

## **EUROPEAN RING-TEST ON THE CHEMICAL ANALYSES OF TOTAL DIETARY FIBRE AND SOLUBLE FIBRE OF COMPOUND DIETS AND RAW MATERIALS FOR RABBITS**

**Xiccato G.<sup>1\*</sup>, Trocino A.<sup>2</sup>, Tazzoli M.<sup>1</sup>, Majolini D.<sup>1</sup>, Carabaño R.<sup>3</sup>, Villamide M.J.<sup>3</sup>, García J.<sup>3</sup>, Nicodemus N.<sup>3</sup>, Abad R.<sup>3</sup>, Blas E.<sup>4</sup>, Cervera C.<sup>4</sup>, Ródenas L.<sup>4</sup>, Martínez E.<sup>4</sup>, Falcao-E-Cunha L.<sup>5</sup>, Bengala Freire J.P.<sup>5</sup>, Maertens L.<sup>6</sup>, Bannelier C.<sup>7</sup>, Segura M.<sup>7</sup>, Gidenne T.<sup>7</sup>**

<sup>1</sup>Department of. Agronomy, Food, Natural Resources, Animal and Environment (DAFNAE), University of Padova  
Viale dell'Università 16 – I-35020 Legnaro, Padova, Italy

<sup>2</sup>Department of Comparative Veterinary Biomedicine and Food Science (BCA), University of Padova,  
Viale dell'Università 16, I-35020 Legnaro, Padova, Italy

<sup>3</sup>Departamento de Producción Animal, ETSI Agrónomos, University of Madrid, Ciudad Universitaria, 28040 Madrid, Spain

<sup>4</sup>Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

<sup>5</sup>Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Tapada de Ajuda, 1399 Lisboa, Portugal

<sup>6</sup>Institute of Agricultural and Fisheries Research, Scheldeweg 68, 9090 Melle, Belgium

<sup>7</sup>INRA, UMR 1289, Tissus Animaux, Nutrition, Digestion, Ecosystème et Métabolisme (TANDEM), BP 52627, F-31326  
Castanet-Tolosan Cedex, France

\*Corresponding author: gerolamo.xiccato@unipd.it

### **ABSTRACT**

Due to the role of soluble fibre (SF) on the digestive physiology and gut health of post-weaning rabbits and to the lack of reference methods for SF determination, in the framework of the harmonization activity on rabbit science methodologies carried out by EGRAN (European Group on Rabbit Nutrition), a collaborative study was achieved on the determination of total dietary fibre (TDF), aNDF corrected for protein and ash (aNDFcorr) and SF calculated as the difference between TDF and aNDFcorr. Five EGRAN laboratories analysed nine samples: four compound diets and five raw materials (alfalfa meal, wheat bran, grape marc, sunflower meal, sugarbeet pulp) with different levels of fibre fractions and SF. Each sample was analysed 3 runs for TDF and aNDFcorr. TDF was analysed according to the AOAC Method 991.43 using the kit and the procedure of Megazyme®.

TDF averaged 48.2% DM, with one laboratory having higher values (49.3% vs 47.9%;  $P < 0.001$ ). The TDF values varied among samples ( $P < 0.001$ ), from about 40% DM for diets to 60% DM for grape marc and sugarbeet pulp. Repeatability ( $S_R = 0.95\%$  DM) and reproducibility of TDF ( $S_L = 1.68\%$  DM) were good with a low coefficient of variation among laboratory:  $CV_L = 3.9\%$ . The aNDFcorr (mean 37.1% DM) significantly differed from laboratories that used Ankom system (36.4% DM) to laboratories that used Fibertech equipment (38.3% DM). The differences among laboratories due to the equipment and to the corrections for ash and protein explained the poorer repeatability and reproducibility of aNDFcorr determination, with  $CV_L = 6.6\%$ . SF values differed ( $P < 0.001$ ) among laboratories (from 9.6% to 12.0% DM) and samples (from 4.0% DM of wheat bran to 8-11% DM of diets and alfalfa meal to 24.3% DM of sugarbeet pulp). The among-laboratory variability of SF was higher ( $S_L = 2.97\%$  DM;  $CV_L = 26.8\%$ ), due to the variability of both TDF and aNDFcorr analyses.

In conclusion, TDF analysis was characterized by good repeatability and reproducibility, but it was less reproducible in case of raw materials with high SF levels. The among laboratory variation increased with aNDFcorr, because of the differences in analytical equipment and the procedure for protein and ash corrections. Finally SF reproducibility appeared rather good for complete diets and raw materials with low or medium concentration (SF 4-10% DM), but it was affected by the analytical errors of both TDF and aNDFcorr and needs a better harmonization.

