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IN VITRO ANALYSIS, AN ACCURATE TOOL TO ESTIMATE DRY MATTER DIGESTIBILITY IN RABBITS. INTRA- AND INTER-LABORATORY VARIABILITY

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Abstract: The aim of the present study was to determine the intra- and inter-laboratory variability of an enzymatic system of *in vitro* analysis for estimating dry matter (DM) digestibility in rabbits and validating the predicted nutritive value of 4 complete diets and 4 raw materials during three different periods of time. Chemical composition, DM digestibility and digestible energy (diets only) were known. *In vitro* DM digestibility (DMd_{inv}) of all samples was determined by 4 laboratories (triplicate analysis) at different times with an interval of one month between analyses. DMd_{inv} variability and chemical parameters were measured in terms of repeatability (S_R: intra-series variability within each laboratory), reproducibility (S_L: intra-series variability among laboratories) and reliability (S_F: variability through time within each laboratory). Both the laboratory and sample affected DMd_{inv} values (P<0.001). The period of time also had a significant effect (P=0.002) on mean DMd_{inv} values (67.4, 66.8 and 67.0% for the 1st, 2nd and 3rd month, respectively). Significant laboratory×sample, time×laboratory and time×sample interaction effects were also observed. Repeatability, reproducibility and reliability values for the diets were better than those obtained for the raw materials (by 2.0, 1.9 and 2.4 times, respectively). Repeatability values were also better than the values obtained for reproducibility and reliability (by 2.2 and 3.6 times, respectively). Repeatability and reproducibility values were consistently worse for raw materials than for complete diets (by 1.5, 4, 2.9 and 1.3, 4.3, 2.8 times for S_R and S_L in period 1, period 2 and period 3, respectively), and were also worse in period 1 with respect to the other two periods (by 2.1 and 2.2 times for S_R and S_L, respectively). Finally, the *in vitro* method always showed better coefficients of variation of repeatability (CV_R) and reproducibility (CV_L) than those of the chemical parameters frequently used as predictors of dietary energy value (acid detergent fibre and crude fibre) (1.73 *vs*. 2.41 and 3.88 for CV_R and 3.24 *vs*. 3.70 and 5.17 for CV_L, respectively). In conclusion, the proposed *in vitro* methodology showed adequate repeatability and reproducibility, being suitable for predictive purposes.

Key words: *In vitro* dry matter digestibility, repeatability, reproducibility, reliability, rabbit.

INTRODUCTION

The use of an enzymatic *in vitro* digestibility technique is an alternative method of evaluating the nutritive value of animal diets, compared to the *in vivo* method, which is expensive, requires facilities, large amounts of feed, considerable numbers of animals, and is highly time-consuming. Ramos *et al*. (1992) developed an enzymatic *in vitro* method to estimate the nutritive value of rabbit feeds based on the method proposed by Boisen (1991) for pigs. This multi-enzyme method showed good accuracy for the prediction

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of nutritive value of rabbit diets (Ramos and Carabaño, 1994) and was validated (Ramos and Carabaño, 1996) using independent values that had not been used previously to obtain the regression models. The index obtained indicated that the equations were robust (prediction errors always below 5%), and so, in practice, could be recommended for nutritive evaluation of rabbits diets. The results of these studies also indicated that the *in vitro* technique was very repeatable and reliable within the same laboratory, which is in agreement with the results obtained by Pascual *et al*. (2000). However, reproducibility values for *in vitro* techniques are scarce in the literature. Xiccato *et al*. (1994) compared the *in vitro* digestibility of organic matter in two laboratories (UPADU and UPM) obtaining differences between *in vitro* values, especially for high fibrous diets. This suggested the importance of performing an inter-laboratory analysis in order to obtain an adequately reproducible value for the parameters studied.

