions were oven dried at 105°C for 3, 6, 12, 16, and 24 h, and results were compared to the reference method. The method based on 3 h of drying provided the closest estimates to those obtained by Karl Fischer titration. The method based on the binomial 105°C × 3 h minimizes the bias in the water recovery, and it is recommended for dry matter analysis in animal feeds. In the second experiment, we evaluated methods for crude ash quantification. Our objective was to evaluate the influence of variations in time and temperature and the use of ashing aids as well, on the crude ash estimates. Twenty-four study materials from eight different feeds were evaluated. The control method was based on a simple ignition time of 3 h at 550°C. The methods variations were: increasing ashing time to 6 h; increasing temperature to 600°C; and using two 3-h ignition cycles at 550°C with ashing aids inclusion between them. The ashing aids were: fresh air supply, fresh air supply plus distillate water, and fresh air supply plus hydrogen peroxide. The crude ash results differed among the method variations, but a consistent decrease in the estimates was observed when liquid aids were applied. The best test results are obtained using water as liquid ashing aid between two ignition cycles at 550°C for 3 h. In the third experiment, we aimed to evaluate the nitrogen recovery in tropical forages by using the Kjeldahl method with modifications involving a pre-digestion step with salicylic acid and, or a partial replacement of copper sulfate by titanium dioxide as a catalyst. The Kjeldahl standard procedure was based on digestion with sodium sulfate and copper sulfate (20:1), followed by steam distillation and titration. The Kjeldahl variations were: the Kjeldahl method with salicylic acid as pre-treatment; the Kjeldahl with titanium dioxide replacing 50% of copper sulfate in the digestion mixture; and the Kjeldahl with salicylic acid and copper sulfate:titanium dioxide (1:1) as a catalyst. We adopted the Dumas method as a standard method. Forty-eight study materials (i.e., tropical forages) were evaluated. Nitrogen recovered was not complete for the Kjeldahl methods, which did not differ to each other. Neither salicylic acid nor titanium dioxide improved the nitrogen recovery. The average nitrogen recovery in tropical forages when analyzed through the Kjeldahl method is 0.974 g/g. The nitrogen recovery of the Kjeldahl method is not influenced by a pre-digestion with salicylic acid or by the partial replacement of copper sulfate with titanium dioxide as a catalyst.

Keywords: Animal feed. Animal nutrition. Feed analysis

RESUMO

QUIRINO, Daiana Francisca, D.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Estudos sobre métodos de análises de matéria seca, cinzas e nitrogênio em alimentos para animais**. Orientador: Edenio Detmann.

Este estudo abrangeu três experimentos para avaliar diferentes métodos para quantificar os teores de matéria seca, cinzas e nitrogênio em alimentos para animais. No primeiro experimento, objetivou-se avaliar a acurácia, repetibilidade e os impactos físico-químicos dos tempos de secagem em estufa nas estimativas de matéria seca obtidas pela secagem a 105°C. Foram avaliadas dezoito amostras de diferentes alimentos que foram divididos conforme o teor de umidade (alto ou baixo). Os métodos foram baseados na secagem em estufa a 105°C, e o método de titulação de Karl Fischer foi adotado como referência. As amostras foram secas em estufa a 105°C durante 3, 6, 12, 16 e 24 h, e os resultados comparados com o método de referência. O método de secagem por 3h foi o que gerou as estimativas mais próximas do método referência. O método de secagem baseado no binômio de 105°C x 3h minimiza o erro na recuperação de água, sendo recomendado para estimar o teor de matéria seca em alimentos para animais. No segundo experimento, métodos para a estimação dos teores de cinzas foram avaliados. Objetivou-se avaliar variações no tempo e temperatura de queima e o uso ou não de aditivos, e suas influências nas estimativas. O método de referência foi baseado em um único ciclo de queima a 550°C por 3h. As variações testadas foram: aumento do tempo de queima para 6 h; aumento da temperatura para 600°C; e o uso de dois ciclos de queima a 550°C por 3 h cada, com a inclusão de aditivos. Dentre eles: ventilação da mufla; ventilação com adição de água destilada, e ventilação com adição de peróxido de hidrogênio. Os resultados de cinzas diferiram em função dos métodos testados sendo que a redução do teor de cinzas foi mais consistente com o uso de aditivos líquidos. As estimativas mais confiáveis foram obtidas utilizando água como aditivo líquido, entre dois ciclos de queima a 550°C por 3h. No terceiro experimento, objetivou-se avaliar a recuperação de nitrogênio pelo método de Kjeldahl e variações envolvendo a pré-digestão com ácido salicílico e/ou a substituição de sulfato de cobre por dióxido de titânio como catalisador. O método Kjeldahl de referência foi baseado na digestão com sulfato de sódio e sulfato de cobre (20:1), seguido por destilação a vapor e titulação. As variações testadas foram: Kjeldahl com a etapa de pré- digestão com ácido salicílico; Kjeldahl com o dióxido de titânio substituindo 50% do sulfato de cobre, e Kjeldahl com ácido salicílico e a mistura catalítica contendo sulfato de cobre: dióxido de titânio (1:1). O método Dumas foi adotado como referência, e quarenta e oito forrageiras tropicais foram avaliadas. A recuperação de nitrogênio pelo método de Kjeldahl foi incompleta, e os métodos de Kjeldahl não diferiram entre si. Nem o ácido salicílico, nem o dióxido de titânio aumentaram a recuperação do nitrogênio. A recuperação média do nitrogênio para as forragens tropicais analisadas pelo método de Kjeldahl é de 0,974 g/g. A recuperação do nitrogênio pelo método de Kjeldahl não é influenciada pela pré-digestão com ácido salicílico ou pela substituição parcial do sulfato de cobre por dióxido de titânio como catalisador.

Palavras-chave: Alimentação animal. Nutrição animal. Análises de alimentos.

SUMMARY

General introduction

The objective of methods for animal feeds evaluation is identify the contribution of each ingredient in diets formulation to attend the animals’ requirements (Mould, 2003). Thus, for an adequate animal performance it is necessary to known the chemical composition of the feed ingredients in order to adjust the diets according to the different developing stages or production phases. Besides nutrient supply, the feed quality can be also accessed through its chemical composition (Nielsen, 2010), ensuring safe feeds for animals.

The feed characterization in animal nutrition is generally done by using the proximal analysis (Malomo and Ihegwuagu, 2017), which encompasses the content estimation of its main constituents like dry matter, mineral matter or crude ash, crude protein, crude fat, and fiber (Horwitz et al., 1990). However, the methods for feed analysis have been continually improved, which demands constant studies regarding optimization and standardization.

The dry matter (DM) can be defined as a sum of the total feed components excluding water. It represents the most routine and important feed analysis, yet the most challenging regarding precision and accuracy (Park, 2008; Bradley, 2010; Pojić et al., 2015; Mauer and Bradley Jr., 2017). On the other hand, the contents of the different nutrients and analytical entities in feeds must be expressed on a DM basis to avoid the diluent effect of water (Thiex and Richardson, 2003; Ileleji et al., 2010), allowing an adequate comparison among feeds, as well as a proper diet formulation. Meanwhile, inaccurate DM estimates affect directly the nutrient supply and animal performance by biasing the estimation of voluntary dry matter intake, digestibility and, feed efficiency (Petit et al., 1997; Thiex and Richardson, 2003).

The terms crude ash or mineral matter refer to the inorganic residue after the complete oxidation of organic matter (OM) in a sample (Ismail, 2017; Liu, 2019). Generally, the ashing procedures comprise vaporizing water and volatile compounds, and converting minerals to silicates, phosphates, oxides, sulfates, and chlorides, besides the burning of organic substances (Liu, 2019). Crude ash plays an important role in the nutritional interpretation of animal feeds by allowing the indirect estimation of total OM, which covers all the potential energy-producing compounds (Souza et al., 2017). Moreover, ash contents are also mandatory to estimate the feed components quantified by difference, such as non-fiber carbohydrates and nitrogen-free extract (Pojić et al., 2010; Detmann et al., 2021). Crude ash estimates have been incorporated as an important input into summative systems for the estimation of energy contents in animal diets (Tedeschi et al., 2005; Detmann et al., 2016; NRC, 2021). Actually, any bias on the crude ash estimates might decrease the accuracy of nutritional evaluation of animal feeds, which in turn may compromise production and culminate in economic losses (Thiex et al., 2012) due to inadequate diet formulation.

The nitrogen (N) analysis is routinely performed in animal nutrition laboratories in order to calculate the protein equivalents in feeds. The Kjeldahl method has been widely used since its introduction in 19th century due to its accuracy, simplicity, and robustness (Sáez-Plaza et al., 2013; Greaser and Guo, 2015). Nevertheless, the Kjeldahl method backbone has been modified over the years intending to simplify the analytical procedures, reduce its hazardous chemicals disposal, and increase the versatility of the method (Thiex et al., 2002).

Overall, the total organic N is supposed to be measured by the Kjeldahl method (Moore et al., 2010). However, the N occurring with N-N and N-O linkages (e.g., nitrate, nitrite, alkaloids, pyridine) are considered resistant to mineralization and might not be totally recovered

(Nelson and Sommers, 1980; Sikorski, 2001; Sáez-Plaza et al., 2013). Particularly, the N in more oxidized forms (e.g., nitrate) may represent a considerable fraction of the total N in plant materials (Simonne et al., 1994). Thus, some nitrate present in plant materials will not be recovered due to incomplete breakdown of N-O bounds during Kejldahl digestion (Nelson and Sommers, 1980), underestimating the N content in plant materials used for animal nutrition.

Thus, due to the importance of feed analysis for diet formulation in animal nutrition, this study aimed to evaluate methods for the quantification of dry matter, crude ash, and nitrogen contents in animal feed.

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Research Article

ANIMAL FOOD, PET FOOD, AND PLANT NUTRIENT METHODS

Evaluation of Heating Times for Loss on Drying at 105°C for Estimation of Laboratory Dry Matter in Animal Feeds

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Abstract

Background: Dry matter (DM) is a routine test for all animal feeds, facilitating feed comparisons and diet formulation. It is the most frequent test, yet the most challenging with respect to precision and accuracy.
Objective: Our objective was to evaluate the accuracy, repeatability, and physicochemical impacts of oven-drying times on LDM test results in animal feeds obtained by loss on drying (LoD) at 105°C.
Methods: Eighteen primary samples collected from different feed sources were grouped into high-moisture (HM) and low-moisture (LM) content materials. The tested methods were based on LoD at 105°C and Karl Fischer titration was adopted as the reference method. Test portions were oven dried at 105°C for 3, 6, 12, 16, and 24 h, and test results were compared to the reference method. Test portions were also subjected to a color evaluation using a colorimetric technique.
Results: The method based on 3 h of drying provided the closest estimates to those obtained by Karl Fischer titration. Extending heating time (i.e., above 3 h) increased the bias, especially for HM feeds, which was attributed to a higher occurrence of non-enzymatic reactions. This was corroborated by the color of the residues, which became darker with increased heating time. The repeatability of LoD methods was considered adequate, ranging from 0.32 to 0.73%.
Conclusion: The LoD method based on the binomial 105°C × 3 h minimizes the bias in the water recovery and causes less non-enzymatic browning in the test portions.
Highlights: The loss-on-drying method recommended for laboratory DM in animal feeds is drying the test portions at 105°C for 3 h.

Dry matter (DM) can be defined as the sum of total feed components excluding water. DM is the single most important and the most commonly performed of all feed tests, and also one of the most challenging regarding precision and accuracy (1–3). Nutrients and analytical entities in feeds are expressed on a DM basis to eliminate the dilution effect of water (4, 5). Expressing nutrients in a DM basis allows their direct comparison in feeds and a means to formulate diets based on nutrient concentration. Inaccurate DM estimates directly affect formulation of the diet, which, in turn, impacts animal performance by biasing the

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estimation of voluntary DM intake, digestibility, and feed efficiency (4, 6).

Laboratory routines for estimating DM in high-moisture feeds (HM) generally involve two drying steps (7, 8). The first is partial drying of the entire laboratory sample, usually in a forced-air drying oven (50–60°C) to approximately 85–90% DM. Drying the feed material facilitates particle size reduction (i.e., grinding), which is necessary to reduce fundamental sampling error (9) and allow for selection of test portions. The second drying step measures the residual DM on a test portion of the partially dried and ground analytical sample and is commonly named laboratory DM (LDM). The total DM is calculated from the values obtained in both steps. For low-moisture feeds (LM), first partial drying is not necessary and LDM represents the total DM of the feed.

Most analytes are measured on test portions selected from the analytical sample after partial drying (as needed) and particle size reduction and test results are converted to a DM basis (10) using the LDM test result. Small differences in LDM estimates can therefore result in large differences in nutrient concentration on a DM-adjusted basis, even when analytical uncertainty is controlled (10, 11).

Gravimetric methods based on heat drying (i.e., loss on drying [LoD] methods) are the most frequently used for LDM, with test results obtained by the difference between the final and initial test portion weights (2). LoD techniques have been applied to a wide range of materials due to their simplicity, high throughput, equipment availability, and low cost (12). However, it is important to note that LoD methods do not measure the water molecule, and the test result is a function of the mass lost under the physical conditions of the method (13). As such, LoD methods are empirical (10), where analytical conditions determine the test result, and any modification in time or temperature might produce different estimates (1, 3, 14, 15).

