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Comparative study of two analytical procedures for the determination of acid insoluble ash for evaluation of nutrient retention in broilers†

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Abstract

Inert markers are routinely used in research to estimate nutrient retention and apparent metabolisable energy nitrogen-corrected (AME_n) content of poultry diets. Acid insoluble ash (AIA) is used as a marker to substitute metal compounds because of environmental concerns. In the current research, two methodologies recommended for determining AIA content in feeds and excretas for the evaluation of total tract apparent retention (TTAR) of nutrients, were compared in 12 broiler diets. The experimental design was completely randomised with 2 AIA analytical techniques and 12 dietary treatments that resulted from a combination of two cereals (corn and rice), two heat processing of the cereal (raw and cooked) and three fiber sources (control with no added fibre, 3% oat hulls and 3% soybean hulls). All diets included 1% celite (diatomaceous earth) as additional source of AIA. The techniques used for AIA determination were coded as VO and GA, respectively. The TTAR of dry matter and organic matter and the AME_n of the feeds differed ($p \leq 0.001$) among diets and were lower when using GA than when using VO ($p \leq 0.05$). However, no interaction between diet and methodology was observed. Moreover, the TTAR of nutrients as determined by both techniques, were highly correlated ($r > 0.98$). We concluded that the GA methodology results in lower retention values than the VO methodology but that both methodologies can be used indistinctly to estimate TTAR of nutrients in broiler feeds.

Additional key words: analytical methodologies; inert markers; metabolisable energy; nutrient digestibility; poultry.

Resumen

Estudio comparativo de dos metodologías analíticas utilizadas para la determinación de cenizas insolubles en ácido para evaluar la retención de nutrientes en pollos de engorde

Los marcadores inertes se utilizan para estimar la retención de nutrientes y energía metabolizable aparente corregida por nitrógeno (EMA_n) en piensos. Las cenizas insolubles en ácido (CIA) son marcadores indigestibles utilizados en investigación animal en sustitución de compuestos metálicos para evitar problemas medioambientales. En este ensayo, para determinar la retención aparente de nutrientes y la EMA_n en pollos, se compararon dos metodologías recomendadas para la determinación del contenido de CIA en piensos y excretas. El diseño experimental fue completamente al azar con dos técnicas analíticas para la determinación de CIA y 12 tratamientos experimentales que consistían en la combinación de dos cereales (maíz y arroz), dos procesados térmicos del cereal (crudo y cocido) y tres fuentes de fibra (control sin fibra añadida, 3% cascarilla de avena, y 3% cascarilla de soja). Las técnicas usadas para la determinación de CIA fueron codificadas como VO y GA, respectivamente. La retención aparente de la materia seca, materia orgánica y EMA_n de los piensos variaron ($p \leq 0{,}001$) con el tipo de pienso y fueron inferiores ($p \leq 0{,}05$) con la metodología GA que con la metodología VO. Sin embargo, no se observó interacción entre tipo de pienso y técni-

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ca experimental. Además, la retención aparente de los nutrientes obtenida mediante ambas técnicas estuvo altamente correlacionada ($r > 0,98$). Se concluye que la metodología GA proporciona valores de retención de nutrientes inferiores a los obtenidos con VO, aunque ambas pueden ser utilizadas indistintamente para estimar la digestibilidad fecal de nutrientes en avicultura.

Palabras clave adicionales: energía metabolizable; marcadores inertes; metodologías analíticas; pollos; retención de nutrientes.