The objective of the present investigation was to determine, during three different periods of time, the intra- and inter-laboratory variability of the *in vitro* analysis associated with estimates of dry matter (DM) digestibility of four complete diets and four raw materials commonly used in rabbit rearing. This study was jointly developed by four European laboratories (ISAL, UPADU, UPV and UPM) within the framework of the Concerted Action FAIR3-1651 "European harmonisation of rabbit feed evaluation-ERAFE" (Gidenne, 1999).

MATERIAL AND METHODS

Samples

The *in vitro* technique and chemical analysis were performed on eight samples: four complete diets (diets 1, 2, 3 and 4) and four raw materials (wheat bran, peas, sunflower meal and barley). These samples had previously been used to perform an analytical ring test (Xiccato *et al*., 1996), so their chemical composition was known (Table 1).

Diet 1 was based on lucerne hay (350 g/kg) and barley (250 g/kg) as main sources of fibre and energy, respectively. The other three diets were formulated by substituting 150 g/kg of sugar beet pulp for 150 g/kg of lucerne hay (diet 2), 150 g/kg of barley (diet 3), and respectively 75 g/kg of lucerne hay and 75 g/kg of barley (diet 4). All diets had similar content of wheat bran (230 g/kg), sunflower meal 36 (50 g/kg), soybean meal 44 (70 g/kg) and molasses (30 g/kg). DM digestibility and digestible energy (DE) of these diets had previously been determined *in vivo* (64.0, 68.1, 61.5, 64.3% and 2746, 2959, 2617, 2772 kcal DE/kg DM for diets 1, 2, 3 and 4, respectively; Trocino *et al*., 1999). Data of *in vivo* energy value for raw materials were not available.

Table 1: Chemical composition of samples (% dry matter).

Sample	Starch	CP[1]	CF[2]	NDF[3]	ADF[4]
Diet 1	18.1	17.7	15.9	38.3	18.5
Diet 2	17.0	17.2	14.3	37.7	17.1
Diet 3	10.7	17.2	18.1	41.7	21.2
Diet 4	13.6	17.2	16.0	39.6	19.1
Wheat Bran	12.5	15.6	12.0	54.4	14.9
Peas	40.3	22.6	6.88	23.5	7.99
Sunflower meal	0.98	31.2	29.2	46.2	31.4
Barley	55.3	10.4	5.20	25.3	6.00

[1]CP, Crude protein; [2]CF, Crude fibre; [3]NDF, Neutral detergent fibre; [4]ADF, Acid detergent fibre.

In vitro analysis

The *in vitro* technique was performed in the four laboratories according to Ramos *et al*. (1992). Samples, previously ground to a pore size of 1 mm, were weighed (1 g) with an accuracy of 0.1 mg and put into 100 mL conical flasks. A small magnetic rod, 25 mL of phosphate buffer (0.1 M, pH 6.0) and 10 mL of 0.2 M HCl solution were added to each flask. The sample and the solutions were mixed carefully by gentle magnetic stirring. The pH was measured and then adjusted to pH 2 with 1 M HCl or 1 M OHNa solutions. Using porcine pepsin (2000 FIP-Units/g protein, Merck n 7190), 1 mL of a freshly prepared pepsin solution (25 mg of pepsin/mL 0.2 M HCl) was added and mixed by gentle magnetic stirring. The flasks were closed with a rubber stopper and the samples incubated in an oven at 40ºC for 1.5 h.

After this incubation, 10 mL of a phosphate buffer (0.2 M, pH 6.8) and 5 mL of a 0.6 M OHNa solution was added to each flask in order to increase the pH to 6.8. The sample and the solutions were mixed carefully by gentle magnetic stirring and pH was measured and then adjusted to pH 6.8 with 1 M HCl or 1 M OHNa solutions. Using porcine pancreatin (grade VI, Sigma n 1750), 1 mL of a freshly prepared pancreatin solution (100 mg of pancreatin/mL phosphate buffer pH 6.8) was added and mixed by gentle magnetic stirring. The flasks were closed with a rubber stopper and the samples incubated in an oven at 40ºC for 3.5 h.

