NUTRITIVE EVALUATION OF RABBIT DIETS BY AN *IN VITRO* METHOD

RAMOS M., CARABAÑO R.

Departamento de Producción Animal. E.T.S. Ingenieros Agrónomos, Universidad Politécnica, 28040 Madrid, Spain.

Abstract - An *in vitro* enzymatic method was carried out to study the relationships between *in vivo* DM (DMd) and GE (GEd) digestibility or digestible energy (DE) and *in vitro* DM digestibility (DMv) in 27 complete rabbit diets. An accurate correlation was found for DMd (R^2=0.84; rsd=1.45), whereas for GEd or DE the correlation was worse (R^2=0.64 and R^2=0.55, respectively). Repeatability and reliability indexes were used to assess the variability of the *in vitro* analysis intra-laboratory and to compare it with that of CF and ADF analyses. A high repeatability was found for all three analyses, although *in vitro* was the most repeatable (CV_r=0.69% *vs.* CV_r=1.78% and 1.72%, for CF and ADF, respectively). Reliability (variability along the time) was also higher for *in vitro* analysis (CV_R=1.77% *vs.* 4.26% and 7.87%).
When 14 more experimental diets, including high amounts of beet pulp (from 10 to 50%) or added fat (3 or 6%), were included in the regression, the resulting prediction equation showed good accuracy (DMd = 4.67 + 0.86 DMv; R^2=0.87; rsd=1.52; p=0.0001; n=41), indicating that the *in vitro* technique is able to estimate DMd of this type of diets.
Prediction equations obtained for DM and GE digestibility depending on *in vitro* DM digestibility have proved to be robust when validated with four independent data sets (92 diets). The prediction errors obtained (lower than 5%) indicate a high prediction ability for evaluation of the nutritive value of rabbit diets.

INTRODUCTION

In situations where facilities to carry out *in vivo* digestibility trials and adequate finance are lacking, *in vitro* studies can be useful in evaluating rabbit diets for their potential utilization.
An enzymatic *in vitro* method has already been developed showing good accuracy of prediction (RAMOS *et al.*, 1992; RAMOS and CARABAÑO, 1994; XICCATO *et al.*, 1994). However, this method had not been sufficiently validated; there was a lack of data about variability of analytical techniques or on predictive ability. Therefore, the objectives of this investigation were:

1. to determine the variability of the *in vitro* analysis intra-laboratory and to compare it with that of CF and ADF analyses, which are the best single predictors of the energy value.
2. to check how the *in vitro* technique is able to predict the nutritive value of diets with an ingredient composition including high amounts of beet pulp or added fat.
3. to appraise the predictive ability of the equations obtained, in order to determine if they can be proposed for practical use or not.

MATERIALS AND METHODS

Diets

A data base of 27 complete rabbit diets were used. Ten commercial diets were provided by several Spanish firms and 17 were experimental diets from the Department of Animal Production of Madrid and the Department of Animal Science of Valencia. Experimental diets were chosen so that, not differing very much from commercial diets, they increased chemical variation ranges. Table 1 shows the average and variation range in chemical composition and digestibility coefficients of the diets.
Another fourteen diets were also analysed: eight diets containing beet pulp, substituted for barley (at a 15, 30, 35 or 50% inclusion level), or for alfalfa hay (10, 20 or 30%); and 6 diets containing added fat (3 or 6%). *In vivo* DMd ranged from 58.8 to 70.3% and from 56.8 to 59.3% for diets containing beet pulp or added fat, respectively. More details about the composition of these diets are described in GARCIA *et al.* (1993), MOTTA. (1990) and FERNANDEZ *et al.* (1994).
To validate the equations obtained, we used 92 complete diets provided by 4 European laboratories: 34 diets had been supplied by the Government Research Station for Small Stock Husbandry (Merelbeke, Bélgium); 19 by INRA (Station de Recherches Cunicoles, Castanet Tolosan, France); 17 by the Dipartimento di Scienze Zootecniche, University of Padova (Italy) and 22 by the Instituto Superior de Agronomía, University of Lisboa (Portugal). All but 3 diets from Italy are experimental diets mostly designed to evaluate different raw materials. Table 2 shows the chemical composition and the *in vivo* DM digestibility coefficients of these 92 diets.