The principle of LoD methods is to subject the test portion to a temperature equal to or higher than the boiling point of water for a time considered sufficient for the total evaporation of water. Since the analysis is normally carried out under normal atmospheric pressure, the oven temperature must be equal to or higher than 100°C. The ideal combination of temperature and time is that which allows water evaporation with minimal losses of non-water volatile compounds and minimal degradation from undesirable non-enzymatic side-reactions, such as Maillard reactions, carbohydrate decomposition, and unsaturated fatty acid oxidation (1, 3, 4, 16). The occurrence of these side-reactions is dependent on time and temperature, as well as on the feed matrix. Bias in LoD methods could be mitigated by choosing the heating time based on the feed matrix (14) or by adjusting the heating time and temperature through validation with non-empirical methods, such as the Karl Fischer titration.

When defining a physical binomial for LoD procedures, an initial reasoning might be the higher the temperature, the shorter the heating time, and vice versa. From this, physical binomials based on higher temperatures and shorter times may seem attractive, as they could allow high operational capacity. Reasoning such as this probably led to the prevalence of the AOAC INTERNATIONAL LDM procedure for animal feed (*Official Method* **930.15**; 135°C/2 h; 17), which was adopted as a National Forage Testing Association (NFTA) method for forages (method 2.2.2.1; 135°C/2 h; 7), and the European Union method for cereals and meals (130°C/2 h; 18). However, the methods based on higher temperatures (i.e., ≥130°C) have been shown to produce high positive biases in terms of water recovery (19, 20).

Methods for LDM using a LoD process based on lower temperatures and longer heating times are commonly used for feeds, such as the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA) method G-003/1 (105°C/16 h; 8), the NFTA method (2.2.2.2; 100°C/24 h or 105°C/16 h; 7), and the method described by Van Soest and Robertson (21) (100°C/overnight). However, excessive heating times, even at lower temperatures, can result in an increased browning of the test portions (19) and increase artificial water formation from non-enzymatic reactions. Additionally, longer heating times result in a decreased operational capacity.

Recent LoD methods have been based on physical binomials with lower temperatures and shorter times to decrease the water recovery biases. That sort of method has been recommended by NFTA method 2.1.4 (105°C/3 h) for forages (22) and AOAC Method **935.29** (104°C/3 h) for malt (17). This type of method has been found suitable to evaluate the LDM in distillers dried grains (DGD) with solubles (19, 20), and presented a lower bias in water recovery for silages, hays, and concentrate feeds (23). If applicable to animal feeds in general, this kind of method could significantly improve laboratory operational capacity and mitigate energy expenditure by increasing the number of samples analyzed daily.

Thus, our objective was to evaluate the accuracy, repeatability, and physicochemical impacts of oven-drying times to estimate the LDM in animal feeds by LoD at 105°C.

Experimental

Experimental Facilities and Study Materials

All analyses were performed at the Animal Nutrition Laboratory of the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Eighteen different primary samples of feeds were collected and clustered into HM and LM feed groups. The HM group included materials that required a partial drying procedure before LDM quantification. The study materials were: HM—corn silage ($n = 5$), sorghum silage, sugarcane silage ($n = 5$), and fresh sugarcane ($n = 2$); LM—grass hay, soybean meal, wheat bran, corn grain, and DDG. The study materials were chosen due to their widespread use in diets for beef and dairy cattle and were obtained from farms and feed mills located in Minas Gerais State, Brazil.

The HM study materials were partially dried using a forced-air oven at 55°C for 48 h (method G-001/2; 8). Then, all study materials were ground in a knife mill (TE-680, Tecnal, Piracicaba, São Paulo, Brazil) to pass through a 1 mm screen sieve. After grinding, all the materials were kept in screw-cap polyethylene flasks. Six replicates (i.e., test portions) were selected for each LDM procedure ($n = 6$).

Dry Matter Evaluation by Loss on Drying

The LoD procedures were performed using a single convective oven (Soc-FABBE Ltda, São Paulo, São Paulo, Brazil) according to the official INCT-CA method G-003/1 (8). Briefly:

- **(a)** Dry dishes at 105°C for at least 2 h, transfer them to a desiccator, and allow them to cool to room temperature (30 min, approximately);
- **(b)** Weigh dishes to the nearest 0.1 mg, removing one at a time from the desiccator and keeping the desiccator closed between dish removals;

(c) Select test portion of approximately 2.0 g as is, place in pre-weighed dish and record the weight to the nearest 0.1 mg;
(d) Insert dishes into the oven at 105°C and dry for 16 h (i.e., overnight);
(e) Transfer the dishes to a desiccator, cool to room temperature, and record the weight to the nearest 0.1 mg.

The modification of oven-drying procedures was performed using the same equipment at the same temperature (105°C), modifying the heating time: 3, 6, 12, and 24 h.

Each run contained one test portion of each study material for each heating time. Thus, 90 test portions (5 heating times × 18 study materials) were analyzed per run, totaling 540 tests. Dishes containing test portions were grouped according to their heating time and placed in the oven together. The heating interval was timed after the temperature stabilization (105°C).

Karl Fischer Titration.—The Karl Fischer titration was performed using an 870 KF Titrino Plus titrator (Metrohm, Herrisau, Switzerland) and followed method I described by Thiex and Van Erem (24). Six test portions of each study material were analyzed using HYDRANAL® reagents as follows: the titrant was the one-component Composite 5 reagent (Honeywell Fluka™, Charlotte, NC, USA); as solvent, an anhydrous methanol–formamide mixture (1 + 1; Honeywell Fluka). The methanol–formamide (1 + 1) mixture is recommended for a feed matrix containing proteins and carbohydrates, the main constituents of feeds. Sodium tartrate dihydrate (Sigma Aldrich, St. Louis, MO, USA) was used as a primary standard. The procedures for titration vessel conditioning, standardization, and measurements were performed according to the manufacturer's recommendations. The Karl Fischer titration procedures were performed concomitantly to the LoD evaluations.

Colorimetric Evaluations

Test portions of the material as is and all residues after oven-drying were color scored according to the Comission Internationale de l'Éclairage (CIE) coordinates (i.e., L* for lightness, a* for redness, and b* for yellowness) using a Hunter MiniScan EZ colorimeter (4500 L; Hunter Associates Laboratory, Inc., Reston, VA, USA). The residues were transferred to a Petri dish, mixed, and then scored. The coordinates were read thrice in each unheated test portion and residue, and the three scores were averaged to obtain the result.

Calculations and Statistical Analysis

The LDM calculations based on LoD and Karl Fischer titration were performed as follows:

$$LDM_{LoD} = \frac{W_2 - T}{W_1 - T} \times 100, \tag{1}$$

$$LDM_{KF} = 100 - H_2O, \tag{2}$$

where LDM_{LoD} = LDM evaluated through a LoD method (% as is); W_1 = weight of dish + test portion as is (g); T = tare or dish weight (g); W_2 = weight of dish + dry material (g); LDM_{KF} = LDM obtained by Karl Fischer titration (% as is); and H_2O = water content obtained by Karl Fischer titration (% as is).

The apparent water recovery for the LoD procedures compared to Karl Fischer titration was calculated as:

$$AWR_{ij} = \frac{100 - LDM_{LoD(ij)}}{-(H_2O)_i}, \tag{3}$$

where AWR_{ij} = apparent water recovery for the test portion j taken from the study material i (g/g); $LDM_{LoD(ij)}$ = LDM evaluated through a LoD method in the test portion j taken from the study material i (% as is); and $-(H_2O)_i$ = average water content in the study material i obtained by Karl Fisher titration (% as is).

The color pattern of residues after LoD was evaluated using the CIE coordinate space using three different approaches. The first was performed by evaluating the lightness coefficient (L*), which ranges from black (i.e., L* = 0) to white (i.e., L* = 100; 25, 26). The second approach was the Chroma C* index, representing the color saturation or intensity, which was calculated as the hypotenuse of a right-angled triangle created by joining points (0, 0), (a*, b*), and (a*, 0) as follows (25):

$$\text{Chroma } C^* = \sqrt{(a^*)^2 + (b^*)^2}. \tag{4}$$

The third colorimetric approach was done by calculating the hue angle (h°), which represents the angle between the hypotenuse and 0° on the a* (bluish-green/red-purple) axis (25):

$$h^\circ = \frac{\text{arctangent}\left(\frac{b^*}{a^*}\right)}{2\pi} \times 360. \tag{5}$$

It was anticipated that all a* and b* results obtained in our work would be positive. Then, Equation (4) could be applied without any further correction and h° will vary between 0° and 90°, which represents a color pattern associated with the red-purple to yellow range.

The CIE coordinates were converted into RGB (i.e., red, green, and blue) coordinates using Coloroid Professional Color Plan Designer software compiling an Excel sheet in which each cell was filled with the corresponding solid color. This procedure was performed to provide a descriptive visual pattern for the study materials.

The LDM obtained by the LoD procedures and Karl Fischer titration and the apparent water recovery obtained with the different heating times were analyzed through an analysis of variance (ANOVA) according to the model:

$$Y_{ijkl} = \mu + G_i + S_{(i)j} + M_k + GM_{ik} + \varepsilon_{ijkl}, \tag{6}$$

where Y_{ijkl} = LDM of the test portion l taken from study material j belonging to the feed group i and evaluated through the method k; μ = general constant; G_i = fixed effect of feed group i (i.e., HM or LM); $S_{(i)j}$ = random effect of study material j nested within the feed group i assumed to be normally, independently and indentically distributed (NIID) (0, $\sigma^2_{S/G}$); M_k = fixed effect of method k, GM_{ik} = fixed effect of interaction between feed group i and method k; and ε_{ijkl} = random error assumed to be NIID (0, σ^2_ε).

After the first ANOVA, data was analyzed again independently for each method according to the model:

$$Y_{ijk} = \mu + G_i + S_{(i)j} + \varepsilon_{ijk}, \tag{7}$$

where Y_{ijk} = LDM of the test portion k taken from the study material j belonging to the feed group i (i.e., HM or LM); μ = general constant; G_i = the fixed effect of feed group i; $S_{(i)j}$ = random effect of study material j nested within feed group i assumed to be NIID (0, $\sigma^2_{S/G}$); and ε_{ijk} = random error assumed to be NIID (0, σ^2_{ε}).

From the adjustment of the model described in Equation (7), the repeatability for each method (i.e., Karl Fischer or LoD) was estimated as:

$$r = \frac{\sqrt{\hat{\sigma}_{\varepsilon}{}^{2}}}{-Y\times}100, \qquad (8)$$

where r = repeatability (%), $\hat{\sigma}^2_{\varepsilon}$ = estimate of error variance [(% as is)^2]; and $-Y$ = average LDM content (% as is).

The color pattern of the test portions and residues after LoD were analyzed according to the model in Equation (6), where values obtained by Karl Fischer titration were replaced by the colorimetric measurements performed on the unheated test portions.

All statistical evaluations were performed using the GLIMMIX procedure of SAS 9.4. The variance components were estimated according to the restricted maximum likelihood method. When necessary, means were grouped using Scheffé's multiple comparison procedure. Statistical significances were stated at $P < 0.05$.

Results

There was an interaction ($P < 0.01$) between method and feed group for the LDM (Table 1). For LM materials, all heating times produced lower LDM test results ($P < 0.05$) compared to Karl Fischer titration, with no difference among oven-drying methods ($P > 0.05$). For HM materials, all applied heating times resulted in LDM test results lower ($P < 0.05$) than those obtained with Karl Fischer titration. Even though statistically different ($P < 0.05$), the LoD method based on 3 h of drying provided the average LDM closest to that obtained by Karl Fischer titration. Longer heating times increased the difference and produced LDM test results in HM materials even lower than those obtained by 3 h of drying ($P < 0.05$). All LDM methods showed acceptable repeatability, which ranged from 0.32 to 0.73%.

Table 1. Average laboratory dry matter (LDM), repeatability, and apparent water recovery evaluated through Karl Fischer titration or loss on drying for different times at 105°C

	LDM, % as is[a,b]			Water recovery, g/g[a,b]	
Method	LM[d]	HM[e]	Repeatability, %[c]	LM[d]	HM[e]
Karl Fischer	90.1a	91.5a	0.33	—	—
3 h	88.7b	90.5b	0.44	1.161a	1.168d
6 h	88.5b	89.7c	0.33	1.181a	1.221c
12 h	88.5b	89.4d	0.60	1.183a	1.257ab
16 h	88.4b	89.4d	0.32	1.191a	1.263a
24 h	88.7b	89.6 cd	0.73	1.164a	1.235bc
SEM	0.306	0.183	—	0.062a	0.038
P-values					
Feed type (F)	0.003		—	0.475	
Method (M)	<0.001		—	<0.001	
F × M	0.001		—	<0.001	

[a] The least-square means of each method correspond to n = 30 for LM and n = 78 for HM study materials. The SEM represents the variability of the method's least-square mean.