After the second incubation, pH were adjusted to 4.8 by adding acetic acid and then 0.5 mL of Viscozyme 120L (120 FBG/G, Novo Nordisk) was added and mixed by gentle magnetic stirring. The flasks were closed with a rubber stopper and the samples incubated in an oven at 40ºC for 16 h (overnight).

After incubation, the undigested residue was collected in a filtration unit (Fibertec System, Tekator) by transferring the sample to a dried and pre-weighed glass filter crucible (poresize no 2). After filtration, the residue was washed several times with distilled water and with ethanol and acetone (50 mL). The residue was then dried at 103ºC until constant weight (24 hours).

The same procedure was followed with a flask without a sample in order to correct the residue due to the reagents (blank).

In vitro dry matter digestibility (DMd_{inv} in percentage) was calculated as follow:

$$DMd_{inv} = (((R_S103 - C) - (R_B103 - C))/ W_S) \times 100$$

where:

R_S103: weight of the crucible and the residue after drying at 103ºC

R_B103: weight of the crucible and the reagent residue after drying at 103ºC (blank)

C: weight of the dried crucible

W_S: weight of the sample on DM

This procedure was repeated 3 times with a time interval of one month in each laboratory. Each time, triplicate *in vitro* determinations were performed.

Chemical analysis

AOAC Procedures (1995) were used for crude protein (CP), crude fibre (CF) and starch (amyloglucosidase-α-amylase method). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to the sequential procedure of Van Soest *et al*. (1991). These procedures were carried out observing the recommendation to harmonize chemical analysis proposed by the European Group on Rabbit Nutrition (EGRAN, 2001). All samples were analysed in triplicate.

Statistical analysis

A statistical analysis was performed using the SAS GLM procedure (1993). Data were analysed as a randomised complete design with a factorial arrangement of treatments: 4 laboratories×8 samples×3 replicates per sample×3 times. The model for measured DMd_{inv} included as main factors the laboratory (labs 1, 2, 3 and 4), the samples (diets 1, 2, 3, 4, wheat bran, peas, sunflower meal, and barley), the different periods of time (periods 1, 2 and 3) and their interactions.

Variability of DMd_{inv} and chemical parameters were measured in terms of repeatability (S_R: intra-series variability within laboratory), reproducibility (S_L: intra-series variability between laboratories) and reliability (S_F: variability through time within a laboratory). The determination of DMd_{inv} and chemical content in each sample was performed in triplicate and duplicate, respectively, in order to determine repeatability. The analyses were performed at the same time on all samples by each of the participating laboratories in order to calculate reproducibility. This procedure was repeated three times at monthly intervals to calculate reliability, but only for DMD_{inv}. Repeatability, reproducibility and reliability were estimated by the SAS VARCOMP procedure (1993) and were calculated as follow:

$$S_R = \sqrt{(S_e)^2}$$

$$S_L = \sqrt{(S_e^2 + S_l^2 + S_{d\times l}^2 + S_{f\times l}^2 + S_{d\times f\times l}^2)}$$

$$S_F = \sqrt{(S_e^2 + S_f^2 + S_{d\times f}^2 + S_{f\times l}^2 + S_{d\times f\times l}^2)}$$

where:

S_e: expected variance of error

S_l: expected variance of the components of the laboratory

S_f: expected variance of components of the time period

$S_{d\times l}$: expected variance of sample×laboratory interaction

$S_{d\times f}$: expected variance of sample×time interaction

$S_{f\times l}$: expected variance of time×laboratory interaction

$S_{d\times f\times l}$: expected variance of sample×time×laboratory interaction

The coefficients of variation of repeatability (CV_R), reproducibility (CV_L) and reliability (CV_F) were calculated as the relation between S_R, S_L, S_F and the mean value of the variable studied, expressed as a percentage.