Table 1: Chemical composition and *in vivo* digestibility of rabbit diets from Spain

		Commercial diets (n=10)				Experimental diets (n=17)			
Chemical composition		min	avg	max	s.d.	min	avg	max	s.d.
Crude protein (CP)	%DM	16.21	17.63	19.35	1.00	15.80	18.28	20.00	1.16
Crude fibre (CF)	%DM	14.33	17.41	18.72	1.42	12.10	15.88	19.60	2.31
Acid-detergent fibre (ADF)	%DM	17.85	20.82	22.96	1.63	14.75	18.97	23.17	2.37
Digestibility									
DM digestibility (DMd)	%	57.38	60.44	63.30	1.89	55.20	63.03	72.00	3.93
GE digestibility (GEd)	%	57.52	60.26	62.50	1.78	55.60	62.49	69.50	3.67
Digestible energy (DE)	MJ/Kg DM	10.31	10.76	11.24	0.30	9.65	11.24	12.47	0.75

Table 2: Chemical composition (% DM) and *in vivo* DM digestibility (DMd,%) of rabbit diets from four European countries

	Belgium (n=34)			France (n=19)			Italy (n=17)			Portugal (n=22)		
	min	avg	max	min	avg	max	min	avg	max	min	avg	max
Crude protein (CP)	13.48	18.52	24.36	15.61	17.96	22.15	14.62	17.80	20.79	14.69	16.86	19.18
Crude fibre (CF)	1.56	15.88	23.04	9.93	15.78	22.66	13.90	15.54	19.50	13.85	15.66	18.65
Acid-detergent fibre (ADF)	14.62	20.01	27.10	10.24	17.58	25.39	17.55	20.03	24.40	17.35	19.33	22.25
DM digestibility (DMd)	48.90	61.00	71.00	60.40	67.46	78.60	54.46	63.03	69.20	60.40	64.01	69.90

In Vivo Digestion Trials

New Zealand x California growing rabbits of an average body weight (BW) 1.329 ± 96.2 g were randomly assigned to 22 diets (commercial and experimental) and were placed in digestibility cages (12 animals per diet) that allowed separation of faeces and urine. After a 7-day adaptation period, the animals were weighed and a minimum of 8-10 animals (BW=1.633 ± 148 g) were kept to continue with the trial. Then, daily feed intake and hard faeces production were recorded individually during a 4-day collection period. A representative sample of the faeces produced daily (30%) was collected in labelled plastic bags and stored at -18°C for its later analysis. *In vivo* digestibility coefficients for DM, GE as well as DE of 5 diets provided by Valencia and 92 diets provided by 4 European laboratories had been obtained in their origin laboratories following the technique normally used for digestibility assays. The procedures used to obtain digestibility coefficients for the 14 diets containing beep pulp or added fat are described in the previously cited papers.

Analytical Methods

Chemical analyses for all diets (n=133) were done in our laboratory. Dry matter (DM), crude protein (CP) and crude fibre (CF) analyses were made as outlined by A.O.A.C. (1984). Acid-detergent fibre (ADF) analysis was conducted following the method of ROBERTSON and VAN SOEST (1981). Gross energy (GE) was determined using an adiabatic calorimeter bomb. Faeces from *in vivo* digestion trials were analysed for DM and GE. All diets were used as substrates to obtain *in vitro* DM digestibility coefficients in our laboratory following the methodology described by RAMOS *et al.* (1992). In order to evaluate the variability of the *in vitro* analysis, one standard sample (CF=17.9%; CP=18.5%) was included in each run. This same standard sample was also subjected to CF and ADF analyses to obtain the variability of these fibre analyses.

Statistical Analysis and Calculations

Regression analysis - Simple regressions between *in vivo* and *in vitro* digestions were calculated using the REG procedure of the S.A.S. program (1991). Comparison of regression equations was done following the procedure outlined by SNEDECOR and COCHRAN (1971).

