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Colorimetric method as alternative to chromium (III) quantification in cattle feces

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ABSTRACT. One of the main factors to establish productivity of grazing cattle is the estimation of forage intake. For this, the most widely used technique is based on the estimation of fecal output using chromium dioxide as external marker. However, quantification can be expensive and sometimes not precise due to the methodology used for this purpose. Therefore, the aim of this study was to validate the colorimetric method for chromium quantification and to implement it in the estimation of fecal output in grazing cattle. The temperature, the digestion time and the wavelength for the measurement were evaluated. The method was validated for selectivity, linearity, detection and quantification limits, precision, accuracy, and stability. Results showed that temperature and digestion time are critical to improve sensitivity and quantification limits. The validation demonstrated that the method is suitable for the quantification of Cr_2O_3 in a wide range of concentrations, being statistically comparable with a reference method, and offering a reliable low cost and easy to implement alternative, to estimate fecal output in bovine digestibility studies.

Keywords: spectrophotometry; fecal output; external marker; chromium dioxide; validation.

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Introduction

The colorimetric method established by Fenton and Fenton (1979) for the quantification of chromium in feces of ruminants has been widely used in the determination of total fecal production and the estimation of digestibility in different animal species. However, the method contains some inaccuracies in the measurement conditions, so it has been questioned regarding its sensitivity (Souza et al., 2013) and poor recovery of the analyte with respect to reference methods (Cabral et al., 2017). Since the method consists in incinerating stool samples with Cr_2O_3 content, which is subsequently taken to an acid digestion process until the development of a yellow or reddish color occurs, the conditions to maximize the absorption are vital to the desired quantification. Conversely, one of the main interferences in colorimetric measurements is the content of organic matter (Li & Hur, 2017), so incineration, digestion, and wavelength values chosen to carry out analyses, are critical components in the quantification of analytes (Shrivastava & Gupta, 2011). The importance of carrying out adequate incineration prior to digestion is related to the high temperature and the reactivity of the free chlorine formed in the final solution, therefore, has been suggested that the ashes are the best way to extract refractory metals like chromium (Hseu, 2004).

It was also found that the Cr quantification methods by colorimetry, in which sulfuric and perchloric acids are used, recover the greatest amount of the analyte present in organic samples (Rocha, Palma, Detmann, & Filho, 2015), meanwhile, the use of nitric acid and nitric-perchloric acid did not differ significantly in terms of Cr recovery in feces (Hseu, 2004). Since the digestion temperature must favor the complete oxidation of Cr_2O_3 and ensure the acid proportion in the solution to achieve the formation of the yellow ion $Cr_2O_7^{-2}$, for this analysis, the anionic polymerization characterized by the reddish color should be avoided. In the case of the quantification of Cr with the colorimetric method, there is an additional factor that could influences the color formation mediated by sodium molybdate ($Na_2MoO_4.2H_2O$). In this sense, it has been reported that molybdenum (Mo) acts as a catalyst for the digestion process (Rocha et al., 2015). On the other hand, the spectrophotometric readings for this method are generally carried out assuming that the

specie formed is $Cr_2O_7^{-2}$ with the reference λ of maximum absorption in the visible at 440 nm. However, different investigations have reported better sensitivity between 350 and 360 nm with differences in the spectral bands due to the pretreatment of the samples and other important bands due to the speciation of Cr, attributed to kinetic conversion, oxidation conditions, and composition of the matrix or acids present in the medium (Szabó et al., 2018). The photometric accuracy standards of $K_2Cr_2O_7$, in general, are performed mainly at 440 nm but readings from 430 to 445 nm have been used to quantify Cr_2O_3 despite the significant spectral differences due to speciation. From the λ tipically used, none is specific for the quantification of the colored species in the reaction with Mo (IV).

Taking this into account, it is necessary to adjust different sample preparation conditions so that the process can be optimized to improve the sensitivity and accuracy of the method in forage intake estimation. From this point, there is a need to reevaluate each of the steps within the procedure, to detect its shortcomings and implement improvements that offer a standardized and reliable method for the quantification of Cr in feces. Therefore, the aim of this research was to establish the optimum digestion conditions for the colorimetric quantification of Cr to have a standardized and validated method for the parameters of selectivity, linearity, intermediate precision, accuracy and stability, as well as to evaluate the effectiveness of this method in the bovine fecal output compared to a conventional atomic absorption method.