**Key words:** Total dietary fibre, soluble fibre, collaborative study, feed composition, EGRAN.

## INTRODUCTION

Since 1992, six laboratories belonging to the European Group on Rabbit Nutrition (EGRAN, <http://www.dcam.upv.es/egran/Default.htm>) have been involved in the harmonisation of scientific methods of research in rabbit nutrition (Perez *et al.*, 1995b; Fernández-Carmona *et al.*, 2005). In this context, different inter-laboratory studies on *in vitro* and *in vivo* evaluation of nutritive value of rabbit feeds (Xiccato *et al.*, 1994; Perez *et al.*, 1995a; Carabaño *et al.*, 2008) have been performed to assess the main causes of variability within and among laboratories. *In vivo* DM digestibility coefficients were in a good accordance among laboratories, but nutrient digestibility coefficients were often very divergent, because of the high analytical variability both in feeds and faeces, especially for fibre and fat determinations (Xiccato *et al.*, 1996). As a consequence, a series of further studies was carried out as well as a detailed revision of analytical methods for rabbit feeds and faeces (EGRAN, 2001).

Recently, the importance of soluble fibre (SF pectins, beta-glucans, etc.) in addition to the insoluble fibre fractions (lignins, cellulose, hemicelluloses) has been emphasised in view of its effects on digestive physiology, growth performance and gut health of post-weaning rabbits (Trocino and García, 2012). In animal feeding, there is a lack of reference methods for these fibre fractions that are quickly fermentable. One solution, compatible with nutrition trials, could be to estimate this fraction by difference between the TDF value (Prosby *et al.*, 1988; Lee *et al.*, 1992) and the aNDFcorr value (corrected for ash and protein content). Thus, EGRAN members have realized a collaborative study to measure the variability within and among laboratories in the determination of total dietary fibre (TDF) and aNDFcorr and in the calculation of the difference to estimate SF.

## MATERIALS AND METHODS

### Samples and analyses

#### Samples

The collaborative study involved five EGRAN laboratories (allocated in Lisbon, Madrid, Padova, Toulouse and Valencia) that analysed nine samples characterized by different levels of insoluble and soluble fibre fractions: four experimental compound diets for growing rabbits (diet 1, diet 2, diet 3, diet 4) and five raw materials (alfalfa meal, wheat bran, grape marc, sunflower meal, sugarbeet pulp). The samples had been also used in previous collaborative studies and stocked under controlled conditions in the EGRAN sample bank (SABA bank: [www.dcam.upv.es/egran/saba.htm](http://www.dcam.upv.es/egran/saba.htm)). The samples were stored and prepared by the INRA team. Each of the laboratories involved in the collaborative study received 50 g per each of the 9 samples (grinded through a 0.5 mm screen and stored in a small plastic bottle with rubber tap). The chemical composition of the diets and raw materials is reported in Table 1.

**Table 1:** Chemical composition of the diets and raw materials (data from SABA bank)

	DM (%)	CP (% DM)	aNDFom* (% DM)	ADF (% DM)	ADL (% DM)
<b>Compound diets:</b>					
Diet 1	91.3	18.4	38.8	19.2	5.1
Diet 2	90.1	18.4	40.2	20.3	3.6
Diet 3	89.7	15.6	33.5	16.5	6.6
Diet 4	90.3	18.2	42.6	22.7	5.1
<b>Raw materials:</b>					
Dehydrated alfalfa meal	89.3	18.5	48.8	32.5	10.8
Wheat bran	86.9	16.7	51.1	18.0	6.2
Grape marc	88.6	10.6	57.6	46.6	33.0
Sunflower meal	89.7	32.2	49.6	30.7	10.4
Sugarbeet pulp	89.4	8.4	37.7	20.9	1.7

\*aNDFom = NDF obtained with an amylolytic pre-treatment and corrected for acid-insoluble ash (AIA)

### Chemical analyses and procedures

The samples were analysed in the same period (March-May 2011) in all laboratories for:

- TDF = residue from enzymatic treatments corrected for protein and ash content (on TDF residue);
- aNDFcorr = aNDF corrected for ash and protein content (on aNDF residue);
- Soluble fibre (SF), calculated as TDF (average of three runs) – aNDFcorr (average of three runs).