RESULTS

The DMd_{inv} results obtained from the different samples evaluated by the four laboratories at three different periods of time are shown in Table 2. Laboratory had a significant effect (P=0.0003) on DMd_{inv} since, in general, laboratories 2 and 4 showed lower mean values (66.7%, on average) than laboratories 1 and 3 (67.3%, on average). As a consequence of the different chemical compositions of the diets and feeds, a sample effect (P=0.0001) was found in DMd_{inv} that varied from 51.4 to 80.6 for wheat bran and peas, respectively. The period of time showed an effect (P=0.002) on mean DMd_{inv} (67.4, 66.8 and 67.0% for the 1st, 2nd and 3rd month, respectively), due to the fact that some laboratories (lab 2 and lab 4) and some samples (diet 3, sunflower and barley) showed differences in their DMd_{inv} values at the different times. Significant laboratory×sample, time×laboratory (P=0.0001) and time×sample (P=0.003) interaction effects were also observed.

Table 2: Effect of laboratory and sample at three periods of time on the average in vitro digestibility of dry matter (DMd_{inv}, %).

	Periods of time		
	Time 1	Time 2	Time 3
Laboratory			
Lab 1	67.4	67.7	67.0
Lab 2	67.4	65.7	67.4
Lab 3	67.3	67.0	67.7
Lab 4	67.5	66.7	65.7
Sample			
Diet 1	65.9	65.7	65.8
Diet 2	69.6	70.1	70.7
Diet 3	64.0	63.5	63.7
Diet 4	66.8	66.6	66.5
Wheat Bran	52.0	51.9	51.4
Peas	80.1	79.9	80.6
Sunflower	60.8	58.9	58.7
Barley	78.7	77.6	78.4
rsd[1]	1.35		
P-value			
Laboratory	0.0003		
Sample	0.0001		
Time	0.002		
Laboratory×Sample	0.0001		
Time×Laboratory	0.0001		
Time×Sample	0.003		

[1] *rsd*: Residual Standard Deviation

Standard deviation within laboratories (repeatability, S_R), between laboratories (reproducibility, S_L) and within laboratories through time (reliability, S_F) is shown in Table 3. Repeatability, reproducibility and reliability values for the diets were better than those obtained for the raw materials (by 51, 52 and 41% for S_R, S_L and S_F, respectively). Repeatability values were also better than those obtained for reproducibility and reliability (by 46 and 28%, respectively). *In vitro* repeatability, reproducibility and reliability, expressed as a percentage of the mean (CV_R, CV_L and CV_L, respectively), showed the same trend as the standard deviations. The values obtained for the diets were better than those for the raw materials (by 2.0, 2.1 and 1.7 times for CV_R, CV_L and CV_L, respectively).

Data in Table 4 show the repeatability and reproducibility for DMd_{inv} at three periods of time. Both indexes were worse for raw materials than for complete diets in each period (by 67, 25, 34% and 74, 23, 36% for S_R and S_L at times 1, 2 and 3, respectively). Repeatability and reproducibility values for raw materials were worse at time 1 than the average of these indexes at the other two time points (by 48 and 45% for S_R and S_L, respectively). Consequently, the standard deviations of all samples for time 1 were increased relative to times 2 and 3 (by 40 and 37%, for S_R and S_L, respectively). A parallel effect for raw

Table 3: Repeatability (S_R), reproducibility (S_L) and reliability (S_F) indexes and coefficient of variation for S_R (CV_R, %), S_L (CV_L, %) and S_F (CV_F, %) for the *in vitro* digestibility of dry matter (DMd_{inv}, %).

	S_R[1]	CV_R	S_L[2]	CV_L	S_F[3]	CV_F
All samples	1.16	1.73	2.17	3.24	1.62	2.41
Complete diets	0.72	1.09	1.37	2.05	1.21	1.82
Raw materials	1.48	2.19	2.88	4.26	2.06	3.05

[1]Within-laboratory standard deviation; [2]Among-laboratories standard deviation; [3]Within-laboratory standard deviation throughout time.

materials was observed on coefficients of variation for S_R (CV_R) and S_L (CV_L) in the first time period, which increased from 1.57 and 3.20 (as average of CV_R and CV_L at times 2 and 3, respectively) to 3.02 and 5.73 for CV_R and CV_L, with respect to time 1. This effect also increased CV_R and CV_L in the first period for all samples (by 40 and 36%, respectively).