Material and methods

Chromium dioxide (Cr_2O_3, Merck, 99%) was used as a marker for animals and for standard laboratory solutions. The digester mixture was prepared with H_2SO_4 (98%, Merck), $HClO_4$ (70%, Panreac) and distilled water in a 3: 3: 4 ratio, respectively, with the addition of 0.02 kg L^{-1} of $Na_2MoO_4.2H_2O$ (99%, Sigma Aldrich). As an analyte-free matrix (H_0), feces taken from six multiparous Holstein cows not dosed with the marker were employed; the animals were maintained in the "Paysandú" Agricultural Station of Universidad Nacional de Colombia, Medellin. The study farm is located at 2,600 m a.s.l., with an average temperature of 14ºC, average precipitation of 2,500 mm and an average relative humidity of 80%; the area is located in the ecological formation bh-MB according to Holdridge's classification. As a reference material, portions of 5.0 g of the free matrix (H_0) enriched with Cr_2O_3 were taken so that their nominal concentration was approximately 15 mg of Cr kg^{-1} of dry matter (DM). For the interference analysis, TiO_2 was used (99%, Panreac).

In order to find the critical parameters, all samples of the reference material and standards were analyzed with the original conditions; furthermore, these were also analyzed with changes in the conditions of sample incineration, temperature and time digestion for the optimization of the Fenton and Fenton method (1979). For the temperature tests, standards of 0.03 g of Cr_2O_3 were taken to Kjeldahl digestion tubes with 15 mL of the mixture of acids and Mo in a digester with raising temperature control. Digestion of each standard was performed at 250, 280 and 300°C, each by triplicate for 15 minutes. The solutions were cooled to room temperature, transferred to 200 mL balloons and filled with distilled water. Then they were centrifuged at 2,000 rpm for 4 minutes and working solutions were prepared with the supernatant. Absorption spectra were taken from 220 to 700 nm with a resolution of 1 nm using quartz cells. The experiment was repeated for a digestion time of 30 minutes. Qualitative spectral analysis and comparison of the signal obtained at different wavelengths reported as a reference were performed. All the spectra were registered using the digester solution as a blank and undergoing the same process.

The matrix effect was evaluated by the standard addition method (Brown & Mustoe, 2014). For this, calibration curves were performed in five levels (k= 2, 6, 12, 15 and 24 mg of Cr kg^{-1} of DM) with standard Cr_2O_3 and calibration curves on the reference material (H_0) enriched with the analyte at the same levels. In the first experiment, both were treated according to the Fenton methodology. For the second experiment, digestion was carried out at 250°C for 30 minutes. The incineration temperature of the enriched matrix was 650°C for 12h (Falk-Windisch, Svensson, & Froitzheim, 2015). A variance analysis was performed between the calibration lines of each experiment with a two-tailed *F* test at a confidence level of 95% and n-2 degrees of freedom (Eq. 1), followed by a significance test for slopes with n_1+n_2-2 degrees of freedom (Eq. 2). The parameters of the regression were calculated using the 'stats' package version 3.5.0 of the R Studio software.

$$F_{calc} = \frac{S_{b1}^2}{S_{b2}^2} \qquad (1)$$

$$t_{calc} = \frac{|b_1 - b_2|}{\sqrt{S_{b1}^2 + S_{b2}^2}} \qquad (2).$$

For stock solutions, standard, analyte-free matrices and reference material were dried at 60°C in a forced air convection oven and milled with a 1 mm sieve (Fritsch Idar Oberstein®-Germany). Subsequently, these were subjected to direct incineration for 12 hours at 650°C in a Terragen muffle with temperature control. The ashes were transferred to Kjeldahl type tubes with the acid mixture and digested for 30 minutes at 250°C. The tubes were allowed to cool to room temperature and the content was transferred to volumetric balloons, filled with distilled water and homogenized with a vortex at 2,000 rpm. Aliquots of 15 mL were placed in Falcon® tubes and taken to centrifugation for 4 minutes at 2,000 rpm. The supernatant solution was taken to quartz cells and the readings were made at 362 nm in a UV-line 900 (Schott) spectrophotometer. The stock solutions were prepared independently for each trial, transferring 0.03 g of Cr_2O_3 to porcelain pots subjected to the same procedure as the samples. Subsequently, they were taken to a final volume of 200 mL. For working solutions, successive dilutions were made from the stock solution using Handystep® electronic micropipettes with sterile disposable tips. All solutions were prepared daily.