Introduction

Inert markers are routinely used in nutrient digestibility studies to eliminate the need of total collection of the excreta produced (Sales and Janssens, 2003a). Chromic oxide (Cr_2O_3) has been the main inert marker used in poultry studies for years (Saha and Gilbreath, 1993; Lázaro *et al*., 2003b). However, Cr_2O_3 might interfere with other minerals present in the diet (Saha and Gilbreath, 1991a) resulting in low fecal recovery rates of Cr. Saha and Gilbreath (1991b) in swine, Hill *et al*. (1996) in dogs and Titgemeyer (1997) in ruminants, have reported recovery rates of Cr of only 93.3%, 87.0% and 94.0%, respectively. In fact, Lázaro *et al*. (2003b) showed an incomplete recovery of the Cr in broilers fed rye-based diets, with values varying between 86.4 and 92.3. The voiding of Cr in feces is not uniform (Prigge *et al*., 1981) with substantial variability in diurnal excretion patterns. In addition, Cr is a heavy contaminant of the environment (Sprinkle *et al*., 1995) and has carcinogenic properties (Peddie *et al*., 1982). Thus, the use of Cr_2O_3 in nutritional research is no longer recommended (Titgemeyer *et al*., 2001). Problems have been observed also with the use of titanium dioxide (Rymer, 2000; Kavanagh *et al*., 2001), as a marker. For example, Yin *et al*. (2000) reported that the recovery of titanium in pigs fed diets high in fibre was very low, ranging between 60.6 and 67.8%, a variability which was probably related to segregation of the marker. Acid insoluble ash (AIA) is a natural alternative to mineral external markers which has shown mean recovery rates close to 100% in several species (Sales and Janssens, 2003a,b). Scott and Boldaji (1997) reported that AIA provided a more accurate estimate of the feeding value of barley in growing chickens than Cr_2O_3. Acid insoluble ash occurs naturally in feed ingredients (McCarthy *et al*., 1974) but the amount of AIA in the final feed is usually low, which makes convenient the inclusion of extra amounts of the marker in the diet to increase the accuracy of the chemical analysis (van Leeuwen *et al*., 1996; Gracia *et al*., 2003). Two assays recommended for the determination of AIA in animal digestibility studies are those reported by Vogtmann *et al*. (1975) and Van Keulen and Young (1977). The Vogtmann *et al*. (1975) technique (VO) has been successfully used to estimate nutrient digestibility in non-ruminant (Batal and Parsons, 2002; Valencia *et al*., 2008, 2009) whereas the Van Keulen and Young (1977) technique (VK) has been used more in research conducted with ruminants (Thonney *et al*., 1979; Sunvold and Cochran, 1991). The VK technique analyses and obtains sequentially, the dry matter (DM), total ash and AIA content of the sample. Consequently, less amount of sample is required. Recently, González-Alvarado *et al*. (2007) (GA) recommended to introduce some modifications to the VK methodology when used in poultry digestibility studies. The technique of GA has been used consistently in recent studies (De Coca-Sinova *et al*., 2008; Jiménez-Moreno *et al*., 2009; González-Alvarado *et al*., 2010). However, no data have been generated comparing the VO and GA methodologies in broilers. This research was conducted to compare two techniques used in research to estimate AIA content of diets and excretas and nutrient retention in broiler fed diets varying widely in ingredient composition.

Material and methods

Experimental diets, husbandry and experimental design

The ingredient composition and nutritive value of the 12 experimental diets used have been reported elsewhere (González-Alvarado *et al*., 2007) and are shown in Table 1. The diets were based on cereals and soy

Abbreviations used: AIA (acid insoluble ash); AME_n (apparent metabolisable energy nitrogen-corrected); CV (coefficient of variation); DM (dry matter); GA (González-Alvarado *et al.* [2007] technique]; N (normal); OM (organic matter); TTAR (total tract apparent retention); VK (Van Keulen and Young [1977] technique); VO (Vogtmann *et al*. [1975] technique).

Table 1. Ingredient and chemical composition of the experimental diets (% as fed-basis, unless otherwise indicated)

	Amount
Ingredient	
Cereal[1]	60.00
Soy protein concentrate, 53% CP[2]	21.96
Fish meal, 72% CP	7.00
Soybean oil	3.80
Sepiolite[3]	3.00
L-Lysine-HCl, 78%	0.10
DL-Metionine, 99%	0.19
Limestone	1.12
Dicalcium phosphate	1.40
Sodium chloride	0.23
Celite[4]	1.00
Vitamin and mineral premix[5]	0.20

Chemical analyses	Corn[6]			Rice[6]		
	Control	OH[7]	SH[8]	Control	OH	SH
Calculated[9]						
ME, kcal kg^{-1}	3,067	3,079	3,091	3,127	3,139	3,150
Crude fibre	2.5	3.3	3.4	1.5	2.3	2.4
Digestible Lysine	1.18	1.18	1.18	1.22	1.22	1.22
Ca	1.00	1.00	1.00	1.00	1.00	1.00
Available P	0.41	0.41	0.41	0.39	0.39	0.39
Determined[10]						
Gross energy, kcal kg^{-1}	3,980	4,118	4,116	3,969	4,078	4,048
Dry matter	90.6	90.8	90.5	90.8	91.1	91.1
Crude protein	21.1	21.6	21.7	21.0	21.4	21.3
Ether extract	6.3	6.6	6.5	4.8	5.1	5.1
Total ash	9.1	6.6	6.9	9.2	6.4	6.2