Repeatability and reliability indexes - The variability of the *in vitro*, CF and ADF analyses was measured in terms of repeatability and reliability. Repeatability (r) is defined as the intra-series variability of an analysis carried out in one laboratory, whereas reliability (R) is the inter-series variability or variability along the time, also in one same laboratory. To calculate repeatability, duplicate analyses of the standard sample were carried out (10 repetitions for *in vitro*, 12 for CF and 12 for ADF); and the coefficient of variation of repeatability (CV_r), calculated as the relation of the average variance of all pairs of values to the mean value of the variable studied, was chosen as the repeatability index. To calculate reliability, single analyses of the standard sample

were carried out along 14 months (30 repetitions for *in vitro*, 17 for CF and 13 for ADF), and the coefficient of variation or reliability (CV_R) was calculated as the relation between the standard deviation of the mean of the single analyses along the time and the mean value.

Validation of regression equations

The predictive ability of the equations obtained was assessed by means of validation with independent data, that is using pairs of values of the dependent and independent variables not used to obtain the regression models. Four independent data sets were used for validation, each one including diets from a different origin (Belgium, France, Italy and Portugal). Three equations were validated: prediction equations of DMd obtained with 27 and with 41 data and prediction equation of GEd obtained with 27 data. The statistical procedure used, as suggested by FUENTES-PILA et al. (1995), was to obtain the MSPE (Mean-Square Prediction Error) defined as follows:

$$MSPE = \frac{\sum(A - P)^2}{n}, \text{ where:}$$

A = actual values for the dependent variable of a data set.
P = predicted values for the dependent variable.
n = pairs of A and P compared.

and then the Prediction Error, defined as follows:

$$\%Error = \frac{\sqrt{MSPE}}{\bar{y}} \times 100, \text{ where:}$$

$\bar{y}$ = mean value of the actual dependent variable of the independent data set.

This permits to obtain an index of the Robustness of a prediction model, with the following criterion:

if % Error < 5% prediction is satisfactory
if 5% < % Error < 10% prediction is acceptable
if 10% < % Error prediction is unsatisfactory

The use of this index is to compare different models in order to select the most suitable for prediction.

RESULTS AND DISCUSSION

Regression analysis

Relationships between DE, *in vivo* digestibility of DM and GE (DMd, GEd) and *in vitro* DM digestibility (DMv) are shown in Table 3.

Table 3: Simple regression equations *in vivo/in vitro* (n=27)

Eq.	y	x	a	b	R^2	rsd	p
1	DMd	DMv	5.49 (±5.02) p=0.2846	0.86 (±0.08)	0.84	1.45	0.0001
2	GEd	DMv	15.74 (±6.87) p=0.0306	0.69 (±0.10)	0.64	1.99	0.0001
3	DE	DMv	591.58 (±372.99) p=0.1253	31.06 (±5.63)	0.55	107.89	0.0001

Model: y=a + b * x

The best fit was obtained for prediction of DMd (equation 1), GEd and DE being worse predicted. Consequently, eq. 1 is considered the « calibration equation ». We verify that this equation is as accurate as the equations obtained in previous works (RAMOS *et al.*, 1992; RAMOS and CARABAÑO,1994).

In order to analyse the performance of calibration equation in the prediction of DMd of diets containing high amounts of added fat or beet pulp, the regression equation of *in vitro* on *in vivo* DM digestibility for 14 experimental diets of this type was first obtained:

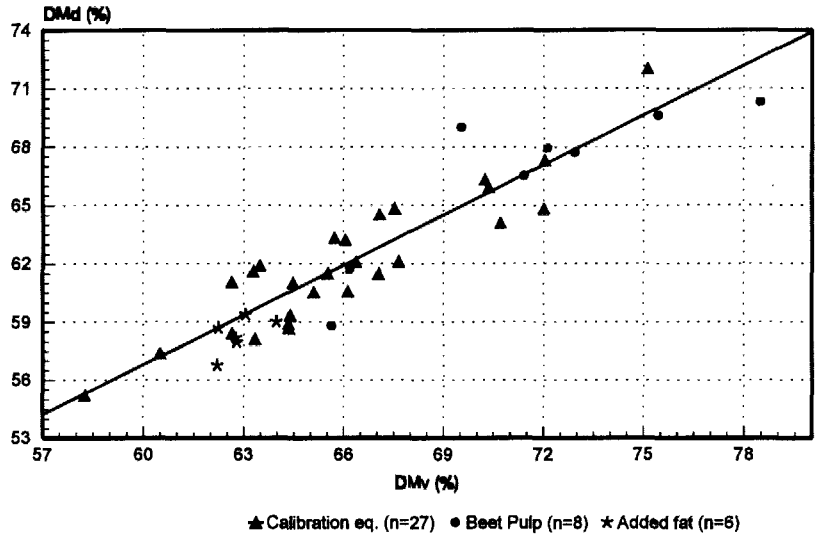
(4) DMd = 2.41 (± 5.82) + 0.89 (± 0.09) DMv R^2 = 0.90 rsd = 1.69 p = 0.0001 n = 14
p = 0.6864

When eq. 4 was compared to eq. 1, no significant differences (p>0.05) were found, neither in residual variances, nor in slopes. Consequently, a new equation including all 41 data was proposed:

(5) DMd = 4.67 (± 3.63) + 0.86 (± 0.05) DMv R^2 = 0.87 rsd = 1.52 p = 0.0001 n = 41
p = 0.2055

When comparing eq. 5 to eq. 1, intercepts were quite similar and slopes were equal, indicating a high stability of the regressor. Consequently, it was concluded that the *in vitro* technique is able to predict diets with high amounts of beet pulp or added fat. Figure 1 shows the fit to the calibration equation (eq. 1).

Figure 1 : Relationship between *in vitro* DM digestibility (DMv) and *in vivo* DM digestibility (DMd) including diets containing beet pulp or added fat to the calibration equation (eq. 1, Table 3)



These type of diets are proved not to be well predicted by fibre parameters. When relationships between DMd and CF or ADF, for the same 41 diets, were obtained (RAMOS,1995), determination coefficients were very low (R^2=0.23 and R^2=0.27, respectively). DE BLAS *et al.* (1992) confirm that exclusion of diets containing added beet pulp or fat from an equation relating GEd and ADF improved the precision (rsd from 3.8 to 2.2).

Repeatability and reliability indexes

Repeatability and reliability indexes for *in vitro*, CF and ADF analyses are shown in Table 4. The results indicate that, although all three analyses are very repeatable in our laboratory, the *in vitro* technique is the most repeatable (CV_r=0.69), whereas CF and ADF analyses present practically the same repeatability (CV_r=1.78 and CV_r=1.72, respectively). The *in vitro* technique has also proved to be the most reliable in our laboratory (CV_R=1.77), showing a big difference respect to CF and ADF analyses (CV_R=4.26 and CV_R=7.87, respectively).

References to repeatability values for *in vitro* techniques or chemical analyses are scarce in the consulted literature. The low CV_r obtained for the *in vitro* analysis in our work is in agreement with the results obtained by ALDERMAN (1985) for a NDF/Cellulase *in vitro* method (CV_r =0,70%). They also agree with some results from a Ring Test carried out by VAN DER MEER (1984), whose CV_r for Pepsine/Cellulase and NDF/Cellulase methods vary in the range 0.62 to 3.43%. The figure obtained in our work for CF is similar to the obtained by ALDERMAN (1985) and XICCATO *et al.* (1996), (CV_r=1.72% and 1.71%, respectively); and the figure

Table 4. Repeatability and Reliability indexes for *in vitro*, CF and ADF analyses

	CV_r (%)	n*	CV_R (%)	n#
DMv	0.69	10	1.77	30
CF	1.78	12	4.26	17
ADF	1.72	12	7.87	13

*: duplicate analyses
#: single analyses

obtained for ADF also agrees with the value given by the last author mentioned (CV_r=1.81%). However, MUÑOZ *et al.* (1994) get higher values for all chemical analyses, indicating a low repeatability (i.e. CV_r for NDF=2.49%). The literature does not show values for reliability. In this work, CV_R are higher than CV_r (two times higher for DMv and CF, and four times higher for ADF). This suggests the importance of performing laboratories analyses along the time in order to obtain a most reliable figure of the parameters analyzed. Several works have reported data about reproducibility (variability among laboratories), always showing higher values than for repeatability. BAILEY and HENDERSON (1990) have found the relation between repeatability and reproducibility to be from 2:3 to 1:2. Therefore, it is necessary to perform a Ring Test in order to obtain reproducibility values for the *in vitro* technique to be compared with literature data.