[b] Means in a column followed by different letters differ at $P < 0.05$.

[c] For details on repeatability calculation, *see* Equations (7) and (8).

[d] LM = Low-moisture materials.

[e] HM = High-moisture materials.

The apparent water recovery of LoD methods compared to the Karl Fischer titration followed the pattern previously described for the LDM, with an interaction between heating times and feed groups ($P < 0.01$, Table 1). For LM materials, the water recovery was constant across all heating times ($P > 0.05$), averaging 1.176 g/g. We observed differences in the apparent water recovery for HM materials ($P < 0.05$). Water recovery closest to 1.0 g/g and closest to the recovery for LM materials was obtained with 3 h of drying. An increase in the heating time increased the water recovery for HM materials, and produced more prominent differences between LM and HM materials.

The descriptive pattern of color for the different study materials is shown in Table 2. There was an interaction between heating time and feed group ($P < 0.01$) for the lightness coefficient (Table 3). For LM materials, no difference among heating times was observed ($P > 0.05$). For HM materials, drying the test portions for 3 or 6 h did not change ($P > 0.05$) the lightness compared to as is material. However, heating times equal to or longer than 12 h produced darker residues ($P < 0.05$). There was no interaction between heating times and feed group ($P > 0.29$) for Chroma C*. On average, HM materials presented higher Chroma C* coefficients ($P < 0.01$) compared to LM materials (26.5 and 22.2, respectively). All heating times decreased ($P < 0.05$) the Chroma C* when compared to as is materials. The difference was smaller and relatively constant when test portions were dried for 3 to 12 h, but became more prominent as the heating time was extended to 16 or 24 h. There was an interaction between heating time and feed group ($P < 0.01$) for the hue angle. For LM materials, no difference among heating times was observed ($P > 0.05$). For HM materials, drying for 3 or 6 h did not change ($P > 0.05$) the hue angle of the residues compared to as is materials. However, heating times equal to or longer than 12 h reduced this characteristic ($P < 0.05$).

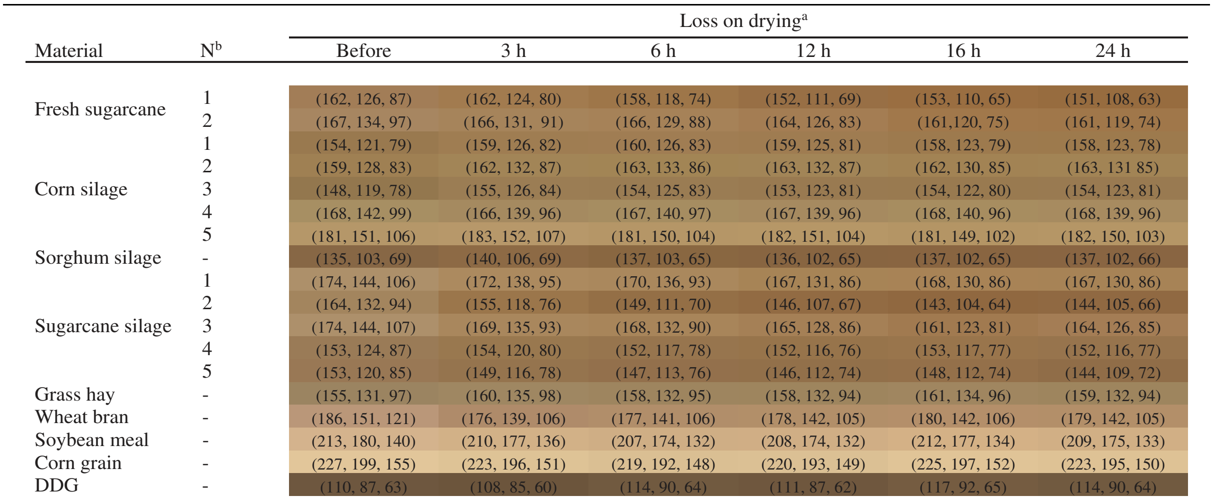
Discussion

The principle of the LoD technique is that the boiling point of water is lower than that of most other feed components, e.g., carbohydrates, lipids, and proteins (27). The ideal LoD conditions remove water without introducing bias due to loss of non-water volatiles or from loss of water due to side-reactions during heating (3, 27). Finding these conditions is extremely difficult and may vary with the feed composition. The validation of any LoD method should examine the damage caused by excessive temperature or time.

Fresh forages and silages, which comprised the group of HM materials, are more susceptible to chemical reactions before or during the drying process which can bias LDM results. Some constituents in the feed matrix are known to be more heat-sensitive, such as carbohydrates and amino acids, which are precursors of Maillard reactions.

The study materials chosen for this work vary in terms of carbohydrate amount and profile. Monosaccharides, disaccharides, oligosaccharides, and polysaccharides are present in grass and fresh forages, whereas monomers, such as pentoses and hexoses, remain as an end product from fermentation in ensiled feeds (28). Soluble nitrogen compounds (i.e., amino acids and small peptides) are present in fresh forages (28) and free amino acids are released by proteolysis during fermentation in the silo (29, 30). Thus, the presence of these types of chemical

Table 2. Color pattern of different study materials before and after the loss on drying procedures for different periods



Material	N[b]	Loss on drying[a]					
		Before	3 h	6 h	12 h	16 h	24 h
Fresh sugarcane	1	(162, 126, 87)	(162, 124, 80)	(158, 118, 74)	(152, 111, 69)	(153, 110, 65)	(151, 108, 63)
	2	(167, 134, 97)	(166, 131, 91)	(166, 129, 88)	(164, 126, 83)	(161,120, 75)	(161, 119, 74)
Corn silage	1	(154, 121, 79)	(159, 126, 82)	(160, 126, 83)	(159, 125, 81)	(158, 123, 79)	(158, 123, 78)
	2	(159, 128, 83)	(162, 132, 87)	(163, 133, 86)	(163, 132, 87)	(162, 130, 85)	(163, 131 85)
	3	(148, 119, 78)	(155, 126, 84)	(154, 125, 83)	(153, 123, 81)	(154, 122, 80)	(154, 123, 81)
	4	(168, 142, 99)	(166, 139, 96)	(167, 140, 97)	(167, 139, 96)	(168, 140, 96)	(168, 139, 96)
	5	(181, 151, 106)	(183, 152, 107)	(181, 150, 104)	(182, 151, 104)	(181, 149, 102)	(182, 150, 103)
Sorghum silage	-	(135, 103, 69)	(140, 106, 69)	(137, 103, 65)	(136, 102, 65)	(137, 102, 65)	(137, 102, 66)
Sugarcane silage	1	(174, 144, 106)	(172, 138, 95)	(170, 136, 93)	(167, 131, 86)	(168, 130, 86)	(167, 130, 86)
	2	(164, 132, 94)	(155, 118, 76)	(149, 111, 70)	(146, 107, 67)	(143, 104, 64)	(144, 105, 66)
	3	(174, 144, 107)	(169, 135, 93)	(168, 132, 90)	(165, 128, 86)	(161, 123, 81)	(164, 126, 85)
	4	(153, 124, 87)	(154, 120, 80)	(152, 117, 78)	(152, 116, 76)	(153, 117, 77)	(152, 116, 77)
	5	(153, 120, 85)	(149, 116, 78)	(147, 113, 76)	(146, 112, 74)	(148, 112, 74)	(144, 109, 72)
Grass hay	-	(155, 131, 97)	(160, 135, 98)	(158, 132, 95)	(158, 132, 94)	(161, 134, 96)	(159, 132, 94)
Wheat bran	-	(186, 151, 121)	(176, 139, 106)	(177, 141, 106)	(178, 142, 105)	(180, 142, 106)	(179, 142, 105)
Soybean meal	-	(213, 180, 140)	(210, 177, 136)	(207, 174, 132)	(208, 174, 132)	(212, 177, 134)	(209, 175, 133)
Corn grain	-	(227, 199, 155)	(223, 196, 151)	(219, 192, 148)	(220, 193, 149)	(225, 197, 152)	(223, 195, 150)
DDG	-	(110, 87, 63)	(108, 85, 60)	(114, 90, 64)	(111, 87, 62)	(117, 92, 65)	(114, 90, 64)

[a] Numbers in parenthesis indicate the RGB coordinates.

[b] N = Number of the primary sample when multiple samples were evaluated in the same study material.

Table 3. Lightness coefficient, Chroma C*, and hue angle in feed samples before and after loss on drying (LoD) for different times at 105°C

LoD	Lightness coefficient, %[a,b]		Chroma C* [b,c]	Hue angle, °h[a,b]	
	LM	HM		LM	HM
Before	67.0a	60.6a	22.7d	76.3a	76.7a
3 h	65.9a	60.0a	24.0c	76.9a	76.5a
6 h	65.7a	59.3ab	24.3bc	77.3a	76.0a
12 h	65.6a	58.5bc	24.7b	77.2a	75.3b
16 h	66.7a	58.1c	25.2a	76.8a	74.7c
24 h	66.2a	58.1c	25.2a	77.2a	74.7c
SEM	3.68	2.26	0.45	1.84	1.14
P-values					
Feed type (F)	0.117		<0.001	0.555	
Method (M)	<0.001		<0.001	<0.001	
F × M	<0.001		0.293	<0.001	

[a] LM = low-moisture materials; HM = high-moisture materials. The least square means of each method correspond to n = 30 for LM and n = 78 for HM study materials. The SEM represents the variability of the method's least-square mean.

[b] Means in a column followed by different letters differ at $P < 0.05$.

[c] The least-square means of each method correspond to n = 108. The SEM represents the variability of the method's least-square mean.

compounds in fresh forages and silages provides precursors for the Maillard reactions (i.e., non-enzymatic browning). In feeds, the formation of furfurals (e.g., hydroxymethylfurfural [HMF]) involves sugars and proteins, and there are two possible paths: the intermediate phase of non-enzymatic browning reactions; and sugar degradation. During the Amadori rearrangement (31), three molecules of water are lost by sugar aromatization, and CO_2 is produced from amino acid decarboxylation (32–34). Caramelization is another path for furfurals and HMF formation, which is characterized by the degradation of reducing sugars under heat treatment (35). Complex carbohydrates are decomposed into monosaccharides (e.g., glucose, fructose, and sucrose), which undergo enolization, dehydration, and cyclization reactions (34, 35). In this sense, those reactions might also be associated with the higher water recovery observed for the HM feeds, mainly as the heating time increases above 3 h.

The more intense browning in HM study materials was corroborated by the darker coloring of the residues as the heating time increases. Brown coloration is produced by combining red, yellow, and black. The greater contribution of the black to the residues dried for longer times was perceived by the decreased values of lightness coefficients (25, 26). On the other hand, all hue angles obtained here were acute angles, meaning a color perception ranging from red-purple (hue angle = 0°) to yellow (hue angle = 90°; 25). As the hue angle decreased as heating time increased in HM materials, there was an increased participation of red instead of yellow in the color perception of residues. This fact along with the increased blackness implied in darker brown residues for longer heating times, which supports a greater occurrence of non-enzymatic reactions.

For LM materials, the concentrations of the chemical precursors aforementioned are not expected to be high, instead they are rich in polysaccharides (e.g., starch in corn and cellulose in wheat bran) and endosperm proteins. Materials such as DDG are subjected to heating during their production, when most of the non-enzymatic reactions are expected to occur and, therefore, less likely to occur during the oven heating in LoD tests. This is supported by the presented results, as DDG presented the darkest coloration among all study materials. Hence, significant and highly prominent chemical modifications are not expected to occur during the heating period at 105°C in LM feeds, which is supported by the lack of effect of heating time length on LDM results, lightness coefficient, and hue angle in LM study materials.

Overall, the 3 h LoD method provided LDM results closest to the Karl Fischer titration results, which was used as a reference method. Our findings are in agreement with the pattern described by Thiex and Van Erem (23) for HM feeds and, as reported by Ahn et al. (19) for LM feeds. It should be noticed that a 3 h LoD test is also advantageous for mitigating energy expenditure and increasing the operational capacity of both equipment and personnel.

Conclusions

The LoD procedures for the evaluation of LDM produce inaccurate results when compared to the Karl Fischer titration. The biases are more prominent in HM materials and increase as the heating time increases at 105 °C. However, when heating time is limited to 3 h, the bias in the water recovery and the post-drying residue browning are minimized. This method is also advantageous for mitigating energy expenditure and increasing the operational capacity of testing laboratories. Thus, 105°C × 3 h should be applied to evaluate the LDM in animal feeds.

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Conflict of Interest

All authors declare no conflict of interest.

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ANIMAL FOOD, PET FOOD, AND PLANT NUTRIENT METHODS

Variations in Methods for Quantification of Crude Ash in Animal Feeds

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Abstract

Background: Crude ash is categorized as an empirical method playing an important role in the nutritional interpretation of animal feeds, allowing indirect estimation of total organic matter (OM).

Objective: Our objective was to evaluate variations in laboratory procedures for crude ash quantification regarding physical parameters (i.e., time, temperature) and ashing aids and their influences on crude ash, repeatability, and discrimination power among feeds.