[1] The cereal used was corn or rice, either raw or heat processed according to treatment. [2] Crude protein. [3] Sepiolite, a complex magnesium silicate clay, was substituted (w/w) by either oat hulls or soybean hulls in the corresponding experimental diets. [4] Acid washed diatomaceous earth (Celite Corporation, Lompar, CA). [5] As indicated by González-Alvarado *et al.* (2007). [6] Means of raw and cooked cereal. [7] Oat hulls. [8] Soy hulls. [9] According to FEDNA (2003). [10] Analysed in duplicate samples.

protein concentrate and resulted from the combination of two cereal sources (corn and rice), two heat processing of the cereal (raw *vs*. cooked) and three fibre sources (control with no additional fibre and 3% of either oat hulls or soy hulls). The fibre sources were included in the diets at expenses (w:w) of sepiolite, a complex magnesium silicate clay added as inert material. Acid washed diatomaceous earth was included as additional inert marker in all the diets. The diets were fed in mash form on *ad libitum* bases to chicks from 1 to 18 days of age.

All procedures used in this research were in compliance with the Spanish Guidelines for the Care and Use of Animals in Research (BOE, 2005). Details on husbandry and care of chicks have been reported elsewhere (González-Alvarado *et al*., 2007). Briefly, a total of 576 one day old male chicks (Cobb 500) were randomly placed in groups of 16 in 36 battery cages (3 cages per treatment). The trial was conducted as a completely randomized design with 12 diets arranged factorially with the two analytical methodologies and the 12 dietary treatments previously described.

Laboratory analyses and acid insoluble ash determination

Details on the analytical procedures used to determine the chemical composition and the nutritive value of diets and excreta have been reported elsewhere (González-Alvarado *et al*., 2007). The AIA of diets and excreta were determined in triplicate as indicated by VO and GA. Both techniques are based on the original analytical method described by Shrivastava and Talapatra (1962) and differ in 1) size of sample (12 g for diet and 5 g for excreta *vs.* 5 g for both diet and excreta), 2) concentration of HCl (4 N *vs.* 2 N), 3) boiling time (30 min *vs.* 5 min), 4) ashing after acid treatment *vs.* before and after acid treatment), 5) duration and temperature applied for ashing of the sample (4 h at 600°C *vs.* 12 h at 600°C for the first ashing and 6 h at 450°C for the second ashing) and 6) number of analysed variables (AIA exclusively *vs.* sequential determination of DM, ash and AIA) (Table 2). Briefly, for the VO methodology, ground samples of feed (12 g) and excreta (5 g) were boiled in a 200 mL Erlenmeyer flask with 100 mL of 4 N HCl that was fitted with a reflux condenser to prevent any HCl loss for 30 min. The slurry was then filtered through ash free filter paper (Whatman Nº 541) and the residue washed hot distilled water (85 to 100°C) until free of acid. Then, the residue was transferred to a tared crucible, dried at 103°C for 48 h and ashed at 600°C for 4 h. After ashing, the crucible was cooled in a desiccator to room temperature and weighed. For the GA methodology, ground samples of feed and excreta (5 g) were dried at 103°C for 24 h in a 100 mL Erlenmeyer flask for DM determination. Then, the dried sample was ashed at 600°C for 12 h for crude ash determination. Afterwards, ashes were hydrolyzed with 40 mL of 2 N HCl, boiled for 5 min and the obtained residues were filtered through ash free filter paper (Whatman Nº 541) and washed until free of acid with hot distilled water (85 to 100°C). The ashes retained in the paper filter were transferred into the same Erlenmeyer flask and dried at 103°C for 48 h. Finally, the ash and paper filter were ashed again at 450°C for 6 h. The flask and its content were cooled in a desiccator to room temperature and weighed to calculate the AIA content.