Validation of regression equations

Results concerning validation with independent data sets, are shown in Table 5. The low prediction errors obtained for the 3 prediction equations validated (always below 5%), in all 4 data sets, indicate that they all show a satisfactory ability for prediction. Therefore, they are very robust equations and so they can be proposed for practical use to predict the nutritive value of complete rabbit diets.

Table 5: Validation of DMd and GEd prediction equations obtained

Data sets	Eq. n	Precision of prediction		
		$MSPE$	$\bar{y}_{actual}$	% Error
Belgium (n=34)	(1)	5,98	60,99	±4,01
	(5)	5,51	60,99	±3,85
	(2)	8,15	60,36	±4,73
France (n=19)	(1)	3,92	67,46	±2,93
	(5)	4,01	67,46	±2,97
	(2)*	5,97	66,02	±3,70
Italy (n=17)	(1)	4,09	63,05	±3,21
	(5)	4,21	63,05	±3,25
	(2)	4,76	62,33	±3,50
Portugal (n=22)**	(1)	9,95	64,01	±4,93
	(5)	10,74	64,01	±5,12

(DMd or GEd), average of the data set; % Error: prediction error, defined as $MSPE$ (%): Mean-Square Prediction Error; $\bar{y}_{actual}$: actual dependent variable $\sqrt{MSPE} / \bar{y}_{actual} \times 100$. ; *n = 15 **: no GEd data of diets from Portugal are available.

CONCLUSIONS

According to the results obtained in this work, the following conclusions can be drawn:

1. The *in vitro* technique studied has proved to be very repeatable and reliable in our laboratory, more than CF and ADF analyses.
2. Diets containing high amounts of beet pulp or added fat can be estimated by the in vitro technique.
3. Prediction equations obtained for DM and GE digestibility depending on in vitro DM digestibility have proved to be robust when validated with independent data. Therefore they can be recommended for nutritive evaluation of rabbit diets in practice

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Valoracion nutritiva de dietas de conejos mediante metodos in vitro - Se ha estudiado un método enzimático de digestión *in vitro* para estimar el valor nutritivo de los piensos de conejos. Los resultados obtenidos indican una buena correlación y precisión para la predicción del coeficiente de digestibilidad de la MS según la digestibilidad *in vitro* de la MS para 27 dietas (R^2=0,84; drs=1,45), estando el coeficiente de digestibilidad de la EB y la ED peor correlacionados (R^2=0,64 y R^2=0,55, respectivamente). Para valorar la bondad de las predicciones se ha tenido en cuenta no sólo el mejor ajuste, sino también los índices de repetibilidad (r) y de fiabilidad (F) del análisis *in vitro* en nuestro laboratorio; índices que se han comparado con los de los análisis de FB y de FAD. La técnica *in vitro* ha demostrado ser más repetible que los análisis de FB y de FAD (CV_r=0,69% *vs.* CV_r=1,78% y CV_r=1,72%, respectivamente), y también más fiable (CV_F=1,77% *vs.* CV_F=4,26% y CV_F=7,87%, respectivamente).

Al incluir en la ecuación de predicción 14 piensos más, con niveles importantes de pulpa de remolacha (de 10 a 50%) o grasa añadida (3 ó 6%), la buena precisión de la ecuación obtenida (dMS=4,67+0,86vMS; R^2=0,87; drs=1,52; p=0,0001; n=41), indica que este tipo de piensos se pueden estimar mediante la técnica *in vitro*.

Las ecuaciones de predicción de dMS y de dEB obtenidas según la vMS se validaron con cuatro conjuntos de datos independientes (92 piensos). Las ecuaciones resultaron robustas (errores de predicción menores del 5% en todos los conjuntos de datos), indicando su alta capacidad predictora del valor nutritivo de piensos para conejos.