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# Technical note: A modified three-step in vitro procedure to determine intestinal digestion of proteins

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**ABSTRACT:** An in vitro, batch incubator (Daisy<sup>II</sup>) was used to simplify the 3-step, in vitro procedure (TSP) to reduce the cost and labor involved in the determination of intestinal digestion of proteins. Four tests were conducted to study the effects of the type of pepsin (P-7012 and P-7000; Sigma, St. Louis, MO), the type of bags used for the incubation of samples (R510 and F57; Ankom Technology, Fairport, NY), the amount of sample per bag (0.5, 1, 2, or 5 g), and the number of bags per incubation bottle (5, 15, 20, or 30 bags) on the estimated intestinal digestion of proteins. A soybean meal sample heated at 170°C for 0, 0.5, 1, 2, 4, 6, or 8 h was used in all preliminary tests to determine the optimum conditions of the technique. The intestinal digestion of 12 protein supplements was determined using the Daisy<sup>II</sup> as well as the proposed TSP techniques. Results using the 2 types of pepsin were highly correlated: P-7012 =  $(0.99 \pm 0.04 \times \text{P-7000}) - 0.29 \pm 2.33$  ( $r^2 = 0.99$ ,  $P < 0.001$ ,

$n = 14$ ). Intestinal digestion of soybean meal samples obtained from the TSP assay were highly correlated with those obtained using the Daisy<sup>II</sup> incubator with Ankom R510 bags:  $\text{Daisy}_{\text{R510}} = (1.37 \pm 0.06 \times \text{TSP}) - 15.45 \pm 3.85$  ( $r^2 = 0.98$ ,  $P < 0.001$ ,  $n = 14$ ); and Ankom F57 bags:  $\text{Daisy}_{\text{F57}} = (1.33 \pm 0.06 \times \text{TSP}) - 15.76 \pm 3.87$  ( $r^2 = 0.98$ ,  $P < 0.001$ ,  $n = 14$ ). Although there was a bias in these equations, when the whole protocol was applied to the determination of intestinal digestion of the 12 protein supplements using the TSP or the Daisy<sup>II</sup> technique with the Ankom R510 bags, the data were highly correlated:  $(0.93 \pm 0.12 \times \text{TSP}) + 6.78 \pm 9.09$  ( $r^2 = 0.84$ ,  $P < 0.001$ ,  $n = 12$ ). The amount of sample per bag and the number of bags per incubation bottle did not affect the estimates of intestinal digestion of proteins. These results indicate that the use of up to 30 nylon bags (Ankom R510) with 5 g of sample in each Daisy<sup>II</sup> incubation bottle could be used to estimate intestinal digestion of proteins in ruminants.

**Key words:** in vitro, intestinal digestion, protein

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## INTRODUCTION

Absorbed AA are the building blocks for the synthesis of tissue and milk proteins (Schwab, 1996; NRC, 2001). Microbial protein synthesized in the rumen has a relatively constant AA profile and digestibility, and supplies 50 to 80% of absorbable AA flowing to the small intestine (Schwab, 1996). Rumen-undegradable dietary protein is the second major source of AA supplied to the small intestine. However, the intestinal digestibility of rumen-undegraded protein is highly variable depending on type of feed and processing. Protein evaluation systems have incorporated the value of intestinal digestion of proteins (INRA, 1989; NRC, 2001). Calsamiglia and Stern (1995) developed a 3-step in vitro proce-

dure (TSP) to estimate intestinal digestion of proteins in ruminants. This procedure was adopted by the NRC (2001) as a reference method. However, the method uses trichloroacetic acid, a highly corrosive and toxic acid for humans and the environment, and could be improved to reduce the cost and labor involved in routine analysis in commercial laboratories. The objective of this study was to modify the TSP of Calsamiglia and Stern (1995), by adapting it to a Daisy<sup>II</sup> incubator, to further reduce the cost and labor involved in the determination of intestinal digestion of proteins.