TDF analysis was performed according to the AOAC Method 991.43 using the kit and the procedure of Megazyme® (<http://secure.megazyme.com/downloads/en/data/K-TDFR.pdf>). The 9 samples were analysed in sequence in 3 successive runs. In each run, samples were analysed in pair (S1 and S2): the S1 residue was analysed for protein correction and the S2 residue for ash correction. One blank sample was analysed every 5 feed samples. The samples were cooked at ~100°C with heat stable  $\alpha$ -amylase to give gelatinization and depolymerisation of starch; incubated at 60°C with protease (to depolymerise and solubilise proteins) and amyloglucosidase (to hydrolyse starch fragments to glucose); and treated with four volumes of ethanol 95-96% to precipitate soluble fibre and remove depolymerised protein and glucose. The residue was filtered, washed with ethanol 78%, pre-dried with ethanol 95% and acetone, then oven-dried (105°C) and weighed. The residue S1 was analyzed for protein while S2 was incinerated at 525°C to determine ash. The TDF was calculated as the weight of the filtered and dried residue minus the weight of the protein and ash.

Similarly, aNDF was determined in 3 runs on the 9 samples in pairs (S1 and S2) for protein (S1) and ash (S2) correction. aNDF was determined by harmonized procedures with sodium lauryl sulphate (EGRAN, 2001), using thermostable amylase. Three laboratories analysed aNDF using nylon bags and Ankom apparatus and 2 laboratories used Fibertech Tecator® equipment with crucibles. aNDFcorr was determined by subtracting to the NDF residue the content of protein and ash measured on S1 and S2 residues, respectively.

### Statistical analysis

Data for TDF and aNDFcorr were analysed considering the laboratory (n=5), the sample (n=9) and their interaction by using the GLM procedure of SAS (1988). The test of Newman and Keuls was used for mean comparison of the main effects. The statistical model for SF included the fixed effects of laboratory and sample. The repeatability (i.e. within-laboratory s.d.,  $S_R$ ) and the reproducibility (i.e. among-laboratory s.d.,  $S_L$ ) were estimated by using the Mixed procedure of SAS:  $SR = \sqrt{(\sigma_e^2)}$  and  $SL = \sqrt{(\sigma_e^2 + \sigma_l^2 + \sigma_l*s^2)}$ , where the expected variance components of the residual ( $\sigma_e^2$ ), the laboratory ( $\sigma_l^2$ ) and the laboratory x sample interaction ( $\sigma_l*s^2$ ) were calculated. The model for TDF and aNDFcorr included the fixed effect of sample and the random effect of laboratory and sample x laboratory interaction. Since SF was calculated as the difference between TDF (means of 3 runs) and aNDFcorr (means of 3 runs), the repeatability and reproducibility for SF were calculated as the sum of variances (i.e for repeatability  $S_{R(SF)} = \sqrt{(S_{R(TDF)}^2 + S_{R(aNDFcorr)}^2)}$ , assuming  $cov(TDF, aNDFcorr)=0$ ).

## RESULTS AND DISCUSSION

The TDF of the 9 samples averaged 48.2% DM, with laboratories 2-5 that provided similar results (Table 2), while laboratory 1 obtained higher values (49.3 vs. 47.9%;  $P<0.001$ ). The TDF values varied among samples ( $P<0.001$ ) from 39-40% DM, for diets 1 and 3, to 58-60% DM, for grape marc and sugarbeet pulp. TDF analysis showed a low variability both within and among laboratory, with a coefficient of variation among laboratories ( $CV_L$ ) of only 3.9%. The interlaboratory variability was higher for grape marc and sugarbeet pulp, probably because of a hard filtration step. The aNDFcorr value averaged 37.1% DM but laboratories 3 and 5 (35.9% DM), that used Ankom system, differed of laboratories 2 and 4 (38.3% DM), that used Fibertech. Laboratory 1 (Ankom system) obtained intermediate results. The aNDFcorr was always lower than the values of aNDFom reported in Table 1, due to the correction for protein. Together with the differences due to analytical apparatus, these corrections were somewhat different among laboratories and explain the poorer reproducibility ( $S_L = 2.46\%$ ) of aNDFcorr determination, with a higher coefficient of variation among laboratory ( $CV_L = 6.6\%$ ). In previous collaborative studies, the  $S_L$  for NDF determination were similar, varying from 2.51% (Perez *et al.*, 1995a) to 1.53% (Xiccato *et al.* 1996).