Methods: The "control" method was based on a simple ignition time of 3 h at 550°C. The variations are briefly described: increasing ashing time to 6 h; increasing temperature to 600°C; and using two 3 h ignition cycles at 550°C with ashing aids inclusion between them: fresh air supply, fresh air supply plus distilled water, and fresh air supply plus hydrogen peroxide. A color evaluation was also performed using a colorimetric technique. Twenty-four study materials from eight different feed types were evaluated.

Results: The crude ash results differed among the method variations, but a consistent decrease in the estimates was observed when liquid aids were applied, which also improved repeatability. Ash residues did not present a consistent color pattern among methods, but the residues were darker when the control method was applied.

Conclusion: The method of obtaining ash residues in animal feeds based on 550°C × 3 h does not have enough robustness and may overestimate crude ash in some feeds. Adjustments in either ignition time or temperature might improve crude ash test results, but the best test results are obtained using liquid ashing aids between two ignition cycles.

Highlights: The recommended method is based on the use of 550°C and two 3 h ignition cycles with water added to the ash residue between cycles.

The terms crude ash or mineral matter refer to the inorganic residue after complete oxidation of organic matter (OM; 1, 2). However, some authors have stated that this residue should be more properly termed as a residue on ignition (3) when oxidation is mostly provided by burning. Generally, the ashing procedures comprise vaporization of water and volatile compounds,

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and conversion of minerals into silicates, phosphates, oxides, sulfates, and chlorides, in addition to oxidizing organic substances (2).

Crude ash plays an important role in the nutritional interpretation of animal feeds by allowing indirect estimation of total OM, which encompasses all potential energy-producing compounds (4). Moreover, ash contents are also mandatory to estimate feed components quantified by difference, such as non-fiber carbohydrates and nitrogen-free extract (5, 6). Crude ash estimates have been incorporated as an important input into summative systems for the estimation of energy contents in animal diets (7–9). Actually, any bias in the crude ash estimates might decrease the accuracy of the nutritional evaluation of animal feeds, which in turn may compromise production and culminate in economic losses (3) due to inadequate diet formulation.

The standard method used to estimate % crude ash in animal feeds was first described in the early 20th century (AOAC Method **942.05**; 10, 11) and is still used worldwide as is or with some minor modifications. Briefly, a 2 g test portion is ignited at 600°C for 2 h and crude ash is reported as the residue on ignition (3).

Nevertheless, the crude ash in animal feeds could be categorized as an empirical or type I method. Thus, it would be an analytical entity that determines a value that can only be achieved in terms of the method per se (12). In this sense, there are no primary reference standards that simulate the complex associations between organic and inorganic compounds, such as those observed in feed materials. Therefore, the method itself cannot be validated for accuracy in determining the "true" value for the constituent. To minimize systematic errors (i.e., bias) among laboratories, empirical methods must be followed exactly as described in the standard manuals. Even minor variations in methodology might result in the measurement of a different constituent (13) and compromise feed interpretations and comparisons among feeds, laboratories, and analysts.

The ashing procedure can be described as the submission of a test portion to a physical binomial based on temperature and time. Despite being originally based on using 600°C, lower ignition temperatures have been suggested for the official AOAC Method since the 1940s (10, 11, 14). Excessive temperatures have been associated with systematic bias caused by the volatilization of several minerals (3, 5, 15, 16). Thiex et al. (3) revisited official AOAC Method **942.05** and suggested temperature adequacy down to 550°C. Some standards for feed analysis have followed a similar pattern of temperature adequacy, such as in ISO method 5984:2002 (17), European Commission (18), or the latest edition of the Brazilian standards for animal feed analyses (method M-001/2; 6).

On the other hand, the second variable of the physical binomial applied to crude ash quantification seems to have a more controversial pattern in the literature. Recommendations for ashing time can range from 1 h (1) to overnight (16, 19) or until a constant weight of residue on ignition is achieved (20). It is known that oxidation power into the muffle furnace results from a balance between temperature and time (21). Thus, in a logical reasoning, the higher the temperature, the shorter the ignition time, and vice versa. However, most methods seem to use modal times ranging from 2 to 3 h as they are based on temperatures between 550–600°C. In general, the use of longer ignition times seems to be an attempt to either avoid or minimize the contamination of residue by a part of the OM that is possibly more refractory to oxidation. The influence of that refractory part has been associated with dark/brown colorations of the residue on ignition (3) even after many hours of ashing.

In this sense, the introduction of ashing aids as a third element contributing to OM oxidation (i.e., besides temperature and time) has been suggested. Accordingly, Thiex et al. (3) recommended a modification to official AOAC Method **942.05**, which should encompass two 3 h ignition cycles at 550°C. The cycles are intercalated by opening the furnace door to guarantee a fresh air supply. In this case, the fresh air would serve as an oxygen supplier and would improve the oxidation of refractory organics. On the other hand, some authors have claimed that the dry ashing process may produce a heavy layer on top of the ash residue, which could hinder its complete combustion and overestimate ash content. In this case, a small amount of water could be poured over the ash residue between two ignition cycles to break up that layer (2, 3). Moreover, some variations in dry ashing methods have been based on the use of chemical substances as ashing aids (3, 18, 22, 23), probably relying on the release of free radicals to speed up oxidation.

However, despite of all the current theoretical knowledge on the dry ashing process, the recommendations regarding laboratory procedures are still highly variable (1–3, 6, 16, 18). Possibly, a "perfect" standard procedure cannot be achieved for all feed materials (3). Notwithstanding, the efficiency of variations in the procedures must be verified to assure adequate levels of method robustness while keeping an optimal ability to discriminate feed materials regarding their different chemical characteristics.

Thus, our objective was to evaluate some variations in laboratory procedures for crude ash quantification regarding physical parameters (i.e., time, temperature) and ashing aids and their influences on crude ash estimates, repeatability, and discrimination power among feed types.

Experimental

Location and Study Materials

All analyses were performed at the Animal Nutrition Laboratory of the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Eight different feed types were chosen aiming to compose a representative set regarding diets offered to beef and dairy cattle: corn silage, fresh sugarcane, sugarcane silage, Tifton-85 hay, soybean meal, corn grain, wheat bran, and dried distillers' grains (DDG). For each feed type, three different unique materials (i.e., field replicates or primary samples) were obtained from feed industries and farms located in Minas Gerais state, Brazil. Therefore, our analytical set encompassed 24 study materials.

The high-moisture feeds (silages and fresh sugarcane) were oven-dried (55°C). Then, all dry study materials were ground in a knife mill (TE-680, Tecnal, Piracicaba, São Paulo, Brazil) to pass through a 1-mm screen sieve. The 24 study materials were then analyzed in triplicate for dry matter (DM) content (dried overnight at 105°C, method G-003/1; 6).

Crude Ash Methods

The "control" procedure herein was based on the official method of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; method M-001/2; 6). Briefly:

(a) Weigh 2.0 g as-is of the test portion into the crucible, recording the weight of crucible and test portion to the nearest 0.1 mg;

(b) Place the crucibles in the muffle furnace;

(c) Ignite in a furnace at 550°C for 3 h. The furnace must be adjusted to reach ignition temperature within 60 min. The ignition time starts counting after ignition temperature is achieved;
(d) Allow the furnace to cool below 200°C, but stay above 150°C. Then transfer crucibles to a desiccator; and
(e) Cool to room temperature and weigh, recording the weight to the nearest 0.1 mg.

For all procedures, the crucibles (5 cm diameter and 30 mL volume) were previously washed in running water, ashed for 3 h at 550°C, and handled and weighed as described above. The same digital muffle furnace was used for all procedures (Fornos Magnus, Belo Horizonte, Minas Gerais, Brazil).

The following variations on the basic method were evaluated:

(1) Increased ignition temperature: all procedures were performed as previously described for the control, but the temperature was increased to 600°C (2, 10);
(2) Increased ashing time: all procedures were performed as previously described for the control, but ashing time was increased to 6 h. This specific time was chosen to allow a direct comparison with method variations that included ashing aids;
(3) Using fresh air as an ashing aid: the procedures were adapted from Thiex et al. (3). The following modifications were added to the control procedure:
    (d) Allow the furnace to cool below 200°C and open the door to ensure a fresh air supply (1-2 min);
    (e) Reignite in a furnace at 550°C for 3 h. The ignition time starts counting after ignition temperature is achieved;
    (f) Allow the furnace to cool below 200°C, but stay above 150°C. Then transfer crucibles to a desiccator; and
    (g) Cool to room temperature and weigh, recording the weight to the nearest 0.1 mg;
(4) Using fresh air and water as ashing aids: the procedures were adapted from Thiex et al. (3) and, besides fresh air supply, they aim at breaking the superficial layer of residue on ignition and allowing more efficient ashing of the bottom layer in the second ignition cycle. The modifications of procedures compared to the control were:
    (d) Allow the furnace to cool below 100°C and open the door;
    (e) Carefully add a few mL of distilled water to the residue to break it up;
    (f) Reignite in a furnace at 550°C for 3 h. The ignition time starts counting after ignition temperature is achieved;
    (g) Allow the furnace to cool below 200°C, but stay above 150°C. Then transfer crucibles to a desiccator; and
    (h) Cool to room temperature and weigh, recording the weight to the nearest 0.1 mg;
(5) Using fresh air and hydrogen peroxide as ashing aids: the procedures were the same as described in the latter variation, except that water was replaced by hydrogen peroxide PA (35% or 130 volume). In this case, besides breaking up the upper layer, we hypothesized the hydrogen peroxide decomposition releases free radicals, which could speed up the oxidation of the residual OM.

Each ashing run contained all 24 study materials. We performed three ashing runs ($n = 3$) for each method, totaling the evaluation of 432 aliquots (i.e., test portions).

Following the recommendations of Thiex et al. (3), we also performed a color evaluation of residues on ignition. Due to the small masses, the residues from the three replicates were pooled and scored for color pattern (L* for lightness, a* for redness, and b* for yellowness), using a Hunter MiniScan EZ colorimeter (4500L; Hunter Associates Laboratory Inc., Reston, VA, USA). Those coordinates were then converted into RGB (i.e., red, green, and blue) coordinates using Coloroid Professional Color Plan Designer software. After that, RGB coordinates were organized in an Excel plan in which each cell was filled with the corresponding solid color. For some materials, the mass of residues on ignition was not sufficient to allow the color evaluation.

Calculations and Statistical Analysis

The calculation of crude ash was performed according to the following equation:

$$\%\mathrm{CA}_{\mathrm{DM}} = \frac{\mathrm{R} - \mathrm{T}}{\mathrm{W} \times \mathrm{DM}} \times 100, \tag{1}$$

where $\%\mathrm{CA}_{\mathrm{DM}}$ = crude ash as a percentage of DM; R = weight of crucible + residue on ignition (g); T = tare (empty) weight of crucible (g); W = weight of test portion (g, as-is); and DM = dry matter content of the sample (g/g).

The crude ash results were analyzed according to the model:

$$\mathrm{Y}_{\mathrm{ijkl}} = \mu + \mathrm{F}_{\mathrm{i}} + \mathrm{S}_{(\mathrm{i})\mathrm{j}} + \mathrm{M}_{\mathrm{k}} + \mathrm{FM}_{\mathrm{ik}} + \varepsilon_{\mathrm{ijkl}}, \tag{2}$$

where $\mathrm{Y}_{\mathrm{ijkl}}$ = crude ash obtained in the test portion l taken from study material j of feed type i and evaluated through method k; μ = general constant; F_{i} = fixed effect of feed type i; $\mathrm{S}_{(\mathrm{i})\mathrm{j}}$ = random effect of study material j nested to feed i assumed to be NIID (0, $\sigma^2_{S/F}$); M_{k} = fixed effect of method k; $\mathrm{FM}_{\mathrm{ik}}$ = fixed effect of interaction between feed type i and method k; and $\varepsilon_{\mathrm{ijkl}}$ = random error assumed to be NIID (0, σ^2_{ε}).

When necessary, means were grouped using Fisher's multiple comparison procedure.

After the first analysis of variance, data was analyzed again in an independent way for each method according to the model:

$$\mathrm{Y}_{\mathrm{ijk}} = \mu + \mathrm{F}_{\mathrm{i}} + \mathrm{S}_{(\mathrm{i})\mathrm{j}} + \varepsilon_{\mathrm{ijk}}, \tag{3}$$

where $\mathrm{Y}_{\mathrm{ijk}}$ = crude ash obtained in the test portion k taken from the study material j of feed type i; μ = general constant; F_{i} = random effect of feed type i assumed to be NIID (0, σ^2_{F}); $\mathrm{S}_{(\mathrm{i})\mathrm{j}}$ = random effect of study material j nested to feed type i assumed to be NIID (0, $\sigma^2_{S/F}$); and $\varepsilon_{\mathrm{ijk}}$ = random error assumed to be NIID (0, σ^2_{ε}).