Variability within and among laboratories and their coefficients of variation for chemical parameters of the samples are shown in Table 5. The *in vitro* method always showed better CV_R and CV_L values (Table 3) than those shown by the chemical parameters frequently used as predictors of dietary energy value (ADF and CF) (1.73 *vs* 2.41 and 3.88 for CV_R and 3.24 *vs* 3.70 and 5.17 for CV_L in all samples, respectively).

DISCUSSION

All the variability factors evaluated in the present work were observed to have a highly significant effect on DMd_{inv}. However, the differences observed among mean values of all samples were relatively low, both

Table 4: Repeatability (S_R) and reproducibility (S_L) indexes and coefficient of variation for S_R (CV_R, %) and S_L (CV_L, %) for the in vitro digestibility of dry matter (DMd_{inv}, %) at three periods of time.

	Periods of time		
	Time 1	Time 2	Time 3
All samples			
S_R[1]	1.53	0.82	1.01
CV_R	2.27	1.23	1.51
S_L[2]	2.78	1.55	1.96
CV_L	4.12	2.32	2.93
Complete diets			
S_R	0.68	0.70	0.79
CV_R	1.02	1.05	1.18
S_L	1.03	1.40	1.61
CV_L	1.55	2.10	2.41
Raw materials			
S_R	2.06	0.93	1.19
CV_R	3.02	1.37	1.77
S_L	3.91	1.81	2.50
CV_L	5.73	2.69	3.72

[1]Within-laboratory standard deviation; [2]Among-laboratories standard deviation.

Table 5: Repeatability (S_R) and reproducibility (S_L) indexes and coefficient of variation for S_R (CV_R, %) and S_L (CV_L, %) for starch , CP, CF, NDF, and ADF analyses of samples.

	Starch	CP[1]	CF[2]	NDF[3]	ADF[4]
All samples					
S_R[5]	1.15	0.34	0.57	1.21	0.41
CV_R	5.46	1.82	3.88	3.15	2.41
S_L[6]	2.47	0.52	0.76	2.41	0.63
CV_L	11.7	2.79	5.17	6.28	3.70
Complete diets					
S_R	0.48	0.45	0.55	0.73	0.43
CV_R	3.24	2.59	3.42	1.86	2.27
S_L	1.91	0.59	0.62	1.29	0.65
CV_L	12.9	3.40	3.86	3.28	3.43
Raw materials					
S_R	1.55	0.14	0.59	1.54	0.38
CV_R	5.68	0.73	4.43	4.12	2.52
S_L	2.92	0.43	0.79	3.17	0.67
CV_L	10.7	2.24	5.93	8.48	4.45

[1]CP, Crude protein; [2]CF, Crude fibre; [3]NDF, Neutral detergent fibre; [4]ADF, Acid detergent fibre; [5]Within-laboratory standard deviation; [6]Among-laboratories standard deviation.

among laboratories and among different time periods (0.6 points on average). When *in vitro* estimates were compared with *in vivo* values for complete diets (Table 6, Trocino *et al*., 1999) we observed that the *in vitro* values overestimate the *in vivo* ones (1.7 as average), as had occurred in previous studies (Ramos *et al*., 1992; Ramos and Carabaño 1996). However, the *in vitro* technique is able to reproduce the variation in digestibility observed in the *in vivo* trial and to predict accurately the energy values determined *in vivo* (according to the equation proposed by Villamide *et al*., 2008) (Table 6). The corresponding *in vivo* determinations for raw materials were not available. However, alternative estimates of expected energy values can be obtained from the chemical composition of raw feedstuffs given in tables of nutritive value (Villamide *et al*., 1998; FEDNA, 2003). When we compared the expected and predicted energy values (Table 6), sunflower meal and wheat bran showed good accuracy, however we observed some discrepancies for barley and peas. These differences could be due to errors in the prediction equation when it is used out of the range of the variables employed (the maximum value for DMd_{inv} in the equation was 75.7%) or to methodological problems of the *in vitro* technique with raw materials of high starch content.