Total tract apparent retention of dietary components

At 18 days of age of the birds, representative samples of excreta produced during the previous 24 h were collected by replicate, mixed, homogenized, oven-dried (60°C for 72 h) and ground with a hammer mill (Model Z-I, Rechst, Stuttgart, Germany) fitted with a 1-mm screen. Total tract apparent retention (TTAR) of DM, organic matter (OM), nitrogen and ether extract, and the apparent metabolisable energy nitrogen-corrected (AME_n) of the diets were estimated according to VO and GA methodologies using AIA as a marker. The TTAR of nutrients was calculated by the following equation:

$$TTAR = 1 - [(Nutrient_e/Nutrient_f) \times (AIA_f/AIA_e)] \times 100$$

where $Nutrient_e$ and AIA_e are the concentrations of dietary components and of AIA in the excreta, respectively, and $Nutrient_f$ and AIA_f represent the concentrations of the same dietary components and the AIA in the feeds, all of them expressed on g kg^{-1} of DM. The AME_n content of the experimental diets was calculated as indicated by Lázaro *et al*. (2003a).

Statistical analysis

All data were analysed as a completely randomised design with treatments arranged factorially with two analytical methodologies and 12 diets using the GLM procedure of SAS (SAS Institute, 1990). The main effects of diet and methodology as well as the interaction between diet and methodology on AIA content in excreta and TTAR of nutrients were evaluated. When significant differences were detected, treatment means were separated using the Tukey's test. The experimental unit was the cage for all traits and differences among

Table 2. Analytical conditions applied for the determination of acid insoluble ash (AIA) in feed and excreta

Methodology	VO (Vogtmann *et al.*, 1975)	GA (González-Alvarado *et al.*, 2007)
Feed, g	10-12	5
Excreta, g	5	5
HCl, Normality	4	2
Boiling time, min	30	5
Determinations in individual samples	AIA	Dry matter, total ash and AIA using the same beaker
Ashing temperature[1],°C	600	600/450
Ashing time[2], h	4	12/6

[1] Ashing temperatures after digestion were 600°C for VO and 600 and 450°C before and after digestion, respectively for GA.
[2] Ashing times after digestion were 4 h for VO and 12 and 6 h before and after digestion, respectively for GA.

treatments were considered significant at $p \leq 0.05$. In addition, the REG procedure of SAS (SAS Institute, 1990) was used to determine linear least squares regressions to provide equations between AIA content of the excreta as determined, and between AIA and total ash and gross energy contents of the excreta by the VO and GA methodologies. The correlation coefficients (r) were also calculated. In addition, the CORR procedure of SAS (SAS Institute, 1990) was used to determine the Pearson's correlation coefficient between TTAR of nutrients and AME_n of the diets as estimated by the two methodologies.

Results and discussion

The AIA content of the excreta differed ($p \leq 0.001$) among diets and was lower ($p \leq 0.001$) when determined by the GA technique than when determined by the VO technique (Table 3). The differences observed in the AIA content of the experimental diets reflected differences in HCl-insoluble ash content of the ingredients used. The main reason for the higher AIA content of the control diets without fibre supplementation was the inclusion of 3% sepiolite in these diets. The negative correlation between AIA and gross energy, and positive between AIA and total ash observed with both methodologies (–0.972 and 0.974 for VO and –0.972 and 0.970 for GA), is confirmed by the Pearson's coefficients values obtained (Table 4). These results suggest that the concentration of HCl used might affect the AIA determination with lower values when lower HCl concentration was used. However, Furuichi and Takahashi (1981) did not find any significant difference in AIA concentration in feeds and faeces of rabbits when the boiling process was performed using 2, 4 or 6 N HCl. Similarly, Bergero *et al*. (2009) did not find any difference in AIA values in feeds and faeces of horses when using 2 N or 4 N HCl. However, Van Keulen and Young (1977) reported in sheep that the use of 4 N HCl provided higher ($p \leq 0.05$) estimates of nutrients digestibility than the use of 2 N HCl. This information suggests that in addition to the concentration of the HCl used, other factors, such as the characteristics of the excreta of the different animal species, could affect the effects of HCl on AIA recovery. When the GA technique is used, samples of diets and excreta are ashed for longer time than when the VO technique is used.