## MATERIALS AND METHODS

### Modified Pepsin-Pancreatin Digestion Procedure

A soybean meal (SBM) sample was heated at 170°C for 0, 0.5, 1, 2, 4, 6, or 8 h, and used in all preliminary tests to establish the optimum conditions of the pepsin-pancreatin digestion technique. Four tests were con-

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ducted to study the effects of the type of pepsin, the type of bags used for the incubation of samples, the amount of sample per bag, and the number of bags per incubation bottle on the estimated intestinal digestion of these SBM samples.

**Type of Pepsin.** The pepsin-pancreatin procedure of Calsamiglia and Stern (1995) is conducted with 15 mL of a solution containing an expensive, high enzymatic activity pepsin (P-7012, Sigma, St. Louis, MO). In contrast, the Daisy<sup>II</sup> requires 2 L of pepsin solution per bottle, making the technique more expensive. However, a less purified pepsin (P-7000, Sigma) is available at a cost 22 times less per unit of activity compared with P-7012, and may also be suitable for the technique.

A comparative study was conducted in 2 replicated periods to determine the effect of the type of pepsin on the intestinal digestion of the different SBM samples. In each period, the pepsin-pancreatin digestion was determined in triplicate following the procedures of Calsamiglia and Stern (1995) using 1 g/L of P-7012 or P-7000 pepsin. Results obtained with the use of the P-7000 pepsin were regressed on estimates obtained with the use of the P-7012 pepsin. Regression analysis was conducted using the GLM procedures of SAS (version 8.2, SAS Inst., Inc., Cary, NC), and the CORR procedure was used to determine the correlation between the 2 types of enzyme.

**Type of Bags.** The pepsin-pancreatin digestion procedure (Calsamiglia and Stern, 1995) was adapted to the Daisy<sup>II</sup> incubator (Ankom, Fairport, NY) using 2 types of bags to determine the intestinal digestion of the different SBM samples. Two replicated periods were conducted. In each experimental period, 0.5-g samples were weighed into bags for fiber analysis (Ankom F57, 50- $\mu$ m pore size) in triplicate, and the same procedure was conducted using nylon bags (Ankom R510, 10  $\times$  5 cm, 50- $\mu$ m pore size) cut in half, resulting in two 5  $\times$  5 cm bags. Three empty bags of each type were used as blanks within each run.

A total of 24 bags were introduced into each incubation bottle containing 2 L of a 0.1 N HCl solution adjusted to pH 1.9 with 1 g/L of pepsin (P-7000), and were incubated for 1 h with constant rotation at 39°C. After incubation, the bags were rinsed with tap water and introduced into the incubation bottles (24 bags per bottle) containing 2 L of a pancreatin solution (0.5 M KH<sub>2</sub>PO<sub>4</sub> buffer adjusted to pH 7.75, containing 50 ppm of thymol and 3 g/L of pancreatin (P-7545, Sigma). Bags were incubated for 24 h with constant rotation at 39°C.

After incubation, bags were rinsed with tap water until the runoff was clear. The original samples and the residue in all bags were analyzed for N content by the Kjeldahl method (AOAC, 1990). Pepsin-pancreatin digestion of N was calculated as the N in the original sample minus the N remaining after pepsin-pancreatin incubation divided by the N in the original sample. The values obtained were compared with those obtained for the same samples using the TSP (Calsamiglia and Stern, 1995) by regression and correlation analyses us-

ing the GLM and the CORR procedures of SAS, respectively.

**Amount of Sample per Bag.** Samples of SBM heated at 170°C for 0, 2, or 8 h were incubated in nylon bags (Ankom R510, pore size 50  $\mu$ m) in the Daisy<sup>II</sup> incubator to determine the effect of the amount of sample per bag on the intestinal digestion of samples with different intestinal digestibilities. Treatments were 0.5 and 1 g of sample weighed into small (5  $\times$  5 cm) nylon bags; and 1, 2, or 5 g of sample weighed into large (5  $\times$  10 cm) nylon bags. Larger bags were considered because they allowed the use of a larger sample size for further analysis after intestinal digestion. Intestinal digestion of the samples was determined in triplicate by the Daisy<sup>II</sup> technique in 2 replicated periods. Results for each treatment were compared within samples by the GLM procedure of SAS.