From the adjustment of the model described in equation (3), the following relative standard deviations were estimated for each method variation:

$$\mathrm{r} = \frac{\sqrt{\hat{\sigma}_{\varepsilon}{}^{2}}}{\overline{\mathrm{Y}} \times} 100, \tag{4}$$

$$\mathrm{RSD}_{\mathrm{F}} = \frac{\sqrt{\hat{\sigma}_{\mathrm{F}}{}^{2}}}{\overline{\mathrm{Y}} \times} 100, \tag{5}$$

$$\mathrm{RSD}_{\mathrm{S}} = \frac{\sqrt{\hat{\sigma}_{\mathrm{S}/}\mathrm{F}^{2}}}{\overline{\mathrm{Y}} \times} 100, \tag{6}$$

where r = repeatability (%); $\hat{\sigma}^2_{\varepsilon}$ = estimate of error variance [(% DM)^2]; $\overline{\mathrm{Y}}$ = average crude ash (% DM); $\mathrm{RSD}_{\mathrm{F}}$ = relative standard deviation among feed types (%); $\hat{\sigma}_F^2$ = estimate of the variance among feed types [(% DM)^2]; RSD_S = relative standard deviation among study materials (%); and $\hat{\sigma}_{S/F}^2$ = estimate of the variance among study materials within feed types [(% DM)^2].

Although some reference values have been established regarding reproducibility (24, 25), it has been difficult to define adequate limits for repeatability. We know that the reproducibility evaluation cannot be performed in our work, as all procedures were performed in one single laboratory. However, we adopted a more functional approach to assess repeatability from the expected reproducibility value, which was calculated as:

$$Re = 2 \times C^{-0.15}, \tag{7}$$

where Re = expected reproducibility (%) and C = average content of crude ash (g/g DM).

According to Horwitz (24), repeatability should ordinarily be one-half to two-thirds of reproducibility. From this, we assumed that the expected conventional limits for repeatability could be established as:

$$L_c = 0.50 \times Re, \tag{8a}$$

$$U_c = 0.67 \times Re, \tag{8b}$$

where L_c and U_c are the lower and upper limits for the expected conventional repeatability (%).

Equation (8) is based on the assumption that reproducibility behaves exactly as expected [Equation (7)]. However, in general, a method is considered reproducible if the actual reproducibility falls between one-half and twice the expected reproducibility (24, 25). From this, the conventional limits for repeatability may be adjusted to a tolerable range according to:

$$L_t = 0.50 \times 0.50 \times Re = 0.25 \times Re, \tag{9a}$$

$$U_t = 0.67 \times 2.00 \times Re = 1.34 \times Re, \tag{9b}$$

where L_t and U_t are the lower and upper limits for the expected tolerable repeatability (%).

From equations (8) and (9) we were able to understand how adequately the method variations behaved regarding precision/repeatability.

All statistical evaluations were performed using the GLIMMIX procedure of SAS 9.4. The variance components were estimated according to the restricted maximum likelihood method. Statistical significances were declared at $P < 0.05$.

Results

There was an interaction ($P < 0.01$) between feed types and methods on the crude ash. The slicing of this effect indicated that for most feed types (corn grain, DDG, wheat bran, grass hay, and fresh sugarcane) the methods did not affect ($P \geq 0.23$) the values of residues on ignition (Table 1). However, differences among methods occurred ($P < 0.01$) for soybean meal, corn silage, and sugarcane silage.

The comparisons among methods within each feed type were generally different from each other and some overlaps were observed (Table 1). For soybean meal, the general pattern indicated that just increasing either time or temperature decreased ($P < 0.05$) crude ash in comparison to the control method. However, a more consistent decrease compared to the control ($P < 0.05$) was seen when ashing aids were applied. All ashing-aid variations clustered together ($P > 0.05$). On the other hand, the mean comparisons for corn silage indicated that increasing neither temperature nor ashing time was sufficient ($P > 0.05$) to decrease crude ash in comparison to the control. Effective decreases were only obtained ($P < 0.05$) when liquid ashing aids were used, which did not differ from each other ($P > 0.05$). The mean comparisons for sugarcane silage were very uninformative and a clear pattern could not be extracted from them.

On average, the overall mean comparisons indicated that increasing either temperature or ashing time caused a consistent decrease in crude ash when compared to the control method ($P > 0.05$, Table 1, Figure 1). In terms of ashing aids, the simple introduction of fresh air was not enough to decrease ($P > 0.05$) crude ash in comparison to simply increasing the temperature or time. However, the use of liquid ashing aids caused an additional decrease ($P < 0.05$) in crude ash compared to the other methods.

The RSDs among feed types and study materials behaved similarly among methods (Table 2) and repeatability was found within the tolerable boundaries for all methods. However, when liquid ashing aids were added, the repeatability was slightly improved.

The color evaluation was performed fully only for four feed types (Table 3). For the others, we faced constraints to obtain residues masses in sufficient quantities to allow an adequate

Table 1. Least-square means for the residue on ignition (% of DM) in different feed types according to the crude ash method

		Method[a]							
	Temperature, °C	550	600	550	550	550	550		
	Time, h	3	3	6	3 + 3	3 + 3	3 + 3		
Feed type	Ashing aid	—	—	—	Fresh Air	Air + H_2O	Air + H_2O_2	SEM	P-value
Corn grain		1.18	1.18	1.17	1.13	1.15	1.13	0.645	0.689
DDG		3.06	3.00	3.11	3.03	3.04	3.04		0.230
Soybean meal		6.67a	6.49b	6.49b	6.40c	6.33c	6.32c		<0.001
Wheat bran		6.48	6.49	6.44	6.43	6.47	6.45		0.679
Grass hay		5.39	5.42	5.41	5.41	5.33	5.35		0.244
Corn silage		4.71a	4.72a	4.70a	4.67a	4.59b	4.57b		<0.001
Fresh sugarcane		2.25	2.15	2.22	2.22	2.17	2.20		0.252
Sugarcane silage		4.20ab	4.24a	4.10c	4.18ab	4.12bc	4.11bc		0.003
Overall		4.24a	4.21b	4.20b	4.18b	4.15c	4.15c	0.228	<0.001

[a] Means in a row followed by different letters differ at $P < 0.05$.

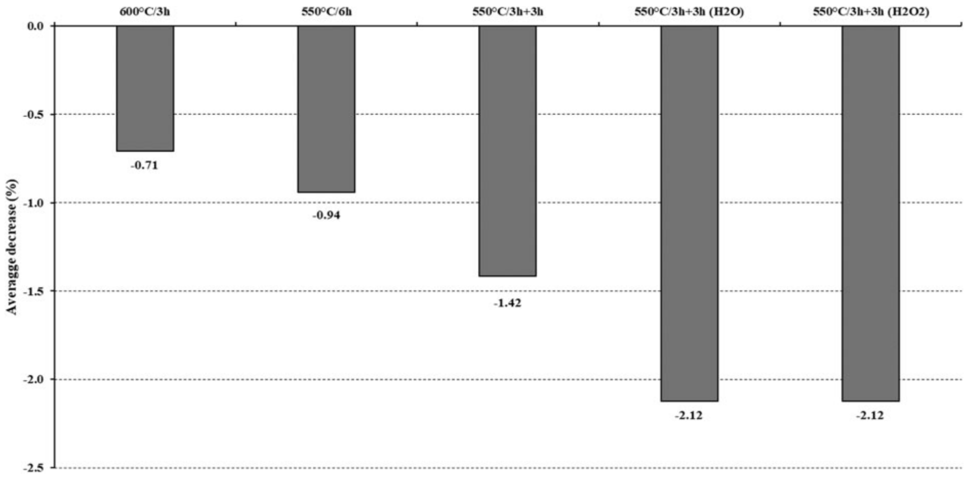

Figure 1. Average percentage decrease in residue on ignition according to the crude ash methods in relation to the control method (i.e., 550°C × 3 h).

Table 2. Descriptive statistics of random variabilities for the residue on ignition according to the crude ash method

		Method					
	Temperature, °C	550	600	550	550	550	550
	Time, h	3	3	6	3 + 3	3 + 3	3 + 3
Item	Ashing aid	—	—	—	Fresh Air	Air + H_2O	Air + H_2O_2
RSD, %							
Among feed types		43.9	44.0	43.4	43.6	43.6	43.6
Among study materials		26.5	26.4	27.1	26.6	26.6	26.5
Repeatability (r)		2.13	2.73	2.80	2.12	1.50	1.24
Expected reproducibility, %		3.21	3.22	3.22	3.22	3.22	3.22
Expected limits for r, %[a]							
Conventional		1.61–2.15	1.61–2.16	1.61–2.16	1.61–2.16	1.61–2.16	1.61–2.16
Maximum tolerable		0.80–4.31	0.80–4.31	0.80–4.31	0.80–4.31	0.81–4.32	0.81–4.32

[a] *See* equations (7), (8), and (9) for more details.

color measurement by the colorimetric method. However, for the feed materials we had, there was no consistent pattern among methods. However, one specific point in the pattern seemed consistent enough to draw some inference. The residues on ignition were darker when the control method was applied, which is in line with the crude ash results. No systematic differences in the color pattern were verified among the other methods.

Discussion

The crude ash in feeds is assessed as the residue on ignition after a complete OM decomposition in a muffle furnace using a time × temperature binomial. Due to their empirical nature, ashing methods have been revised (3, 26) aiming at minimizing their associated biases and seeking a balance between complete oxidation and loss of volatile minerals. In general, biases might be generated from incomplete OM decomposition, volatilization of some mineral compounds under specified temperatures, or inappropriate method application (1, 3, 16, 26).

Despite being independent physical parameters, time and temperature do not act independently, and interactions between them will define the amount of crude ash obtained from a specific feed material (21). Once the adequate temperature is established, adjustments on ignition time would provide some fine-tuning on the analytical entity's estimates and vice versa. On the other hand, temperatures below the minimum required for adequate ignition of the OM may not be compensated by extending ignition time. An equilibrium between the physical parameters of the process must be achieved to allow an appropriate quantification of crude ash in feeds.

The results here confirmed that increases in either ignition time or temperature led, on average, to decreases in the % crude ash. They bring evidence that the physical binomial 550°C × 3 h is not the best option to quantify the crude ash in feed materials. This was the only clear pattern to emerge from the colorimetric evaluation of residues on ignition. Some feed types were not sensitive to the variations in the physical parameters; however, others were. Thus, to achieve more adequate robustness, the chosen method should focus on the feed types that are particularly sensitive to the method.

An ideal ignition temperature should be as low as possible to reduce volatile compound losses, yet high enough to ensure total carbon loss (23). A recurrent issue associated with excessive

Table 3. Color pattern of the residue on ignition in different feed types according to the crude ash method

		Method					
	Temperature, °C	550	600	550	550	550	550
	Time, h	3	3	6	3+3	3+3	3+3
Feed type[a]	Ashing aid	—	—	—	Fresh Air	Air+H_2O	Air+H_2O_2
Corn grain	1	NA[b]	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA
DDG	1	(160,158,156)	(156, 154, 153)	(172, 170, 168)	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA
Soybean meal	1	(164, 151, 134)	(205, 181, 154)	(185, 169, 147)	(210, 188, 159)	(213, 188, 160)	(213, 188, 160)
	2	(161, 150, 133)	(211, 185, 156)	(186, 170, 147)	(200, 178, 151)	(219, 194, 167)	(213, 188, 160)
	3	(174, 160, 141)	(216, 190, 162)	(201, 181, 155)	(215, 190, 160)	(218, 191, 165)	(211, 187, 161)
Wheat bran	1	(110, 104, 100)	(119, 117, 115)	(118, 112, 108)	(122, 177, 112)	(114, 111, 108)	(122, 118, 113)
	2	(114, 108, 103)	(120, 117, 116)	(123, 118, 113)	(128, 123, 119)	(118, 115, 113)	(151, 147, 142)
	3	(130, 125, 122)	(164, 160, 157)	(150, 146, 143)	(161, 157, 153)	(166, 159, 154)	(177, 170, 165)
Grass hay	1	(141, 134, 127)	(154, 145, 136)	(141, 133, 123)	(149, 143, 133)	(161, 150, 138)	(165, 154, 142)
	2	(145, 144, 142)	(161, 157, 152)	(152, 149, 148)	(158, 155, 154)	(157, 153, 149)	(170, 165, 160)
	3	(148, 144, 141)	(160, 154, 148	(156, 152, 150)	(155, 151, 149)	(143, 138, 134)	(155, 149, 144)
Corn Silage	1	(125, 119,109)	(139, 130, 118)	(133, 125, 115)	(129, 121, 110)	(145, 130, 112)	(141, 126, 109)
	2	(142, 143, 145)	(152, 151, 150)	(144, 145, 146)	(152, 152, 153)	(126, 122, 120)	(143, 140,138)
	3	(112, 117, 122)	(127, 131, 135)	(118, 122, 128)	(117, 122, 128)	(135, 134, 135)	(119, 120, 122)
Fresh sugarcane	1	(137, 136,135)	(142, 141, 141)	(143, 141, 141)	(144, 143, 144)	(132, 128, 126)	NA
	2	(150, 147, 145)	(162, 157, 152)	(161, 157, 153)	NA	NA	NA
	3	(152, 146, 139)	(163, 153, 140)	(149, 143, 134)	(160, 152, 142)	NA	NA
Sugarcane silage	1	(160, 155, 149)	(175, 166, 155)	(167, 161, 154)	(172, 165, 155)	NA	NA
	2	(107 ,105, 105)	(122, 120, 119)	(118, 116, 116)	(108, 107, 107)	(118, 113, 109)	(122, 116, 112)
	3	(121, 113, 104)	(139, 128, 118)	(136, 126, 116)	(128, 118, 108)	(133, 118,104)	(136, 121, 107)

[a] The numbers 1 to 3 indicate the different study materials (i.e., field samples) evaluated within each feed type.