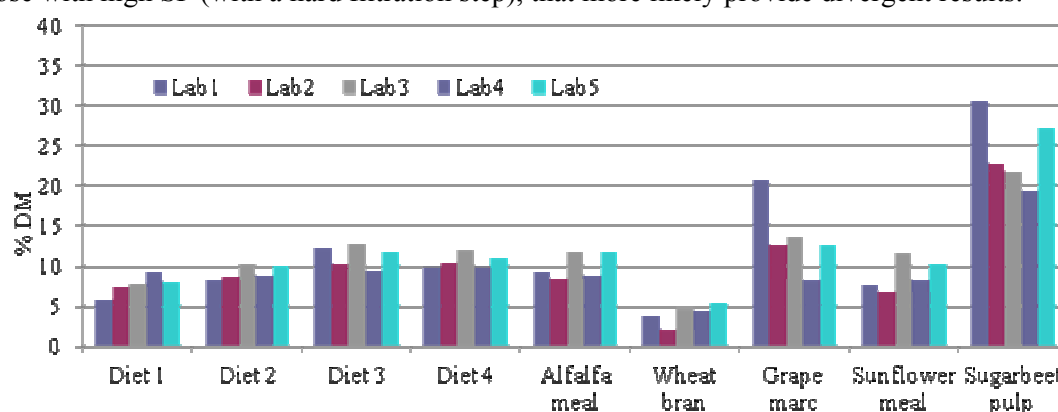
**Table 2:** TDF, aNDFcorr and SF values (%DM) of the 9 samples and interlaboratory variations (% DM)

	TDF	aNDFcorr	SF
Replicates	135	135	45
Laboratory effect:			
Laboratory 1	49.3 <sup>a</sup>	37.3 <sup>b</sup>	12.0
Laboratory 2	48.0 <sup>b</sup>	38.0 <sup>a</sup>	9.9
Laboratory 3	47.7 <sup>b</sup>	35.9 <sup>c</sup>	11.8
Laboratory 4	48.1 <sup>b</sup>	38.5 <sup>a</sup>	9.6
Laboratory 5	47.9 <sup>b</sup>	35.9 <sup>c</sup>	12.0
Samples effect:			
Diet 1	40.7 <sup>g</sup>	31.1 <sup>f</sup>	7.7 <sup>cd</sup>
Diet 2	44.2 <sup>f</sup>	35.1 <sup>e</sup>	9.2 <sup>bc</sup>
Diet 3	39.2 <sup>h</sup>	28.0 <sup>g</sup>	11.3 <sup>bc</sup>
Diet 4	46.4 <sup>e</sup>	35.8 <sup>e</sup>	10.6 <sup>bc</sup>
Dehydrated alfalfa meal	51.3 <sup>c</sup>	41.2 <sup>c</sup>	10.0 <sup>bc</sup>
Wheat bran	46.1 <sup>e</sup>	42.1 <sup>b</sup>	4.0 <sup>d</sup>
Grape marc	58.1 <sup>b</sup>	44.4 <sup>a</sup>	13.6 <sup>b</sup>
Sunflower meal	48.2 <sup>d</sup>	39.3 <sup>d</sup>	8.9 <sup>bcd</sup>
Sugarbeet pulp	59.5 <sup>a</sup>	35.2 <sup>e</sup>	24.3 <sup>a</sup>
Mean	48.2	37.1	11.1
F lab*	11.8	84.8	2.5**
F sample*	833	853	30.7
F lab x sample*	7.0	31.1	-
<i>Repeatability and reproducibility:</i>			
Within laboratory s.d. (repeatability): S <sub>R</sub>	0.95	0.68	1.17 <sup>(1)</sup>
Among laboratory s.d. (reproducibility): S <sub>L</sub>	1.68	2.45	2.97 <sup>(2)</sup>
Variation coefficient among lab. (S <sub>L</sub> /Mean x100): CV <sub>L</sub> (%)	3.9	6.6	26.8

Means with different letters on the same column differ at P=0.05. \*All F values are significant for P<0.001 except \*\* P=0.06. <sup>(1)</sup>S<sub>R(SF)</sub> =  $\sqrt{(S_{R(TDF)}^2 + S_{R(aNDFcorr)}^2)}$ , assuming cov(TDF, aNDFcorr)=0. <sup>(2)</sup>S<sub>L(SF)</sub> =  $\sqrt{(S_{L(TDF)}^2 + S_{L(aNDFcorr)}^2)}$ , assuming cov(TDF, aNDFcorr)=0.

The SF of the 9 samples averaged 11.1% DM and different among them (P<0.001), with the lowest values for wheat bran (4.0% DM) and the highest for sugarbeet pulp (24.3% DM), while diets and other raw materials had intermediate values (8-14% DM). However, the repeatability of SF data is rather complex to be determined because SF was calculated as difference (TDF-aNDFcorr). As TDF and aNDFcorr analyses are independent and there is no run sequence, SF was calculated as the difference among the average of the 3 runs of TDF and aNDF, respectively, corresponding to 45 values. The among-laboratory s.d. resulted higher for SF (2.97% DM), due to the accumulation of variability of both analyses. The CV<sub>L</sub> was even higher (26.8%) due to ratio with the low SF mean.