The modified method for the determination of Cr in feces was validated for the parameters of selectivity, linearity, intermediate precision, accuracy, limits of detection, quantification, and stability. The selectivity was tested through the study of maximum absorption found in the absorption spectra between 200 and 700 nm in order to confirm that there are no interferences associated with the matrix or the possible presence of the Ti marker in test samples. For this, the recovery of Cr was evaluated in six reference material samples with a nominal concentration of 15 mg kg^{-1} of Cr and enriched with the same concentration of Ti (HCr-Ti), The Cr recovery was evaluated by an *F*-test of equality of variances between groups (with and without Ti enrichment), assuming there are no significant differences between them (H_0: $\sigma_1^2 = \sigma_2^2$). To establish if the variation found corresponded to random errors, a *t*-test of significance was made for inequality variances under the same null hypothesis.

The linearity was evaluated in five levels (3.0, 6.0, 12.0, 24.0 and 30 mg Cr kg^{-1}), each in triplicate, by an ANOVA for the regression, using the *t* statistic for the evaluation of the slope (b_w) and the intercept (a_w) with a degree of significance α = 0.05. The limits of detection (LOD) and quantification (LOQ) were calculated from the parameters of the regression model (Eq. 3 and 4)

$$LOD = \frac{3 * S_y}{b_w} \qquad (3)$$

$$LOQ = \frac{10 * S_y}{b_w} \qquad (4)$$

The intermediate precision was evaluated between analysts and days for three different stool samples (H_0), enriched with Cr individually at three concentration levels corresponding to 80, 100 and 120% of the expected central value in the test samples according to the concentration radius for Cr in bovine feces from animals that have been dosed with amounts of marker between 10 and 20 g Cr_2O_3 day^{-1}, each in duplicate. The procedure was repeated by each analyst on five different non-consecutive days (p=5). It is established that the method is accurate for an RSD >5%. For repeatability, the acceptance criterion is that the values are within the range $\overline{X} \pm tS/\sqrt{n}$, where $\overline{X}$ is the average of the series of results obtained at the same level, with *t* for n-1 degrees of freedom, α = 0.05 and *S* the standard deviation. The accuracy was evaluated as the percentage of recovery of Cr in three different samples of reference material with a nominal concentration of 15.73, 16.33 and 15.43 mg Cr kg^{-1} of DM, each in duplicate. The calculation of the Cr concentration in the reference material was made with the linear regression model. For the recovery analysis, an ANOVA was used for unequal variances ($p < 0.05$) and a *t*-test, taking as a null hypothesis that the difference between the means is zero at the 95% confidence level. All the validation analyzes were carried out using the Statistica® software version 7.1.

The stability test of the solutions was carried out for six samples of H_0 with contents of 15 and 25 mg/kg of Cr stored at 4ºC during 180 days in predefined periods of time (1, 30, 60 and 180 days). It was evaluated whether there was a significant difference in the absorption over time with Tukey's multiple HSD comparison test for a level of significance of 95% (Hayden, 2012) employing the algorism from the package 'stats' version 3.5.0 of R Studio.

For fecal output estimation, stool samples were collected from eight multiparous Holstein cows with variable lactation periods, and average live weight of 653 ± 71.2 kg LW; these were maintained under

grazing conditions with *ad libitum* consumption of water, mineralized salt and Kikuyu grass (*Pennisetum clandestinum*) with regrowth days between 32 and 40 days and offering between 2.6 and 4 kg of DM 100 kg^{-1} LW. The external marker was supplied during the milking periods in two daily doses of 10 g of Cr_2O_3, offered during the supplementation with commercial concentrates (Ramírez & Giraldo, 2017). For each animal, control stool samples (H_0) were taken prior to the delivery of the marker. The experimental period was 23 days of adaptation to the diet with the marker and seven days of measurement, stage in which the collection of samples was carried out. The sampling and quantification of Cr by atomic absorption as reference method, was carried out according to the procedure described by Correa, Pabón, and Carulla (2009). All protocols for the use of experimental animals were followed according to the regulations of the Research Ethics Committee of Universidad Nacional de Colombia, Medellin Headquarters.

The estimation of fecal output (FO) was made according to Lippke's model, where the percentage of Cr recovery in feces of 74.9% was used (Correa et al., 2009). The FO was estimated with the Cr values found for the test samples with the colorimetric method (A) and contrasted with the atomic absorption method used as a reference (B). The methodological comparison was carried out using the BlandAltmanLeh package from R studio following the statistical procedure described by Giavarina (2015).