[b] NA = Not available. The residue on ignition was insufficient for the color evaluation using the colorimeter.

temperatures is the loss of minerals through volatilization (26, 27). From this, it could be speculated that increasing the temperature to 600°C could have decreased ash content due to increased volatilization. However, the decreased ash content obtained by extending ashing time seems to allow a different explanation for that. Considering that the simple extension of either time or temperature led to the same average decrease in % crude ash, the most probable cause was an improvement in the elimination of some refractory OM, rather than an increase in volatilization. Once more, the inadequacy of the control procedure is evident.

However, despite the aforementioned likely improvement the % crude ash was, on average, further reduced with the use of liquid ashing aids. In this particular case, any loss increased by volatilization has no physical or chemical reasons to occur. However, the pattern obtained with the different ashing aids was not consistent among feed types.

Despite clustering along with the liquid ashing aids for soybean meal and sugarcane silage, on average, the simple fresh air supply was not enough to reduce the crude ash to the same levels observed when liquid aids were applied. It has been stated that a fresh air supply between two ignition cycles could renew the oxygen supply inside a muffle furnace. Consequently, it could improve the release of carbon that might remain in the sample after the first ignition cycle (3, 28). However, such a pattern did not correspond to what was observed here.

Further improvements in crude ash estimates were obtained only when liquid ashing aids were added between the two ignition cycles. During the ashing procedure, a heavy layer might be formed on the top of the residue interfering with carbon release (2). Then, adding liquid aids between ignition cycles might improve OM decomposition by mechanical action, crushing the crust eventually formed in the previous ignition cycle and improving the degradation of refractory compounds in the second ignition cycle (27). Hydrogen peroxide might accelerate OM combustion in dry ashing methods (23). The decomposition of hydrogen peroxide basically occurs when its highly unstable oxygen–oxygen bond is broken, which can release free radicals, such as hydroxyl and hydroperoxyl (29). Those are highly reactive and may improve OM decomposition. However, we did not observe differences between using water and hydrogen peroxide as ashing aids, indicating that their effects were similar and most likely associated with the physical breakdown of the heavy layer on the top of the ash residue, allowing better oxidation of material below the crust during the second ignition cycle. Considering the similarity of both liquid ashing aids, water is recommended, mainly due to its lower cost and ease of use.

Several authors have considered that complete ashing is reached when heating is continued until the residue on ignition achieves a uniform color and is free from unburned particles (30, 31). In this sense, the color should be as light as possible (i.e., white, light gray; occasionally reddish or green). Accordingly, if the residue is dark or brown, it could indicate an undesirable carbon presence (3, 28, 31, 32).

However, any visual color evaluation can be biased, as it depends on the subject's judgment. There is no standard color chart available for color evaluations of ash residue, which makes visual evaluations subjective and imprecise. Color scoring would be the result of a light interaction between the object and the observer's eyes, and can also vary according to the ambient lighting (33). That is the reason why we decided to perform a color evaluation using a colorimetric technique, which would avoid any subjectivity when scoring the residue coloration. In general, the control method (i.e., 550°C × 3 h) presented the darkest residues, but no clear pattern among feeds was observed for the other methods.

Despite confirming the inadequacy of the control method, the inconsistent pattern among methods indicates that ash color seems to be more a characteristic of the feed material itself rather than particularly useful information to evaluate the ashing quality. Even though improvements in crude ash had been obtained using liquid ashing aids, none of the ash residues showed a color pattern close to white or light gray. Thus, the results here obtained agreed with St. John (30), who stated that neither macroscopic observations nor an analyst's judgment for carbon presence in crude ash are useful in determining the optimal ashing methodology.

Precision in our study was represented by repeatability, which is also known as within-laboratory variation (24). Repeatability is based on random residual variance, and lower values indicate a more reliable procedure for feed evaluation (34). Repeatability limits were proposed and calculated based on the expected reproducibility and all method variations exhibited repeatability within tolerable limits. However, the use of liquid ashing aids improved repeatability. Such a pattern reinforces our previous discussion. Besides overestimating crude ash, the OM retained in the bottom layer of ash residues seems to be variable among test portions. Thus, the action of the liquid ashing aids also improves the precision of the procedures by decreasing random variation among replicates.

In terms of feed analysis, an ideal method must also be able to allow adequate discrimination among and within feed types. That characteristic is expressed here by the RSDs among feed types and study materials. Unlike repeatability, it is desirable that those RSDs be maximized under any given evaluation. Hence, the capability to discriminate feeds with different characteristics, as well as to cluster similar feeds would be surely guaranteed. Regarding those requisites, all methods evaluated herein performed similarly.

Conclusions

The method of obtaining residues on ignition in animal feeds based on the binomial 550°C × 3 h does not have sufficient robustness and may overestimate crude ash in some feeds. Adjustments in either ignition time or temperature appear to improve crude ash test results, but the best results are obtained using liquid ashing aids between two ignition cycles. The recommended method is based on the use of 550°C and two 3 h ignition cycles with water added to the ash residue between cycles.

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Conflict of Interest

All authors declare no conflict of interest.

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Variations in the Kjeldahl method for the evaluation of nitrogen content in tropical forages

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Abstract

Background: Nitrogen is one of the most important components of feeds and its quantification allows estimating of protein equivalents in feeds, an important characteristic for diet formulation in animal nutrition.

Objective: We aimed to evaluate the N recovery in tropical forages by using the Kjeldahl method with modifications involving a pre-digestion step with salicylic acid and, or a partial replacement of copper sulfate by titanium dioxide as a catalyst.

Method: The Kjeldahl standard procedure was based on digestion with sodium sulfate and copper sulfate (20:1), followed by steam distillation and titration. The Kjeldahl variations were: the Kjeldahl method with salicylic acid as pre-treatment; the Kjeldahl with titanium dioxide replacing 50% of copper sulfate in the digestion mixture; and the Kjeldahl with salicylic acid and copper sulfate:titanium dioxide (1:1) as a catalyst. We adopted the Dumas method as a standard method. Forty eight study materials (i.e., tropical forages) were evaluated.

Results: Nitrogen recovered was not complete for the Kjeldahl methods, which did not differ to each other. Neither salicylic acid nor titanium dioxide improved the nitrogen recovery.

Conclusions: The average N recovery in tropical forages when analyzed through the Kjeldahl method is 0.974 g/g. The N recovery of the Kjeldahl method is not influenced by a pre-digestion with salicylic acid or by the partial replacement of copper sulfate with titanium dioxide as a catalyst.

Highlights: The pre-treatment using salicylic acid, or titanium dioxide partialy replacing cooper sulfate did not improve the nitrogen recovery by Kjeldahl methods in tropical forages.

Introduction

The nitrogen (N) analysis is routinely performed in animal nutrition laboratories in order to calculate the protein equivalents in feeds. The Kjeldahl method has been widely used since its introduction in the 19th century due to its accuracy, simplicity, and robustness (Sáez-Plaza et al., 2013; Greaser and Guo, 2015). Nevertheless, the Kjeldahl method backbone has been modified over the years intending to simplify the analytical procedures, reduce its hazardous chemicals disposal, and increase the versatility of the method (Thiex et al., 2002).

The Kjeldahl method presents three basic steps: digestion, distillation, and titration. First, organic N is mineralized in a boiling mixture containing sulfuric acid along with salt and a catalyst agent and converted to ammonium sulfate. Then, the mineral N solution is alkalized to free up the ammonia, which is steam distillate and quantified through acid titration (Thiex et al., 2002; Dierckx et al., 2006; Buffler, 2017; Detmann et al., 2021).

Overall, the total organic N is supposed to be measured by the Kjeldahl method (Moore et al., 2010). This group of N compounds encompasses mainly protein- and nucleic acid-based N (e.g., amino-N, free amino- N, nucleic acids, phospholipids), which is assumed to be quantitatively recovered (Mariotti et al., 2008; Moore et al., 2010). However, there is no distinction between the non-protein N from protein-N (Sáez-Plaza et al., 2013) and the N occurring with N-N and N-O linkages (e.g., nitrate, nitrite, alkaloids, pyridine) are considered resistant to mineralization and might not be totally recovered (Nelson and Sommers, 1980; Sikorski, 2001; Sáez-Plaza et al., 2013).

Particularly, the N in more oxidized forms (e.g., nitrate) may represent a considerable fraction of total N in plant materials (Simonne et al., 1994), mainly under heavy fertilizer applications (Du Preez and Bate, 1989). Thus, some nitrate present in plant materials will not be recovered due to incomplete breakdown of N-O bounds during Kejldahl digestion (Nelson and Sommers, 1980), which avoids conversion to ammonia during the alkalinization and steam distillation (Buffler and Mühleis, 2018). However, nitrate can be important for ruminant feeding, as it can act as an alternative electron sink in the rumen, being reduced to ammonia. In these dynamics, nitrate may help mitigating methane emissions and be incorporated into microbial protein after its reduction to ammonia (Leng, 2014). Therefore, to some extent, the nitrate-N in forages can be useful for ruminant nutrition and feeding.

To evaluate the total N in materials containing nitrate, it has been suggested the utilization of a pre-digestion step with reductants for nitrate (NO_3^-) reduction to ammonium (NH_4^+; Sikorski, 2001; Dierckx et al., 2006; Sáez-Plaza et al., 2013), which could improve the N recovery by Kjeldahl method. One of the reductant agents used to access the N from highly oxidized compounds is salicylic acid. Under acidic conditions (i.e., sulfuric acid), the nitrate from the test portion reacts with the phenolic group present in salicylic acid to form a nitro phenolic compound, resulting in 5-nitrosalicylic acid (Buffler and Mühleis, 2018). Hence, it has been suggested that salicylic acid used as a pretreatment in the Kjeldahl method could improve the recovering of nitrate-N (Bremner, 1965; Du Preez and Bate, 1989).

Another limitation associated with the Kjeldahl method is the foam formation during the digestion step. Depending on the sample size and matrix, the foam formed might prevent complete digestion due to spattering, or acid volatilization (Nelson and Sommers, 1980). It has been shown that titanium dioxide addition as a catalyst to the digestion mixture might reduce the foam occurrence, as well as improve the N recovery (Simmone et al., 1993; Buffler, 2017). Generally, the association of copper sulfate and titanium dioxide (catalysts) with potassium or sodium sulfate (salts) in the digestion mixture is adopted when the sample composition is high in organics or for liquid samples (Buffler, 2017). As titanium dioxide is a non-toxic catalyst, the partial replacement of copper sulfate in the digestion mixture can decrease the chemical hazardous disposal.

In tropical regions, several species can be used for ruminant feeding. As the forage composition is highly variable, the knowledge of its N content is necessary for proper diet formulation. To our knowledge, there are no studies evaluating the N recovery in tropical forages using a pre-digestion step with salicylic acid or titanium dioxide as a catalyst in the Kjeldahl method.

Thus, we aimed to evaluate the N recovery in tropical forages by using the Kjeldahl method with modifications involving a pre-digestion step with salicylic acid and, or a partial replacement of copper sulfate by titanium dioxide as a catalyst.

Experimental

Location and study materials

All analyses were performed in the Animal Nutrition Laboratory at the Animal Science Department of Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Forty eight different primary forage samples were collected in the Viçosa municipality as follows: corn silage [*Zea mays* (n = 5)], sorghum silage [*Sorghum bicolor* (n = 1)] sugarcane silage [*Saccharum officinarum* (n = 5)], fresh sugarcane (n = 2), Tifton hay [*Cynodon* sp. (n = 3)], and 34 different fresh forages [*Pennisetum purpureum* Schum (n = 3), *P. purpureum* Schum cv. BRS Capiaçu (n = 2), *Uruchloa decumbens* (n = 14), *U. brizantha* cv. Marandu (n = 1), *Uruchloa* sp. cv. Mulato (n = 1), *Cenchrus ciliaris* (n = 1), *Cynodon* sp. cv. Tifton 85 (n = 2), *C. dactylon* (n =1), *Megathyrsus maximus* cv. Mombaça (n = 1), *M. maximus* cv. BRS Tamani (n = 1), *M. maximus* cv. BRS Zuri (n = 1), *Andropogon* gayanus (n = 1), *Stylosanthes guianensis* cv. Campo Grande (n = 1), and *Arachis pintoi* (n = 2)]. Samples were chosen in order to include forages with different chemical compositions and a wide range of N content.

All samples were partially dried in a forced-air drying oven at 55°C for 24-72 h (Detmann et al., 2021; method G-001/2) and were ground to pass through a 1-mm screen sieve using a knife mill (TE-680, Tecnal, Piracicaba, São Paulo, Brazil).