The repeatability of DMd_{inv} for all samples studied was better than the fibre analysis frequently used as predictors of digestible energy in rabbit diets (from 1.5 to 2.2 times, for NDF, ADF and CF). These results agree with those reported by Ramos and Carabaño (1996), who observed that chemicals analyses, such as CF or ADF, are less repeatable than *in vitro* determinations. The repeatability values were better for complete diets than for raw materials. These results again suggest possible methodological problems involved with the *in vitro* technique in the digestion of some of the major components of certain raw materials, as mentioned above. The repeatability value of DMd_{inv} for complete diets was slightly worse (CV_R =1.09) than those reported by Ramos and Carabaño (1996) and Pascual *et al*. (2000) in rabbit diets

Table 6: *In vitro* dry matter (DM) digestibility (DMd_{inv}, %), *in vivo* digestible energy (DE, MJ/kg DM) and DM digestibility (DMd, %) and predicted value of DE (MJ/kg DM) from *in vitro* DM digestibility.

		In vivo		
Sample	DMd_{inv}	DMd	DE	Predicted DE[1]
Diet 1	65.3	64.0	11.4	11.4
Diet 2	69.2	68.1	12.4	12.1
Diet 3	64.7	61.5	11.0	11.3
Diet 4	65.9	64.6	11.6	11.5
Wheat Bran	51.7		9.28[2]	9.38
Peas	80.2		15.0-15.3[2]	13.7
Sunflower	59.5		10.1- 10.6[2]	10.4
Barley	78.2		14.2- 14.6[2]	13.3

[1]According to the equation DE (MJ/kg DM)=1.63+15.0 DMd_{inv} (Villamide *et al*., 2008); [2]According to Villamide *et al.* (1998) and FEDNA (2003).

(0.69 and 0.65 %, respectively), and by Noblet and Jaguelin-Peyraud (2007) for the *in vitro* digestibility of organic matter (0.9 % CV) in pig diets.

The variability observed through time (reliability) in the present study was worse than repeatability, showing similar values to previous studies for complete diets [CV_F = 1.77 and 1.43 for Ramos and Carabaño, (1996) and Pascual *et al*. (2000), respectively]. This enzymatic technique showed better results for reliability than those that use caecal or faecal inoculates, or fibre analysis, which shows values of CV_F that vary from 5 to 8% (Ramos and Carabaño, 1996; Pascual *et al*., 2000), suggesting great stability through time of the enzymes used in this technique.

The highest variability was observed among laboratories (reproducibility), both for chemicals and *in vitro* DM digestibility, being around twice that of repeatability. Similar results were reported by Bailey and Henderson (1990) for different chemicals. They found a relationship between repeatability and reproducibility values of from 2:3 to 1:2. This suggests that both chemical and *in vitro* DM reproducibility were within the normal range of variation. However, the reproducibility of *in vitro* DM digestibility showed better figures than chemicals.

Neither repeatability nor reproducibility were constant through time, and improved as time progressed. In our study the results obtained at time 1 were worse than times 2 and 3, when similar values were found. According to Alderman (1985) both parameters depend on the experience of the operator and the concentration of the compound to be determined. The accuracy of an analysis improves with the experience of the operator until reaching the precision of the method. In our study, the 3 laboratories had previous experience in this *in vitro* technique, but in all cases a different operator took part in each trial. This could partially explain the improvement of accuracy with time.

In conclusion, the proposed *in vitro* methodology provides adequate repeatability and reproducibility and being suitable for use in different laboratories as a useful predictive tool.

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