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Comparison of Techniques to Determine the Ruminal and Post-Ruminal Protein Disappearance of Various Oilseed Meals

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Abstract: Ruminal, post-ruminal and total tract crude protein disappearance of cottonseed meal containing 105 (CSMH) or 65 (CSML) g oil per kg of dry matter (DM), soybean meal (SBM), rapeseed meal (RSM) and sunflower meal (SFM), originating from Iranian plant varieties, were measured using *in situ* mobile bag, three-step *in situ*/*in vitro* (3-step) and *in vitro* enzymatic procedures. For the *in situ* mobile bag technique, 4 Holstein steers (450±50 kg, body weight) fitted with ruminal fistulae and T-shaped intestinal cannulae were used. Three-step procedure was followed by rumen incubation of samples for 12 h and enzymatic incubation of ruminal undegradable samples. *In vitro* enzymatic technique was followed by 1 h borate- phosphate buffer incubation, then 4 h in protease solution as ruminal disappearance and enzymatic (pepsin and pancreatin) incubation of ruminal undegradable samples. A significant ($p<0.01$) effect was found between the procedures to measure ruminal, post-ruminal and total tract CP disappearance. The protein disappearance of oilseed meals in the rumen and post-rumen determined *in vitro* enzymatic procedure tended to be higher than 3-step technique. Post-ruminal protein disappearance measured with *in vitro* procedure was higher ($p<0.01$) than *in situ* mobile bag and 3-step techniques (*In vitro* = 0.75, *In situ* mobile bag = 0.74 and 3-step = 0.60; S.E.M = 0.023, respectively). There was a significant ($p<0.01$) effect of feeds on ruminal, post-ruminal and total tract CP disappearance. The CSMH had a lower ($p<0.01$) ruminal CP disappearance, while the SFM had higher value (0.58 and 0.91, respectively). The CSMH had lower ruminal CP disappearance compared with CSML (0.58 vs. 0.68, respectively). The CSML had a lower ($p<0.01$) post-ruminal CP disappearance, while the SBM had higher value (0.59 and 0.81, respectively). In addition, our results indicated that when used to assess post-ruminal disappearance of oilseed meal protein, the 3-step method can underestimate the disappearance of protein.

Key words: Protein disappearance, 3-step procedure, *in vitro* enzymatic procedure, oilseed meal

INTRODUCTION

The current feed evaluation systems recognize the need to estimate the protein value as the amount of ruminal and post-ruminal protein disappearance (CNCPS, 1992; NRC, 2001). *In vivo* and *in vitro* techniques to measure ruminal and intestinal protein disappearance have been markedly developed (Calsamiglia and Stern, 1995; Gargallo *et al*., 2006, Hvelplund *et al*., 1994; McNiven *et al*., 2002). The Mobile Nylon Bag technique (MNB) is easy to use and provides an estimation of ruminal, post-ruminal and total tract Crud Protein (CP) and amino acid disappearance (Fathi Nasri *et al*., 2008; Riasi *et al*., 2008; Taghizadeh *et al*., 2005) and correlated well with *in vivo* true intestinal nitrogen disappearance (Hvelplund *et al*., 1994). However, the cost and requires access to ruminal fistulaed and intestinal cannulated steers or cows, as well as animal welfare issues and time consuming for routine feed evaluation, have led to develop various laboratory methods (Calsamiglia and Stern, 1995; Gargallo *et al*., 2006; McNiven *et al*., 2002; Tejido *et al*., 2002). Numerous of *in vitro* enzymatic procedures are currently used to estimate ruminal and post-ruminal protein disappearance (Calsamiglia and Stern, 1995; Danesh Mesgarana and Stern, 2005; Gargallo *et al*., 2006; McNiven *et al*., 2002). In addition, these methods estimate true digestibility of feed protein (Danesh Mesgarana and Stern, 2005). Calsamiglia and Stern (1995) suggested a three step *in situ*/*in vitro* enzymatic procedure (3-step). This method has been applied by some researchers to numerous feeds (Calsamiglia and Stern, 1995; Danesh Mesgarana and Stern, 2005; Danesh Mesgaran *et al*., 2007; Jahani-Azizabadi *et al*., 2007; Yu *et al*., 1999).