Kjeldahl procedures

The basic reagents were used to perform the Kjeldahl procedures: concentrated sulfuric acid (H_2SO_4), hydrochloric acid 37% (HCl), boric acid (H_3BO_3), sodium hydroxide (NaOH), sodium sulfate (Na_2SO_4), copper sulfate pentahydrate ($CuSO_4$ $5H_2O$), bromocresol green, methyl red, titanium dioxide (TiO_2), and salicylic acid ($C_7H_6O_3$). Only pure reagents (i.e., analytical grade) were used. The following equipment was utilized for the analyses: digestion block (TE-040, Tecnal, Piracicaba, São Paulo, Brazil), steam distillation apparatus (TE-036/1, Tecnal, Piracicaba, São Paulo, Brazil), and a fume hood.

To perform the standard Kjeldahl method (**KJ**), we followed the official method adopted by the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; method N-001/2, Detmann et al., 2021), which is briefly described:

a. Weigh 200-mg as-is test portion in a test tube;

b. Add 2 g of the digestion mixture (sodium sulfate:copper sulfate, 20:1) and 5 mL of concentrated sulfuric acid;

c. Place the tubes in the digestion block within a fume hood and slowly heat it up until it reaches the temperature of 400°C. Keep them at this temperature until the solution becomes translucent;

d. Remove the tubes and allow them to cool down inside the fume hood;

e. When the tube temperature is below 100°C, add a small portion of distilled water (approximately 10 mL) and homogenize it to avoid or minimize the solution crystallization;

f. Add 10 mL of a boric acid solution (20 g/L) to a 250-mL Erlenmeyer flask.

g. Adapt the Erlenmeyer flask to the distillation apparatus to receive all distilled ammonia;

h. Transfer the digestion tube with the digested sample to the distillation set and add 25 mL of a sodium hydroxide solution (400 g/L);

i. Perform steam distillation while maintaining the condenser terminal immersed in the receiver solution until all the ammonia is released. The total volume of the distillate should be 100 mL;

j. Remove the Erlenmeyer flask and titrate it with a hydrochloric acid solution until the indicator color changes (from green to light pink).

For each digestion run, two blank tubes (without sample) go through all steps (digestion, distillation, and titration) aiming at quantifying and eliminating interferences. All the procedures to prepare the different solutions can be found in Detmann et al. (2021).

We evaluated the following variations of the standard Kjeldahl method:

1- Kjeldahl with salicylic acid (**KJS**): besides digestion mixture, a pre-treatment with salicylic acid adapted from Simonne et al. (1994). Briefly, 0.5 g of salicylic acid was added to the test tube containing the sample, 5 mL of concentrated sulfuric acid were added, and the mixture was shaken each 30 min. After two hours, we added 2 g of the digestion mixture (sodium sulfate:copper sulfate, 20:1) using a funnel, and the mixture was digested as previously described.

2- Kjeldahl with titanium dioxide (**KJT**): titanium dioxide was used as a catalyst in the digestion procedure. It replaced 50% of the copper sulfate in the digestion mixture (Buffler, 2017);

3- Kjeldahl with salicylic acid and titanium dioxide as catalyst (**KJST**): this method was performed by taking into account both modifications presented above.

We anticipate that the KJS method caused intense foaming during the digestion step, which triggered the solution to boil over the test tubes. Thus, the digestion had to be interrupted even before the digestion block reaches the planned final temperature. Hence, we concluded that the KJS method is not feasible for forage samples, and results obtained with KJS were neither shown nor discussed in this work. Nevertheless, this constraint caused by salicylic acid addition was not observed when copper sulfate was partially replaced by titanium dioxide as a catalyst.

The N contents obtained by all Kjeldahl methods were obtained according to the following equation:

$$N=\frac{(V_{sample}-V_{blank})\times N_{acid}\times 14\times 1000}{W} \quad (1),$$

where N = N content (g/kg as is); V_{sample} = volume of the hydrochloric acid solution (mL) used for titrating the test portion solution (mL); V_{blank} = volume of the hydrochloric acid solution (mL) used for titrating the blank; N_{acid} = true normality of the hydrochloric acid solution; 14 = atomic weight of N; and W = weight (mg) of the test portion.

The hydrochloric acid solution was prepared to present 0.02 N. The true normality was checked through titration with a standard sodium carbonate solution, as described by Detmann et al. (2021).

Two replicates (i.e., test portions) of each study material were analyzed according to each Kjeldahl method. The N contents were expressed on as-is basis to avoid introducing bias coming from laboratorial dry matter quantification (Mertens, 2003).

Dumas procedure

A Dumas method was used as a reference to quantify the N recovering obtained with the different Kjeldahl methods. A 2 to 3-mg test portion was wrapped in tin foil, and introduced at a furnace for elemental analyzer model 2400 Series II CHNS/O Analyzer (Perkin Elmer, Shelton, CT, USA), using helium as carrier gas and acetanilide OAS (SC0381 - SerCon, Crewe, England) as a analytical standard. Standardization and programming were conducted as specified by the manufacturer. The results were expressed on as-is basis as previously described.

Statistical analyses

The mean N content of each material was calculated by averaging the results obtained through the replicates. All statistical analyses were performed using a single N content for each study material within each N analysis method. The Dumas method was considered the standard procedure.

The similarity between the different Kjeldahl methods (dependent variables) and the Dumas method (independent variable) was evaluated through the adjustment of a linear regression model, according to the equation:

$$Y_i=\beta_0+\beta_1\times X_i+\varepsilon_i \qquad (2),$$

where Y_i = N content (g/kg as is) obtained through a Kjeldahl method in the study material i; β_0 = intercept; β_1 = slope; X_i = N content (g/kg as is) obtained through the Dumas method in the study material i; ε_i = random error assumed to be NIID $(0, \sigma^2_\varepsilon)$.

The model (2) was individually adjusted for each Kjeldahl method (i.e., KJ, KJT, and KJTS) and the following hypotheses were tested:

$$H_0: \beta_0 = 0 \text{ vs. } H_a: \beta_0 \neq 0 \qquad (3),$$

$$H_0: \beta_1 = 0 \text{ vs. } H_a: \beta_1 \neq 1 \qquad (4).$$

The methods were assumed to produce similar test results when both null hypotheses were not rejected. When at least one of the hypotheses above was rejected, the average N recovery of the Kjeldahl method was estimated through the adjustment of a linear regression model without intercept, according to the equation:

$$Y_i= \beta\times X_i+\varepsilon_i \qquad (5),$$

where Y_i = N content (g/kg as is) obtained through a Kjeldahl method in the study material i; β = slope or average N recovery (g/g); X_i = N content (g/kg as is) obtained through the Dumas method in the study material i; ε_i = random error assumed to be NIID $(0, \sigma^2_\varepsilon)$.

The comparison between the different Kjeldahl methods regarding the N contents in the study materials was performed by adjusting a linear regression model using Dummy variables (Draper and Smith, 1966) according to the equation:

$$Y_{ij}= \beta_{01}\times D_1+ \beta_{02}\times D_2+\beta_{03}\times D_3+ \beta_{11}\times D_1\times X_j+\beta_{12}\times D_2\times X_j+\beta_{13}\times D_3\times X_j+\varepsilon_{ij} \qquad (6),$$

where Y_{ij} = N content (g/kg as is) obtained through a Kjeldahl method i in the study material j; β_{0i} = intercept regarding the Kjeldahl method i (i = 1 for KJ, i = 2 for KJT, and i = 3 for KJTS); β_{1i} = slope regarding the Kjeldahl method i; X_j = N content (g/kg as is) obtained through the Dumas method in the study material j; Di = dummy variable associated with the Kjeldahl method i (for KJ, D_1 =1 and D_2 and D_3 = 0; for KJT, D_2 = 1 and D_1 and D_3 = 0; for KJTS, D_3 = 1 and D_1 and D_2 = 0); ε_{ij} = random error assumed to be NIID $(0, \sigma^2_\varepsilon)$.

The Kjeldahl methods were compared by using the following hypotheses:

$$H_0: \beta_{0i}- \beta_{0i'} = 0 \text{ vs. } \beta_{0i}- \beta_{0i'} \neq 0 \qquad (7),$$

$$H_0: \beta_{1i}- \beta_{1i'} = 0 \text{ vs. } \beta_{1i}- \beta_{1i'} \neq 0 \qquad (8),$$

where $i \neq i'$.

We declared that two Kjeldahl methods were different to each other when at least one of the null hypotheses above was rejected.

All statistical procedures were performed using the REG procedure of SAS 9.4. Statistical significances were declared at $P < 0.05$.

Results

The N contents of the study materials (Dumas method) varied from 3.1 to 26.5 g/kg as is (Table 1). The broad range of N content of the study materials indicates that the set of evaluated samples consisted of low- to high-quality forages, which in turn represents a necessary feature for the inference of N analysis methods. We found that all the methods were strongly and positively associated with each other ($P<0.01$; Table 2; Figure 1).

All the Kjeldahl methods produced N contents that differed from those obtained with the Dumas method ($P<0.01$; Table 3; Figure 1). The N recovery varied from 0.971 to 0.980 for the different Kjeldahl methods, averaging 0.974 g/g. We did not find any difference between the different Kjeldahl methods regarding the N contents in the study materials ($P\geq0.89$; Table 4).

Discussion

The N content in animal feeds is routinely obtained through two different core methods: the Kjeldahl distillation method and its variations, and the Dumas combustion method and its variations. The ammonia N and total organic N (e.g., amino-N, free amino-N, nucleic acids, and phospholipids) are supposed to be quantitatively recovered by the Kjeldahl method (Mariotti et al., 2008; Moore et al., 2010). On the other hand, in the combustion method, both organic and inorganic N compounds are converted to gases and reduced to N_2, which is quantified by thermal conductivity (Simonne et al., 1998). Due to its capability to quantify total N, the combustion method has been considered the standard method for N content quantification in different organic matrixes. In this sense, it is not expected a total N recovery when a Kjeldahl method is applied to feed matrixes, once this method and its different variations should quantify only the ammonia and the partially oxidized N compounds (i.e., N bounded to organic compounds). Highly oxidized chemical forms of N compounds (e.g., nitrate, nitrite) are not able to be reduced during the Kjeldahl digestion and, therefore, they could not be quantified. Several researchers have compared the N contents obtained by the combustion and Kjeldahl methods in plant materials (McGeehan and Naylor, 1988; Simonne et al., 1994, 1997; Watson and Galliher, 2001), and the results corroborated the incomplete N recovery of Kjeldahl method in different proportions according to the study materials, which agrees with the results obtained herein.

At least partially, the incomplete N recovery in our samples of tropical forages obtained by all the Kjeldahl methods could be associated with the nitrate-N present in plant materials (McGeehan and Naylor, 1988; Simonne et al., 1994; 1998; Watson and Galliher, 2001; Guo et al., 2007). For vegetal leaves from agricultural crops (e.g., tomato, cucumber) up to 0.25 g/g of N may not be quantified by Kjeldahl methods(Nelson and Sommers, 1980; Simonne et al., 1998). The proportion of N that was not quantified by the Kjledahl methods in our samples was less than 0.03 g/g, which is lower than the aforementioned values. However, the high amount of nitrate-N in plant materials from agricultural crops could be due to its natural higher nitrate accumulation during plant growth under high N soil availability (Du Preez and Bate, 1989), mainly in non-tropical regions. In tropical regions, the soils present a lower N availability. Thus, the nitrate-N content accumulated in tropical forages would tend to be lower than those observed by Nelson and Sommers (1980) and Simonne et al. (1998), which would represent a lower contribution of nitrate-N in total N in tropical forages compared to non-tropical agricultural crop materials.

Moreover, all Kjeldahl methods here evaluated produced similar values for N recovery. In disagreement with our expectations, the pre-treatment with salicylic acid before digestion did not affect the N recovery in tropical forages. The key chemical reaction is the nitration of aromatic compounds, in which nitrate from the test portion reacts with the phenolic group present in salicylic acid, resulting in 5-nitrosalicylic acid under acidic conditions (Buffler and Mühleis, 2018). The product of this reaction would be easily separated and quantified (Olah et al., 1982). However, it has been stated that this product could only be recovered after reduction by a compound like sodium thiosulphate, which reduces 5-nitrosalicylic acid into an amino group (Du Preez and Bate, 1989). In our study, sodium thiosulphate was not used, which probably contributed to the absence of the effect of salicylic acid on N recovery.

It has been stated that nitrate-N content alone could not be responsible for the incomplete N recovery of the Kjeldahl method (Simonne et al., 1998; Guo et al., 2007). Differences concerning feed matrix, the content of cyclic N compounds (e.g., nucleic acids), etc., may also contribute to incomplete N recovery (Simonne et al., 1998). Several researchers have suggested an improvement in N recovery when TiO_2 is added as a catalyst to the digestion mixture (Kane, 1986; Simonne et al., 1993; Buffler, 2017). The TiO_2 is non-toxic, which has positive implications for the analytical routine of the Keldahl method. In our study, the TiO_2 partially substituted the $CuSO_4$, which agrees with the recommendation of Buffler (2017). However, no improvement in N recovery was observed.