Results and discussion

In the digestion carried out between 10 and 15 minutes and up to 300°C (Fenton & Fenton, 1979) great variability in the color of the final solution was found, so the signal response was evaluated with the different digestion conditions to maximizing the absorption of the samples. It was found that the variation of the digestion temperature has an influence on the intensity of color development and, in addition, time is a factor that affects absorption. With heating at 250°C for 15 minutes, the development of a yellow color occurs, which intensifies in the following 15 minutes. This change between 15 and 30 minutes is not visually perceptible; however, the variation in the signal is approximately 5% in all reference λ (Table 1). When the temperature was increased to 280°C, the yellow color remained the first minutes. During cooling, the color was lost and the solution turned brown, notably decreasing the absorption intensity. At 300°C, the volume ratio of the solutions is not maintained, and the desired color was lost. At temperatures below 250°C, the solutions turned reddish and there was no effective development of the expected color. In all cases, a negative trend in intensity is observed with the increasing temperature. When the intensity of the signal was evaluated with respect to the time of digestion, it was found that for 250°C better signal strength is obtained with 30 minutes of digestion, meanwhile, from 280°C, the intensity decreases with time. With longer digestion times, no reproducible results were found.

Table 1. Absorption mean values on representative wavelengths varying digestion parameters.

Digestion parameters		λ (nm)					
Time (min)	Temperature (°C)	362	363	364	430	440	465
15	250	2,233	2,211	2,180	0,332	0,335	0,259
	280	2,061	2,044	2,012	0,316	0,322	0,249
	300	0,739	0,732	0,722	0,162	0,153	0,113
30	250	2,333	2,314	2,281	0,343	0,344	0,269
	280	1,629	1,613	1,590	0,276	0,272	0,215
	300	0,904	0,897	0,884	0,172	0,171	0,136

The results obtained indicate that the control of the digestion conditions have an inference in the development of the color, attributable in acidic conditions, to the equilibrium between the ions $HCrO_4^-$ and $Cr_2O_7^{-2}$. An inadequate control of these parameters, leads to the polymerization of the ions in tri- and tetra-chromate ($Cr_3O_{10}^{-2}$ and $Cr_4O_{13}^{-2}$), whose main characteristic in solution is a reddish coloration (Şahan et al., 2014), that is, if the conditions for the coexistence of all these ions in solution are given, variations in absorption frequencies and intensities will be generated, which directly affects the colorimetric quantification. This is related to the equilibrium that occurs in very acid media (pH < 3), where it has been proven that the absorption peaks for these species are codependent of pH (Szabó et al., 2018). Therefore, changes in the composition of the digester mixture due to temperature affect the intensity of the signals, being responsible not only for the changes observed in the absorption bands of the complex formed with

respect to the reference ($K_2Cr_2O_7$), but also for the low sensitivity that has been previously reported for the colorimetric method (Souza, 2013). Another important factor is the presence of $Na_2MoO_4.2H_2O$ which is used for the development of color. In general, although this compound has been referred to as a catalyst, Mo has been recognized for forming polyoxanions with metals due to its availability of empty *d* orbitals, leading to the formation of complexes with other anions, including the hexavalent forms of chromium, so it could play a much more important role in this reaction, because in the absence of this compound, color does not develop. Although this particular reaction has not been studied from the structural point of view nor the species in solution have been fully characterized, thermodynamic studies of Cr-Mo binary systems have evaluated the phase equilibrium for the liquid state, finding models consistent with the experimental data found in the literature that evidences the existence of these complexes (Hu, Wang, Wang, Du, & Zhu, 2016).

Likewise, the spectral study carried out allowed defining with precision that the optimum λ of analysis is 362 nm, finding that the sensitivity is linked to it, and that contrary to what is usually reported for this method, the reference λ for dichromate is not adequate to predict Cr content in samples treated under this procedure. Although the solution is colored, its intensity at 440 nm is not sufficient for the concentration ranges of Cr found in feces of animals dosed with Cr_2O_3 in digestibility tests. These findings are consistent with Cr measurements in feces using DPC colorimetry where the samples were subjected to the same oxidation treatment (Fernández, Díaz, Velurtas, & Fenucci, 2009).