The differences in SF among laboratories were lower in diets than in raw materials (Fig. 1), especially for those with high SF (with a hard filtration step), that more likely provide divergent results.

**Figure 1:** Interlaboratory variation of SF (%DM) values of the compound diets and raw materials

## CONCLUSIONS

The analysis of TDF was characterized by a good repeatability and reproducibility (1.68% DM), but the analysis of raw materials with high SF level (grape marc and sugarbeet pulp) was affected by a higher variability. The interlaboratory variability increased in the case of aNDF<sub>corr</sub>, probably because of the different apparatus (Fibertech vs Ankom) and some differences in protein and ash corrections among the laboratories. Finally SF precision was rather good for compound diets and raw materials with low or intermediate concentration (SF 4-11% DM), while it was affected by the analytical errors of both TDF and aNDF<sub>corr</sub>. These analyses need a better harmonization for raw materials with high SF concentration.

## REFERENCES

- Carabaño R., Nicodemus N., García J., Xiccato G., Trocino A., Pascual J.J., Falcao-e-Cunha L., Maertens L. 2008. In vitro analysis, an accurate tool to estimate dry matter digestibility in rabbits. Intra- and inter- laboratory variability. *World Rabbit Sci.*, 16, 195-203.
- E.G.R.A.N. 2001. Technical note: Attempts to harmonize chemical analyses of feeds and faeces for rabbits feed evaluation. *World Rabbit Sci.*, 9, 57-64.
- Fernández-Carmona J., Blas E., Pascual J.J., Maertens L., Gidenne T., Xiccato G., García J. 2005. Recommendations and guidelines for applied nutrition experiments in rabbits. *World Rabbit Sci.*, 13, 209-228.
- Lee S.C., Prosky L., Vries J.W. 1992. Determination of total, soluble, and insoluble dietary fiber in foods-enzymatic-gravimetric method, MES-TRIS buffer: collaborative study. *J. Assoc. Anal. Chem.*, 75, 395-416.
- Perez J.M., Cervera C., Falcao E Cunha L., Maertens L., Villamide M.J., Xiccato G. 1995a. European ring-test on in-vivo determination of digestibility in rabbits : reproducibility of a reference method in comparison with domestic laboratory procedures. *World Rabbit Sci.* 3, 171-178.
- Perez J.M., Lebas F., Gidenne T., Maertens L., Xiccato G., Parigi-Bini R., Dalle Zotte A., Cossu M.E., Carazzolo A., Villamide M.J., Carabaño R., Fraga M.J., Ramos M.A., Cervera C., Blas E., Fernández J., Falcao-E-Cunha L., Bengala Freire J. 1995b. European reference method for in-vivo determination of diet digestibility in rabbits. *World Rabbit Sci.* 3, 41-43.
- Prosky L., Asp N.G., Schweizer T., Vries J., Furda I. 1988. Determination of insoluble, soluble and total dietary fibre in foods and foods products: interlaboratory study. *J. Ass. Off. Anal. Chem.*, 71, 1017-1023.
- SAS 1998. SAS/STAT User's Guide (Release 6.03). *SAS Inst. Inc., Cary NC, USA.*
- Trocino A., García J. 2012. Role of soluble fibre in growing rabbits. *In Proc. "10<sup>th</sup> World Rabbit Congress", Sharm-El-Sheik (Egypt), 2012 September, in press.*
- Xiccato G., Cossu M.E., Carazzolo A., Carabaño R., Ramos M. 1994. Evaluation in vitro de la valeur nutritive des aliments pour lapins: efficacité de différents enzymes digestifs. *In Proc. "6<sup>èmes</sup> Journées de la Recherche Cunicole", La Rochelle (France), 1994 December, ITAVI publ., Paris, Vol. 2, 375-383.*
- Xiccato G., Carazzolo A., Cervera C., Falcao E Cunha L., Gidenne T., Maertens L., Perez J.M., Villamide M.J. 1996. European Ring-test on the chemical analyses of feed and faeces: influence on the calculation of nutrient digestibility in rabbits. *In Proc. "6<sup>th</sup> Congress of the World Congress", Toulouse (France), 1996 July, ITAVI publ., Paris, Vol. 1, 293-297.*