However, one positive aspect can be connected with TiO_2 utilization. When salicylic acid was used without TiO_2, the method variation showed to be unfeasible, as the intense foaming occurred during the initial phase of digestion, which triggered the solution to boil over the test tubes. Nevertheless, the utilization of salicylic acid along with TiO_2 allowed controlling the foam formation, which indicates a potential antifoaming effect of TiO_2, without any decrease in the N recovery. Some specific N analysis in animal feeds through the Kjeldahl method are known to present constraints associated with excessive foaming, such as neutral detergent insoluble N. In these cases, the partial replacement of copper sulfate with TiO_2 could be helpful to decrease the loss of test portions caused by excessive foaming and improve the feasibility of the Kjeldahl method.

Conclusions

The total N recovery in tropical forages when analyzed through the Kjeldahl method is 0.974 g/g. The average N recovery of the Kjeldahl method is not influenced by a pre-digestion step with salicylic acid or by the partial replacement of copper sulfate with titanium dioxide as a catalyst. However, titanium dioxide exhibits an antifoaming effect. This characteristic can be useful when study materials that present intense foaming during digestion are analyzed.

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Table 1. Descriptive statistics for the nitrogen content in the forage samples (g/kg as is) according to the analytical method

	Method[a]			
Item	D	KJ	KJT	KJTS
Mean	12.8	12.7	12.7	12.8
Minimum	3.1	3.3	3.2	3.4
Median	12.2	11.8	12.1	11.9
Maximum	26.5	24.7	24.7	24.7
s	6.10	5.46	5.50	5.46
n	48			

[a] D, Dumas method; KJ, Kjeldahl method; KJT, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide; KJTS, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide and addition of salicylic acid.

Table 2. Pearson´s linear correlations between nitrogen contents in the forage samples obtained through different analytical methods

	Method[a, b]		
Method	D	KJ	KJT
KJ	0.976	-	-
KJT	0.964	0.994	-
KJTS	0.978	0.999	0.994

[a] D, Dumas method; KJ, Kjeldahl method; KJT, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide; KJTS, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide and addition of salicylic acid.

[b] All correlations were different from zero ($P<0.01$).

Table 3. Characteristics of the regression model for the relationship between the nitrogen contents in the forage samples (g/kg as is) evaluated through the different Kjeldahl methods (dependent variable) and the Dumas method (independent variable)

	Complete model[a]						Reduced model[b]
Method[c]	Intercept	*P*-value	Slope	*P*-value	S_{xy}	r^2	Slope
KJ	1.53±0.403	<0.001	0.873±0.0285	<0.001	1.19	0.953	0.971±0.0138
KJT	1.57±0.493	0.002	0.871±0.0349	<0.001	1.46	0.931	0.971±0.0163
KJTS	1.62±0.383	<0.001	0.876±0.0271	<0.001	1.13	0.958	0.980±0.0135

[a] For details, see Equations (2), (3), and (4).

[b] For details, see Equations (5).

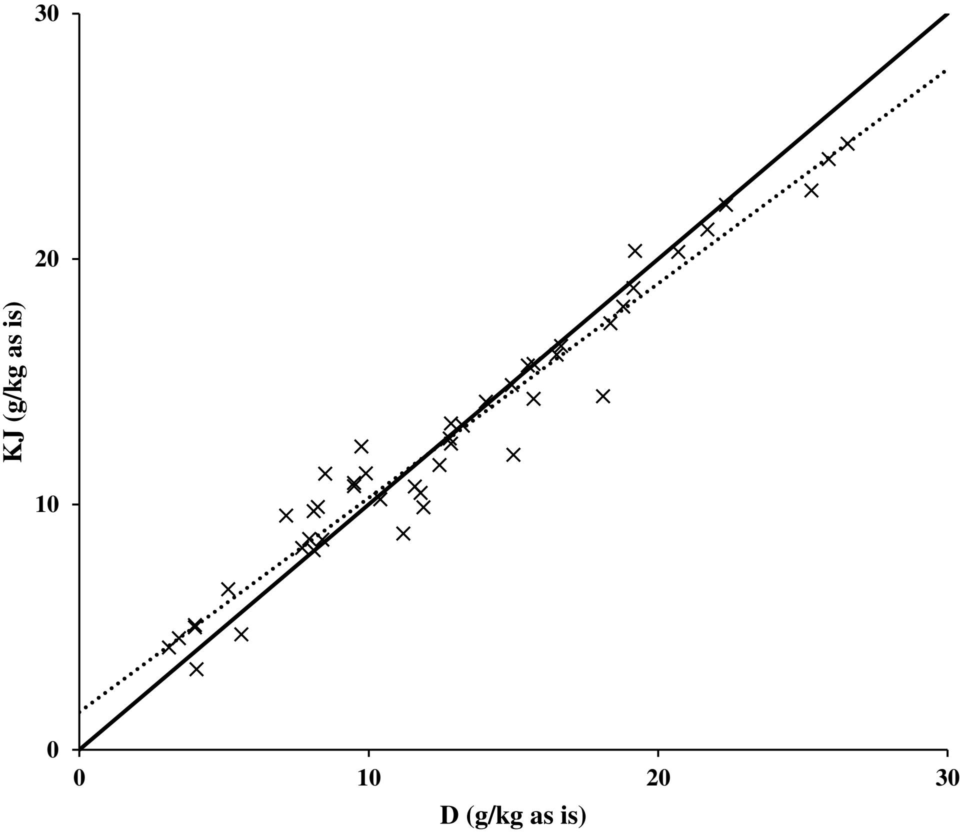
[c] KJ, Kjeldahl method; KJT, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide; KJTS, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide and addition of salicylic acid.

Table 4. Descriptive levels of probability (P-values) for the comparison between regression parameters for the relationship between the nitrogen contents in the forage samples evaluated through the different Kjeldahl methods (dependent variable) and the Dumas method (independent variable)

	Method[a, b]	
Method	KJ	KJT
	Intercept	
KJT	0.953	-
KJTS	0.930	0.930
	Slope	
KJT	0.945	-
KJTS	0.947	0.890

[a] KJ, Kjeldahl method; KJT, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide; KJTS, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide and addition of salicylic acid.

[b] For details, see Equations (6), (7), and (8).


KJ (g/kg as is)
D (g/kg as is)
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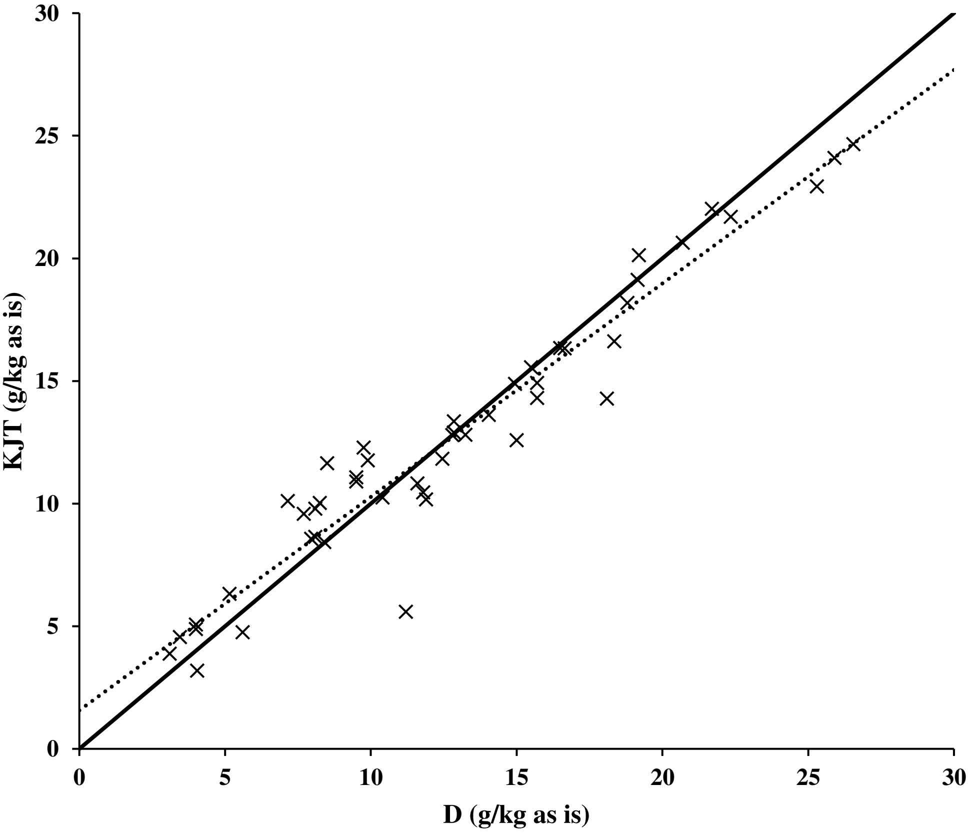
10
20
30
0
10
20
30


KJT (g/kg as is)
D (g/kg as is)
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15
20
25
30
0
5
10
15
20
25
30

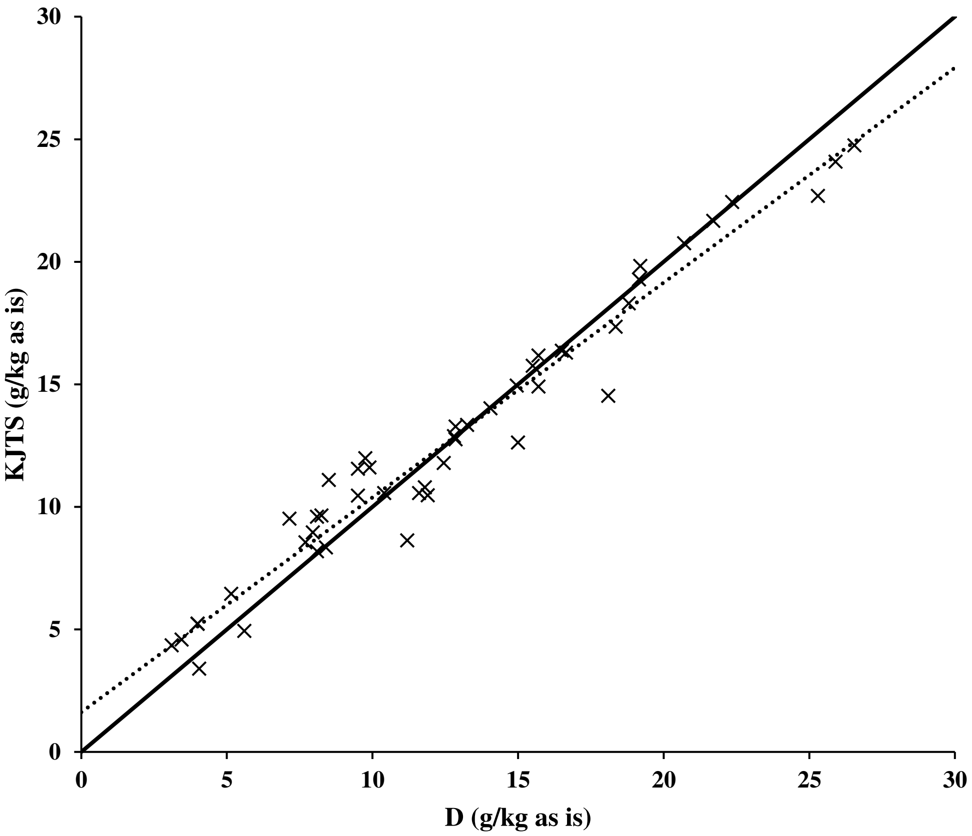

Figure 1. Relationships between the nitrogen contents in the forage samples (g/kg as is) evaluated through the different Kjeldahl methods and the Dumas method [D, Dumas method; KJ, Kjeldahl method; KJT, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide; KJTS, Kjeldahl method with the partial replacement of copper sulfate by titanium dioxide and addition of salicylic acid. The solid and dashed lines correspond to the equality line (Y = X) and least square straight line, respectively. For details, see Tables 3 and 4].

General Conclusions

Based on the results obtained in this thesis, we conclude:

1. The loss on drying procedures for the evaluation of laboratory dry matter produce inaccurate results when compared to the Karl Fischer titration. The biases are more prominent in high moisture materials and increase as the heating time increases at 105°C. However, when heating time is limited to 3 h, the bias in the water recovery and the post-drying residue browning are minimized. This method is also advantageous for mitigating energy expenditure and increasing the operational capacity of testing laboratories. Thus, 105°C 3 h should be applied to evaluate the laboratory dry matter in animal feeds;
2. The method of obtaining residues on ignition in animal feeds based on the binomial 550°C 3 h does not have sufficient robustness and may overestimate crude ash in some feeds. Adjustments in either ignition time or temperature appear to improve crude ash test results, but the best results are obtained using liquid ashing aids between two ignition cycles. The recommended method is based on the use of 550°C and two 3 h ignition cycles with water added to the ash residue between cycles; and
3. The total nitrogen recovery in tropical forages when analyzed through the Kjeldahl method is 0.974 g/g. The average nitrogen recovery of the Kjeldahl method is not influenced by a pre-digestion step with salicylic acid or by the partial replacement of copper sulfate with titanium dioxide as a catalyst. However, titanium dioxide exhibits an antifoaming effect. This characteristic can be useful when study materials that present intense foaming during digestion are analyzed.