Regarding the matrix effect, it was established that the variances are homogeneous with a critical value ($F_{crit\ 95\%,3,3}$ = 15.44) higher than the test values for both experiments (F_{calc} =13.5 and 1.43, respectively). Under this premise, the comparison of slopes by a t-test (t_{crit}=2.31) showed that slopes are not significantly different ($p > 0.05$) and therefore, there is no matrix effect (t_{calc} de 0.34 and 0.93). The parameters of the regressions for both experiments are presented in Table 2. In all cases, R^2 was higher than 0.999.

Table 2 Linear regression parameters for the evaluation of matrix effects on two digestion conditions.

T (°C)			Coefficients	Error	t	Probability	CI low. 95%	CI up. 95%
300°C	External calibration	Intercept	-0.0803	1.04E-03	-77.4	4.75E-06	-0.0836	-0.077
		Slope	0.0177	7.33E-05	241.7	1.56E-07	0.0175	0.0179
	Standard addition	Intercept	-0.0374	3.81E-03	-9.8	2.24E-03	-0.0495	-0.0253
		Slope	0.0176	2.69E-04	65.4	7.87E-06	0.0168	0.0185
250°C	External calibration	Intercept	-0.0307	7.26E-03	-4.2	2.41E-02	-0.0538	-0.0076
		Slope	0.0266	5.07E-04	52.5	1.52E-05	0.025	0.0282
	Standard addition	Intercept	-0.0518	8.68E-03	-6	9.41E-03	-0.0794	-0.0242
		Slope	0.0273	6.06E-04	45.1	2.40E-05	0.0254	0.0293

CI low.: lower confidence interval; CI up.: upper confidence interval.

Notwithstanding the matrix does not present significant effects in the original method nor with the optimized digestion parameters, when the calibration curves are contrasted for the two methods (Figure 1), it is observed that the slopes differ, being significantly higher for the second case, which implies that at 250°C, the sensitivity increases and the analyte recovery is optimized when the temperature and the digestion time is controlled. This result is one of the main advantages of the optimized method with controlled digestion parameters since, in recent years, the use of Cr_2O_3 as a marker has been relegated due to the incomplete recovery of Cr associated with the method and its subsequent sensitivity problems (Souza et al., 2013).

Although in the original method the incineration of the samples at 450°C is proposed, in this work it was found that the data were not reproducible when ashes obtained at this incineration point were used. This is because in the resulting solutions and after the digestion, there were particles in suspension that was not possible to precipitate with centrifugation. For this reason, all the matrix samples used for the optimization analyzes were incinerated at 650°C according to the thermal stability scales for Cr and the suggested temperature by the Colombian Technical Standard for the Study of minerals in agricultural products and derivatives. Previous studies reported for the handling of stool samples with Cr content also show better recoveries when the samples are properly incinerated (Kavouras et al., 2015). Therefore, the establishment of the required oxidation point of organic matter (OM) depends on the analyte that wants to be recovered in the ashes (Verbinnen, Billen Van Coninckxloo, & Vandecasteele, 2013). The thermal stability scale for Cr has been established at 700°C in an oxygen atmosphere; in this point the OM disappears completely, so this is the ideal incineration point to carry out its subsequent recovery in ashes (Falk-Windisch et al., 2015).

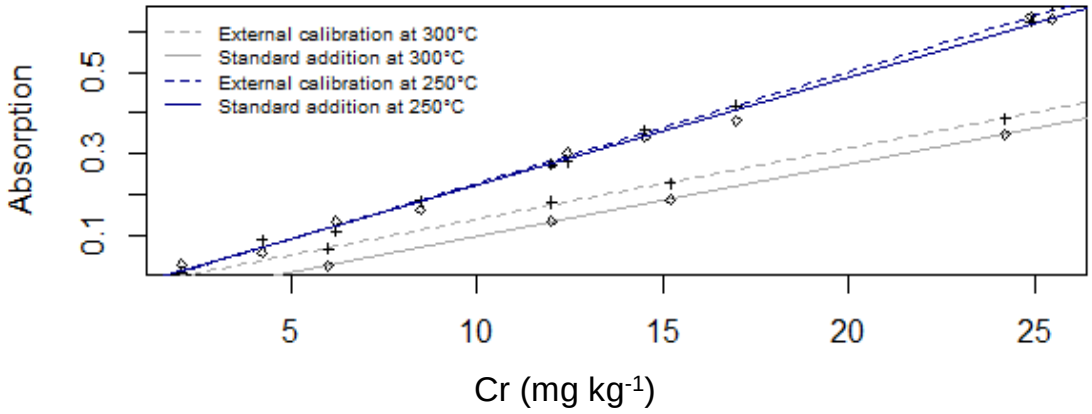

Figure 1. Matrix effect evaluation.