**Number of Samples per Incubation Bottle.** Heat-treated (170°C for 1 h) SBM was used for this test. Treatments consisted of introducing 5, 15, 20, or 30 nylon bags (Ankom R510, 5  $\times$  10 cm) filled with 5 g of sample into each incubation bottle. The pepsin-pancreatin digestion was conducted in the Daisy<sup>II</sup> incubator in 2 consecutive periods following the procedure explained previously. After incubation, 5 bags of each treatment were randomly selected to obtain the pepsin-pancreatin digestibility values. Treatment differences were compared using the GLM procedure of SAS.

### Ruminal Incubation

The research protocol was approved by the Institutional Animal Care and Use Committee of the Universitat Autònoma de Barcelona.

Calsamiglia and Stern (1995) justified the need for a ruminal incubation of samples before the pepsin-pancreatin digestion. Therefore, after determining the optimal conditions of the pepsin-pancreatin test, an experiment was conducted to determine the effect of ruminal preincubation on pepsin-pancreatin digestion of feed protein determined by the Daisy<sup>II</sup> technique.

The protein supplements used for this test were blood meal, fish meal, green peas, lupin seeds, whole cottonseed, corn gluten meal, alfalfa pellets, heat-processed SBM, sunflower seeds, barley dried distillers grains, corn dried distillers grains, and corn gluten feed. Approximately 5 g of each protein source (ground through a 2-mm screen) was weighed into 5  $\times$  10-cm nylon bags (Ankom R510, pore size 50  $\mu$ m). Depending on N content and CP degradability of the feed tested, 25 to 33 bags per feedstuff were suspended in the rumen of a cannulated multiparous Holstein cow. An incubation of 12 h was selected because this length of incubation represents the residue that escapes ruminal degradation (Maiga et al., 1996; O'Mara et al., 1997). In addition, Calsamiglia and Stern (1995) detected no differences in pepsin-pancreatin digestion of proteins when samples were preincubated in the rumen for 12 to 18 h.

After 12 h of ruminal incubation, the bags were rinsed 3 times (5 min each) in an automatic washing machine. Because the microbial contamination of feeds may be important in high-fiber, low-protein feeds, these should be processed so that microbial contamination is reduced (Huntington and Givens, 1995). Therefore, the bags were suspended in a 0.1% methylcellulose solution, and incubated in a shaking water bath at 37°C for 30 min to remove solid, attached bacteria (Whitehouse et al., 1994). The bags were stored at -18°C until subsequent analyses.

Before the pepsin-pancreatin incubation step, the bags were thawed and machine-washed 3 times (5 min each) to remove detached bacteria. The bags were oven-dried at 55°C for 48 h. Remaining residues were composited within feedstuff. Intestinal digestion of the residual CP was determined by quadruplicate analysis using optimal conditions of the Daisy<sup>II</sup> pepsin-pancreatin procedure. Results were compared with those obtained when the same protein supplements were processed without ruminal exposure. Differences between the intestinal digestion of samples with or without ruminal exposure were analyzed by the GLM procedure of SAS. Significance was declared at  $P < 0.05$ , and the Tukey's test (Tukey, 1953) was used to separate the means.

### *Comparison of TSP and Daisy<sup>II</sup> Protein Digestibility Values*

The rumen-incubated residues of the same 12 protein supplements used in the previous test were processed by the TSP of Calsamiglia and Stern (1995), and the results were compared with those obtained by the Daisy<sup>II</sup> technique using the GLM procedure of SAS. The correlation between the 2 techniques was determined by the CORR procedure.

### *Chemical Analyses*

All the SBM samples used in the preliminary tests and all the protein supplements and rumen and pepsin-pancreatin incubation residues obtained in the ruminal incubation test were analyzed for DM (24 h in a 103°C forced-air oven) and N (Kjeldahl method; AOAC, 1990) content.