Table 3. Acid insoluble ash content (AIA, % dry matter) in feeds and excreta as determined by VO and GA methodologies[1] in broilers at 18 days of age

Cereal	HP[2]	Fibre inclusion	Diet		Excreta	
			VO	GA	VO	GA
Corn	Raw	—	2.938	2.840	10.286	9.916
	Raw	3% OH[3]	1.275	1.232	4.537	4.265
	Raw	3% SH[4]	1.266	1.238	4.221	4.063
	Cooked	—	2.843	2.747	10.419	9.947
	Cooked	3% OH	1.233	1.188	4.632	4.429
	Cooked	3% SH	1.146	1.125	4.252	4.064
Rice	Raw	—	3.004	2.833	12.521	12.079
	Raw	3% OH	1.012	0.975	5.607	5.236
	Raw	3% SH	1.061	1.023	5.029	4.742
	Cooked	—	2.941	2.903	11.476	11.111
	Cooked	3% OH	1.191	1.148	5.900	5.542
	Cooked	3% SH	1.069	1.065	4.650	4.471
		Mean	1.748	1.693	6.961	6.655
SEM[5]					0.106	
Coefficient of variation, %					2.7	
Source of variation					Probability	
Methodology					0.0001	
Diet					0.0001	
Methodology × Diet					0.8874	

[1] See Table 2. [2] Heat processing (60 min at 104 ± 3°C for corn and 45 min at 90 ± 3°C for rice). [3] Oat hulls. [4] Soy hulls. [5] Standard error of means (3 replicates of 16 chicks each per treatment).

Table 4. Regression equations between acid insoluble ash (AIA, % dry matter) and ash and gross energy (GE) contents of the excreta according to the methodology[1] used

Equation	r	SE_b[2]	RSD[3]	p-value
Methodology VO				
AIA = 0.643 × Ash – 8.991	0.974	± 0.0258	± 0.7190	0.0001
AIA = –0.015 × GE + 63.561	–0.972	± 0.0006	± 0.7458	0.0001
Methodology GA				
AIA = 0.624 × Ash – 8.816	0.970	± 0.0265	± 0.7390	0.0001
AIA = –0.015 × GE + 61.740	–0.972	± 0.0006	± 0.7224	0.0001

[1] See Table 2. [2] Standard error of estimated slope. [3] Residual standard deviation.

Therefore, the conditions applied when using the GA technique might be better adapted to all types of excreta than the VO technique, ensuring the complete ashing of the organic material contained in the original samples. For AIA content of the excreta the regression equation between the two techniques was: y = 0.972x (± 0.0066) – 0.112 ($R^2 = 0.998$; residual standard deviation = 0.1219; $p < 0.0001$) where y and x were the percentage of AIA in the excreta on DM basis using the GA and the VO technique, respectively (Fig. 1). The correlation coefficient for AIA content of the excreta between VO and GA techniques was high ($r = 0.999$) suggesting that both techniques can be used indistinctly for the determination of AIA and the estimation of nutrient retention in broiler studies.

Nutrients digestibility differed among diets and in general was higher with diets containing 3% oat hulls than with diets containing 3% soy hulls (Table 5). Similarly, nutrient retention values were higher when the VO methodology was used. However, no interactions between methodology and diet were detected for any of the variables studied. The TTAR of nutrients as determined by both techniques was highly correlated ($r > 0.98$; data not shown). The TTAR of DM, OM and the AME_n of the diets were lower ($p \leq 0.05$) with the GA than with the VO technique. The CV values of CTTAR of DM and OM and of AME_n of the diets were low (0.92%, 1.01% and 0.71%, respectively) for both methodologies. However, for ether extract (1.20%) and especially for total nitrogen retention (4.43%), the CV values were less acceptable. Probably, these high CV values precluded the detection of significant differences between methodologies for such fractions (ether extract and nitrogen).

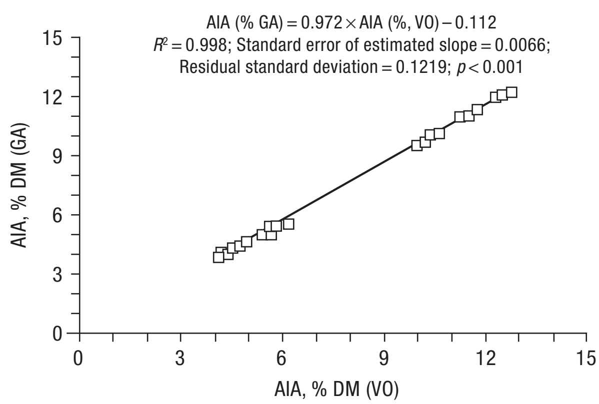

Figure 1. Relationship between acid insoluble ash content of the excreta [AIA, % of dry matter (DM)] as obtained by the VO (Vogtmann *et al.,* 1975) and the GA (González-Alvarado *et al.,* 2007) methodologies.