With the spectral study, it was found that the method is selective for quantification of Cr at the chosen wavelength, even in the presence of titanium. In the group of samples of the reference material enriched with Ti ($H_{Cr\text{-}Ti}$), an average recovery of 14.86 mg Cr kg^{-1} was calculated, meanwhile for the undoped reference material (H_{Cr}) the average recovery was 14.70 mg Cr kg^{-1}. The *F*-test showed that the variances between the groups H_{Cr} and $H_{Cr\text{-}Ti}$ are uneven ($p = 0.079$). Moreover, the *t*-test of significance for this condition indicates that the variation between groups corresponds to random errors since the calculated *t* did not exceed the critical value, so it is accepted that there is no significant difference between means ($p > 0.05$). Thus, the scope of the method in the estimation of stool production with the use of Cr_2O_3 is extended to digestibility studies with TiO_2, becoming a reliable tool when both markers are required to be assessed simultaneously. It was also found that the adequate treatment of the sample eliminates any interference due to OM and that the presence of inorganic compounds in the solution due to the ashes of the samples does not affect the recovery of Cr for its quantification.

For linearity, the analysis of residuals of the regression showed heteroscedasticity, for which a weighted regression adjustment was made with a weighting factor $wi = xi^{-1}$. Linearity was evaluated through Fisher's test for a level of significance $\alpha = 0.05$ with n calibration samples and k concentration levels ($F_{\alpha, n-2, n-k}$), finding a value $F > F_{crit}$ which indicates that the variance is heterogeneous and there is significant linearity ($p < 0.05$). The analysis of variance showed that the slope is statistically different from zero ($p < 0.05$) with a goodness of fit R^2 of 0.996. The parameters of the regression are shown in Table 3. The LOD and LOQ were 0.98 and 3.27 mg Cr kg^{-1} respectively, which established the quantification range for a 95% confidence between 3.3 and 30 mg Cr kg^{-1}.

Table 3. Statistical parameters of the weighted linear regression.

	Estimate	Std. Error	t-value	Pr (> |t|)
Intercept (a_w)	-0.0427	8.17E-03	-5.23E+00	1.63E-04
Slope (b_w)	0.0241	4.26E-04	5.67E+01	<2e-16
Residual standard error:	0.01783 on 13 degrees of freedom			
Multiple R-squared:	0.996			
Adjusted R-squared:	0.9957			

*F statistic of 3214 on 1 and 13 D.F., p-value: < 2.2e-16.

This concludes that the linearity is acceptable, the confidence intervals offer satisfactory goodness of fit and that the *LOD* and *LOQ* are suitable for work at low concentration levels. Since fecal output estimation problems have been reported due to the presence of basal chromium in the feces of test animals that have been dosed in previous experiments with the marker (Sprinkle et al., 2018), the quantification limits and the sensitivity of the method here described, facilitates the determination of basal chromium so that, at the moment of making fecal production estimates, this is not a factor that prints variability or overestimation of the marker.

Regarding the intermediate precision, a relative standard deviation (% RSD) of 3.63% and deviation values between concentration levels lower than 0.6 were obtained, so it is concluded that the results do not show significant differences between days or analysts ($p < 0.05$); so when the test is performed under different conditions, the variability is not attributable to different combinations between analysis day and analyst (Table 4). The value of the mean for each level of concentration evaluated was found within the limits established as acceptance criteria; therefore, the method is satisfactory for repeatability.

Table 4. Intermediate precision and repeatability.

Reference level (mg Cr kg^{-1})		12,0	15,0	18,0
Mean (X)		12,15	15,51	17,91
Upper limit		12,42	15,74	18,13
Lower Limit		11,88	15,29	17,69
S		0,58	0,48	0,47
% RSD		4,79	3,12	2,62
% RSD Global			3,63	
CV (%) *	Analist 1	4,17	3,19	2,27
	Analist 2	5,39	3,22	2,53

*CV (%) measured between three independent samples of reference material per day (p = 5).

For the accuracy analysis, the calculated statistic (t = 0.966) was lower than the critical value (t 0.95 = 1.943) with p = 0.1858. It was concluded that there is no significant difference between the means of the reference and the calculated concentration (Table 5). The average recovery percentage was 98.9% with an error of 1.84%, which satisfies the criterion of accuracy set.