## RESULTS AND DISCUSSION

The SBM samples processed at 170°C for different times provided a wide range of pepsin-pancreatin digestions (0 h = 82.2%, 0.5 h = 78.5%, 1 h = 78.4%, 2 h = 57.4%, 4 h = 29.7%, 6 h = 23.9%, and 8 h = 10.7%) when using the procedure of Calsamiglia and Stern (1995).

### *Modified Pepsin-Pancreatin Digestion Procedure*

**Type of Pepsin.** Results obtained with the use of the pepsin of lower enzymatic activity were highly corre-

lated ( $P-7012 = [0.99 \pm 0.04 \times P-7000] - 0.29 \pm 2.33$ ;  $r^2 = 0.99$ ,  $P < 0.001$ ,  $n = 14$ ) to results obtained with the use of the high enzymatic activity pepsin, suggesting that P-7000 could be used in the TSP of Calsamiglia and Stern (1995) without affecting the results and at a lower cost (P-7000 is 22 times cheaper than P-7012 per unit of enzymatic activity).

**Type of Bags.** The results obtained with the Daisy<sup>II</sup> technique using nylon bags (Ankom R510) were correlated [ $TSP = (0.713 \pm 0.032 \times Daisy_{R510}) + 12.50 \pm 2.14$ ;  $r^2 = 0.98$ ,  $P < 0.001$ ,  $n = 14$ ] to results obtained with the TSP. A relation was also observed when using Ankom F57 bags for fiber analysis [ $TSP = (0.731 \pm 0.034 \times Daisy_{F57}) + 12.38 \pm 2.22$ ;  $r^2 = 0.98$ ,  $P < 0.001$ ,  $n = 14$ ]. Although the intercept was different from 0 ( $P < 0.05$ ) and the slope different ( $P < 0.05$ ) from 1, the relationship suggests that intestinal digestion of proteins estimated with the Daisy<sup>II</sup> technique using either type of bag would rank feeds according to their intestinal digestibility, but actual values would need to be transformed using the equation provided. In practical conditions, the use of the larger nylon bags (Ankom R510) allows for the determination of intestinal digestion in the same bag used for the preincubation in the rumen and the incubation of a larger sample (up to 5 g), reducing the labor involved. In addition, the determination of the digested protein by filtration eliminates the need to use trichloroacetic acid, an acid that is classified as highly corrosive, toxic, and environmentally unfriendly International Chemical Safety Cards (WHO/IPCS/ILO; ICSC-0586).

**Amount of Sample per Bag.** The pepsin-pancreatin digestion of SBM protein in the Daisy<sup>II</sup> was not affected by the amounts of sample per bag tested. Estimated intestinal digestion obtained with SBM was 97.9 ( $\pm 0.40$ ), 97.5 ( $\pm 0.17$ ), 97.5 ( $\pm 0.48$ ), 97.3 ( $\pm 0.18$ ), and 97.3 ( $\pm 0.21$ ) %, respectively, for small nylon bags containing 0.5 or 1.0 g of sample, and large nylon bags containing 1.0, 2.0, or 5.0 g of sample. Intestinal digestion obtained with SBM heated at 170°C for 2 h was 58.6 ( $\pm 1.76$ ), 59.1 ( $\pm 1.40$ ), 60.3 ( $\pm 1.72$ ), 57.3 ( $\pm 1.5$ ), and 56.4 ( $\pm 1.29$ ) %, respectively, for small nylon bags containing 0.5 or 1.0 g of sample, and large nylon bags containing 1.0, 2.0, or 5.0 g of sample, respectively. Results obtained for SBM heated at 170°C for 8 h were 9.2 ( $\pm 1.17$ ), 9.0 ( $\pm 0.90$ ), 8.2 ( $\pm 0.65$ ), 9.1 ( $\pm 1.02$ ), and 8.3 ( $\pm 0.43$ ) %, respectively, for small nylon bags containing 0.5 or 1.0 g of sample, and large nylon bags containing 1.0, 2.0 or 5.0 g of sample.