The sequential analysis of DM, total ash and AIA content, as recommended by González-Alvarado *et al.* (2007), minimizes sample losses that inevitably occur when analysis is not performed using the same flask. Therefore, GA might be the technique of choice for estimating AIA when the size of the sample is very small. Thus, the GA methodology might have advantages over the VO methodology in ileal digestibility studies carried out in chicks, in which AIA and nutrient contents have to be determined in the digesta from different intestinal segments of individual birds and in which the amount of collected sample is generally small. Under these circumstances, the GA technique allows to analyse more variables per sample or to perform more analyses per each variable of interest. Therefore, the GA technique might improve the assessment of the feeding value of ingredients or diets, over the VO technique.

We conclude that nutrient retention values are higher when the VO methodology is used in comparison to the GA methodology. However, both techniques are highly correlated and can be used indistinctly for efficient quantification of AIA in comparative studies on nutrient retention in broilers. Moreover, the GA me-

Table 5. Total tract apparent retention (%) of nutrients and apparent metabolisable energy nitrogen-corrected (AME_n, kcal kg^{-1}) of the diets as estimated by VO and GA methodologies[1] in broilers at 18 days of age

Cereal	HP[2]	Fibre inclusion	Total tract apparent retention								AME_n	
			Dry matter		Organic matter		Nitrogen		Ether extract			
			VO	GA	VO	GA	VO	GA	VO	GA	VO	GA
Corn	Raw	—	71.4	71.4	77.7	77.5	69.7	69.6	81.5	81.4	2,872	2,870
	Raw	3% OH[3]	71.9	71.1	75.9	74.9	67.4	66.6	84.3	83.9	2,904	2,881
	Raw	3% SH[4]	70.0	69.5	74.2	73.6	64.6	64.0	84.4	84.1	2,879	2,863
	Cooked	—	72.7	72.4	79.0	78.4	70.0	69.6	84.9	84.8	2,976	2,967
	Cooked	3% OH	73.4	73.1	77.3	76.8	69.0	68.8	87.4	87.4	3,066	3,062
	Cooked	3% SH	73.1	72.3	76.8	76.0	67.1	66.2	85.9	85.5	3,025	3,001
Rice	Raw	—	76.0	76.5	81.9	82.3	66.4	67.3	85.7	86.1	3,065	3,081
	Raw	3% OH	82.0	81.4	85.3	84.8	76.6	76.1	90.3	90.1	3,264	3,253
	Raw	3% SH	78.8	78.4	82.3	81.9	69.8	69.3	87.9	87.7	3,131	3,121
	Cooked	—	74.4	73.9	80.3	80.0	66.2	65.7	85.5	85.3	2,988	2,978
	Cooked	3% OH	79.8	79.3	83.4	82.9	75.1	74.8	90.6	90.4	3,165	3,156
	Cooked	3% SH	77.0	76.2	80.5	79.7	67.9	66.8	87.4	87.0	3,056	3,033
Mean			75.0^{A}	74.6^{B}	79.5^{A}	79.1^{B}	69.1	68.7	86.3	86.1	3,033	3,022
SEM[5]			0.40		0.47		1.76		0.60		12.4	
Coefficient of variation, %			0.92		1.01		4.43		1.20		0.71	
Sources of variation			Probability									
Methodology			0.0159		0.0150		0.5712		0.4563		0.0441	
Diet			0.0001		0.0001		0.0001		0.0001		0.0001	
Methodology × Diet			0.9361		0.9881		1.0000		1.0000		0.9455	

[1] See Table 2. [2,3,4,5] See Table 3. [A,B] Means values within a row not sharing a common superscript differ ($p \leq 0.05$).

thodology requires less time and effort and less sample size to perform the same number of analytical measurements per sample. Therefore, this technique may be more convenient in nutrient ileal digestibility studies than the VO technique.

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