Table 5. Accuracy test on chromium (Cr) recovery.

	Cr (mg kg^{-1})		
	Reference material	Calculated value	Recovery (%)
	15.73	15.70	99.81
		15.74	100.04
	16.33	15.58	95.37
		15.72	96.26
	15.43	15.45	100.10
		15.79	102.34
Mean	15.83	15.66	98.99
Variance	0.17	0.02	

Finally, the stability assessment of the solution's absorption over time employing Tukey's multiple HSD comparison test, showed no statistical difference between the absorption values obtained the day when the samples were prepared and the other measurements during the period evaluated, with standard errors (RSE) for the evaluated levels of 0.0082 and 0.0053, respectively. It is observed that, statistically, the absorption does not change significantly with time (p > 0.05), however, the distribution of the variance shows that for the level corresponding to the target of the samples (Figure 2a), the differences between the groups regarding storage days have radius of variation values lower than the highest concentration level (Figure 2b). In the first level, the difference between the signal obtained the first month and the second is very similar, meanwhile, for the second level of variation, a radius less close to the central value of the null difference is observed.

Although statistically there is no significant variance in the signal over the study period, a tendency of increase in the variation of the signal with time is observed, so the stability period with a lower radius of variation is considered as the criterion for storage and stability. This coincides with the first concentration level that corresponds to the expected central value in the test samples. Under this criterion, a period of maximum 60 days is fixed as stability criterion in the conditions previously indicated. Since this increase in variation cannot be associated with random errors without further study, it is recommended that samples are not stored for periods longer than recommended to ensure the analytical quality of the results.

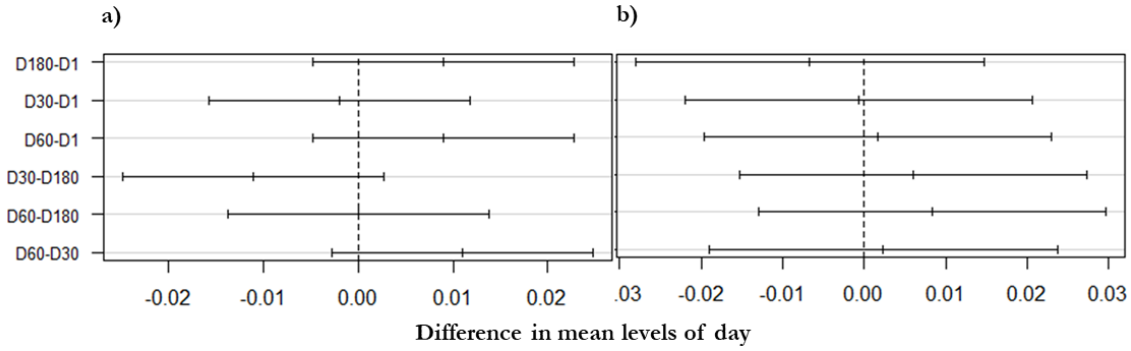

Figure 2. a) Differences in means of the first level (15 mg Cr kg^{-1}). **b)** Differences in means for the second level (25 mg Cr kg^{-1}).

The percentages of Cr obtained by both methods were evaluated by a Pearson's test, finding positive correlated values (r = 0.9847, 95% CI = 0.9732- 0.9914, p < 0.001, t = 39.279). However, although the data shows correlation, the *t* contrast is not adequate for the methodological comparison because the samples from the same animal correspond to different treatments and, intra-subject effects or between treatments were not considered. Therefore, it is necessary to have an additional criterion that allows deciding if the established method is concordant with the reference one. For this, the Bland-Altman mean difference contrast method was used (Abu-Arafeh, Jordan, & Drummond, 2016) to establish the *bias* between the methods with a 95% confidence interval. The variabilities between the paired individual data were determined and used as a criterion to establish whether the methods can be used indistinctly for the quantification of Cr in the samples.

Under this premise, a concordance test was performed between paired data under the hypothesis that similar values have a mean difference of zero. Since the slope of the regression does not differ significantly from 1 (p < 2e-16), it is concluded that there is an agreement between the methods, so that the percentages of Cr calculated by atomic absorption and by the colorimetric method, do not differ in *FO* estimation. The parameters of the regression are shown in Table 6.

Table 6. Bland-Altman parameters for concordance of methods for the percentage of chromium content comparison in fecal samples.