**Number of Samples per Incubation Bottle.** The number of bags introduced per incubation bottle had no effect on the pepsin-pancreatin digestion of a heat-treated SBM sample estimated by the Daisy<sup>II</sup> technique (55.6  $\pm$  1.01, 56.6  $\pm$  0.92, 55.9  $\pm$  1.80, and 55.9  $\pm$  1.66% for bottles containing 5, 15, 20, or 30 bags, respectively). This result suggests that the use of up to 30 bags per incubation bottle is possible when estimating the intestinal digestion of proteins by the Daisy<sup>II</sup> technique.



**Table 1.** Intestinal CP digestion (%) determined by the Daisy<sup>II</sup> technique of original protein supplement samples (O) and of rumen preincubated samples (R)

Protein source	Type of sample		SEM	P-value
	O	R		
Blood meal	69.61 <sup>b</sup>	45.74 <sup>a</sup>	1.26	***
Green peas	72.57 <sup>a</sup>	82.45 <sup>b</sup>	0.31	***
Lupin seeds	78.34 <sup>a</sup>	88.20 <sup>b</sup>	0.16	***
Whole cottonseed	84.96 <sup>b</sup>	35.62 <sup>a</sup>	0.26	***
Corn gluten meal	93.20 <sup>b</sup>	79.22 <sup>a</sup>	1.90	**
Alfalfa pellets	84.74 <sup>b</sup>	59.70 <sup>a</sup>	0.39	***
Heat-treated soybean meal	98.12 <sup>b</sup>	96.33 <sup>a</sup>	0.17	***
Sunflower seeds	93.57 <sup>b</sup>	82.99 <sup>a</sup>	0.10	***
Barley dried distillers grains	95.60 <sup>b</sup>	65.60 <sup>a</sup>	0.18	***
Corn gluten feed	89.53 <sup>b</sup>	70.49 <sup>a</sup>	0.18	***
Corn dried distillers grains	76.78 <sup>b</sup>	56.32 <sup>a</sup>	0.67	***
Fish meal	93.98 <sup>b</sup>	91.46 <sup>a</sup>	0.64	*

<sup>a,b</sup>Means within the same row lacking common superscripts differ ( $P < 0.05$ ).

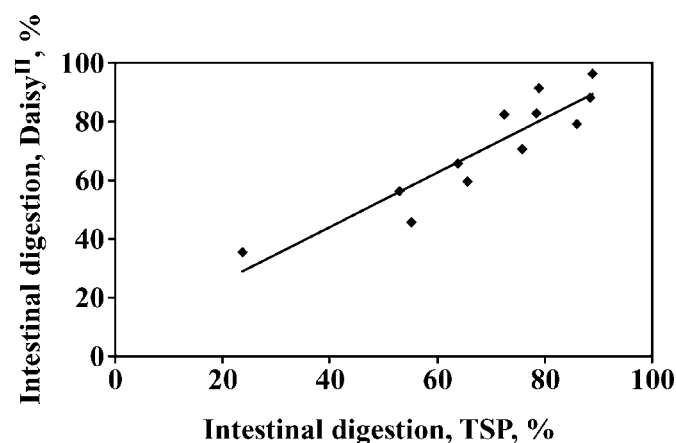
\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

### Ruminal Incubation

Pepsin-pancreatin digestion of CP remaining after 12 h of ruminal incubation was lower ( $P < 0.05$ ) compared with digestion of samples not incubated in the rumen for all protein supplements except for green peas and lupin seeds (Table 1). The reduction in pepsin-pancreatin digestion after ruminal preincubation of some feedstuffs has been reported previously (de Boer et al., 1987; Calsamiglia and Stern, 1995). Results of this experiment confirm that preincubation of feeds in the rumen is recommended to estimate intestinal digestion of the rumen-undegradable protein fraction of feeds by the Daisy<sup>II</sup> technique.

### Comparison of TSP and Daisy<sup>II</sup> Protein Digestibility Values

In this experiment, 12 protein supplements (including the unprocessed SBM and 1 heat-processed SBM



**Figure 1.** Regression showing the relationship between the intestinal digestion (%) of 12 protein supplements determined by the Daisy<sup>II</sup> technique and by the 3-step procedure (TSP);  $TSP = (0.905 \pm 0.12 \times \text{Daisy}^{\text{II}}) + 4.75 \pm 9.09$ ;  $r^2 = 0.84$ ,  $P < 0.001$ ,  $n = 12$ .

from the previous experiment) were used to represent common feeds in the field. Intestinal CP digestion values determined by the Daisy<sup>II</sup> technique are shown in Table 1. The average estimate of intestinal CP digestion in the Daisy<sup>II</sup> (71.2 %) was similar to the TSP (69.2%), and these values were correlated [ $TSP = (0.905 \pm 0.12 \times \text{Daisy}^{\text{II}}) + 4.75 \pm 9.09$ ;  $r^2 = 0.84$ ,  $P < 0.001$ ,  $n = 12$ ], with the slope not different from 1 and the intercept not different from 0 (Figure 1). This relationship indicates that the Daisy<sup>II</sup> technique can be used to predict the intestinal digestion of proteins, reducing the cost and labor involved in the process.

### IMPLICATIONS

The Daisy<sup>II</sup> technique can be used to determine intestinal digestion of crude protein. Compared with the original 3-step procedure, the modifications introduced resulted in substantial reductions in cost and labor. In addition, this procedure eliminated the need to use trichloroacetic acid, an acid that is classified as highly corrosive, toxic, and environmentally unfriendly.

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### APPENDIX 1. PROTOCOL OF THE DAISY<sup>II</sup> PROCEDURE

1. Weigh approximately 5 g of feed (ground through a 2-mm screen) into 5 × 10-cm nylon bags (Ankom R510, pore size 50 μm; Ankom, Fairport, NY) and suspend them in the rumen for 12 h. The number of bags required to provide enough residue per feedstuff will depend on the N content and CP degradability of the feed tested.
2. After ruminal incubation, rinse the bags for 5 min 3 times in an automatic washing machine (or until the runoff is clear). To determine AA concentration in high-fiber, low-protein feeds, suspend bags in a 0.1% methylcellulose solution and incubate in a shaking water bath at 37°C for 30 min to help in the detachment of particle-associated bacteria. Rinse bags with tap water and store them at –18°C until subsequent analyses. Thaw and wash all bags for 5 min 3 times in an automatic washing machine (or until the runoff is clear). Allow to drain, and dry in an oven at 55°C for 48 h. Pool the residues from the bags, composite them by feedstuff and determine the N content by the Kjeldahl method (AOAC, 1990).
3. Weigh 0.5 to 5 g of rumen-exposed residue into nylon bags (Ankom R510, pore size 50 μm) and heat-seal. Place up to 30 bags in each incubation bottle of a Daisy<sup>II</sup> incubator (Ankom). Add 2 L of a prewarmed 0.1 N HCl solution (pH 1.9) containing 1 g/L of pepsin (P-7000, Sigma, St. Louis, MO) and incubate with constant rotation at 39°C for 1 h.
4. After incubation, drain all liquid, and rinse the bags with tap water until the runoff is clear.
5. Introduce the bags into the incubation bottles (a maximum of 30 bags per bottle), and add 2 L of a prewarmed pancreatin solution (0.5 M KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.75, containing 50 ppm of thymol and 3 g/L of pancreatin; Sigma P-7545). Incubate bags for 24 h with constant rotation at 39°C.
6. After incubation, drain all liquid, and rinse the bags with tap water until the runoff is clear.
7. Allow the bags to drain, and dry in an oven at 55°C for 48 h. Record the dry weight. Analyze the residue in all bags for N content by the Kjeldahl method (AOAC, 1990).
8. Calculate the pepsin-pancreatin digestion of protein as the amount of the sample N (rumen-exposed residue) minus the N remaining after pepsin-pancreatin incubation divided by the amount of sample N.

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