Residuals	Min	1Q	Median	3Q	Max
Residuals	-0.268178	-0.135277	-0.005666	0.139153	0.292898
Residual standard error: 0.1551 on 48 degrees of freedom					
Coefficients	Estimate	Std. Error	t	Pr(>|t|)	
(Intercept)	0.003758	0.022068	0.17	0.865	
Slope	0.995555	0.025346	39.28	<2e-16	
Multiple R-squared: 0.9698					
F-statistic: 1543 on 1 and 48 D.F., p-value: < 2.2e-16					
Summary of Bland-Altman regression	Parameter value	Lower limit	Upper limit		
SD	0.1662				
Mean A*	0.0932				
Mean B**	0.0965				
Bias	-0.0033	-0.3044	0.2977		
LoA sup (d+1.96SD)	0.3236	0.2461	0.4098		
LoA inf (d+1.96SD)	-0.3279	-0.4055	-0.2418		

*Mean value determined by the colorimetric method A - mean value determined by the reference method B. **Mean value determined by the reference method B - mean value determined by the colorimetric method A.

According to the criteria of the chosen statistic, it is concluded that there is no overestimation of the Cr values found with the colorimetric method versus the reference method as reported by different authors for the original Fenton method (Cabral et al., 2017). In addition, the analysis showed a systematic and proportional *bias* (Figure. 3) without trend and on the zero line, with limits lower than ± 0.05, which means that the methods are correlated, the data are concordant with each other, and there is no significant difference between them. Likewise, there is no significant difference between the stool production calculated with the paired data obtained through the two methods (p > 0.05).

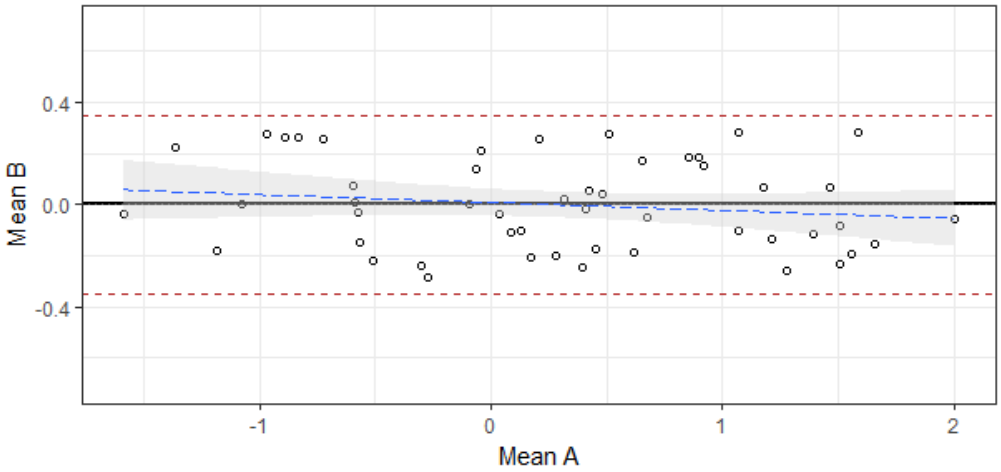

Figure 3. Bland-Altman concordance method analysis.

For all cases, the average fecal output values estimated within digestibility trials with this marker are close to the reference values reported by different researchers for the forage evaluated under similar grazing conditions, which range between 3.30 and 4.70 kg DM day^{-1} (Ramírez & Giraldo, 2017), indicating that the implementation of the colorimetric method for the estimation of *FO* under grazing in supplemented animals turns out to be satisfactory. Although it has been found in studies of comparative evaluation of markers that with Cr_2O_3 intake overestimation occurs, this problem is often attributed to the traditional analytical technique (Canesin, Fiorentini, & Berchielli, 2012) or other factors related with the internal markers (Guzman et al., 2017), so optimization of this method constitutes an important advantage for the use of the technique in digestibility tests.

Conclusion

The experiments showed that inaccuracies in the colorimetric method have led to disregard the relevance of its use. The assessment of the parameters showed that fixing the conditions improves sensitivity. The analytical validation proved that the method presents a good performance within the range of concentrations expected in forage intake tests and have not important interferences that could affect the chromium recovery. This advantage makes the method a useful tool to guarantee the assurance of results and a reliable alternative to higher cost instrumental methods and operational requirements such as atomic absorption for estimating fecal output in ruminal studies.

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