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McNiven *et al.* (2002) recommended an *in vitro* enzymatic procedure to estimate ruminal, post-ruminal and total tract CP disappearance. However, in these methods factors that might influence the extent of ruminal and post-ruminal protein disappearance such as nature of feed and protein fractions are not considered (Mathis *et al.*, 2001; Merkel *et al.*, 1999; Palmer and Jones, 2000; Stern *et al.*, 1997). Oilseed meals are a major source of protein supplement in ruminant diets (Danesh Mesgarana and Stern, 2005). These feed sources have different oil content because of processing method to use to extract the oil from seeds. Previous results indicated that unsaturated fatty acids, released from triglycerides during microbial hydrolysis in the rumen, might have negative effects on ruminal degradation (Maczulak *et al.*, 1981). Danesh Mesgaran *et al.* (2007) showed that ruminal, post-ruminal and total tract CP disappearance values of cottonseed meal reduced with increase in oil content. The aim of the present study was to compare the procedures including MNB, 3-step and *in vitro* enzymatic to determine the ruminal and post-ruminal protein disappearance of various oilseed meal [cottonseed meal containing 105 (CSMH) or 65 (CSML) g oil per kg of DM, sunflower meal (SFM), soybean meal (SBM) and rapeseed meal (RSM)].

MATERIALS AND METHODS

Experimental feeds and chemical analysis: Oilseed meal samples, produced in various Iranian oil industries, were cottonseed meal [105 and 65 g oil kg^{-1} dry matter (DM); CSMH and CSML, respectively], rapeseed meal (RSM), soybean meal (SBM) and sunflower meal (SFM). Samples were dried using a forced-air oven at 60°C for 48 h. All feed samples were ground to pass through a 2-mm screen, then, analyzed for total N (Kjeldahl method, Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden), Neutral Detergent Fiber [NDF] (Van Soest *et al.*, 1991), Ether Extract [EE] (AOAC, 2000, ID 920.39) and ash (AOAC, 2000, ID 942.05).

***In situ* and *in vitro* protein disappearance:** *In situ* mobile nylon bag technique (Subuh *et al.*, 1996), *in vitro* enzyme procedure (McNiven *et al.*, 2002) and three-step *in situ*/*in vitro* enzyme procedure (Calsamiglia and Stern, 1995) were used to estimate ruminal, post-ruminal and total tract protein disappearance of the samples.

Mobile nylon bag technique: Disappearance of protein from the samples was determined using *in situ* mobile bag procedure as described by Subuh *et al.* (1996). Four Holstein steers (395±13 kg) fitted with ruminal fistulae and T-shaped intestinal cannulate were used. Animals were fed 5.1 kg of DM of alfalfa hay, 3.2 kg of DM maize silage and 2.5 kg of DM concentrate (170 g CP kg^{-1} of DM) per head per day, at 8.00 and 18.00 h. Samples were ground to pass a 2-mm screen. Approximately 6 g, DM, of each sample (6 bags per each feed) were placed in polyester bag (9×17 cm, with pore size of 50 μm) and incubated in the rumen for 12 h. All bags were placed simultaneously in the rumen before the morning feeding. After removal from the rumen, they were washed with tap water and subsequently dried using a forced-air oven (60°C, 48 h). A part of the feed residual of each rumen incubated bag was taken and 1 g was placed in mobile bag (3 × 6 cm, 52 μm pore size, 8 bags per sample). Bags were closed by heat sealing and inserted into the small intestine via the intestinal cannulae at the rate of one bag every 30 min, then, removed from the voided faces and rinsed in tap water. The bags were dried using a forced-air oven (60°C, 48 h) and weighed to determine DM disappearance. Nitrogen (N) concentration of un-incubated, rumen and post- rumen incubated samples was determined by kjeldahl method (Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden).

***In vitro* procedure:** This part of the experiment was followed by using the *in vitro* enzymatic procedure of McNiven *et al.* (2002). Approximately, 1 g of each sample was weighed into polyester bags with a pore size of 22 μm (6 bags per sample) and then heat-sealed. Thirty bags were placed into a bottle (2.4 L volume), containing 1.6 L borate–phosphate buffer [2-sodium tetraborate (Na2B4O7, 10 H2O; 13.17 g L^{-1}) + 1-monosodium phosphate (NaH2PO4, H2O; 7.604 g L^{-1})]. Bags were incubated in the buffer for 1 h in a shaking incubator at 38.6°C (Parsazma, Iran). Then, 400 mL of protease (Sigma P-5147, St. Louis, MO, USA) solution (1980 unit protease in 400 mL of borate–phosphate buffer) were added. The incubation was continued for four additional h at 39 °C in the shaking incubator. Liquid from each bottle was decanted off and bags were rinsed three times with distilled water and 3 bags from each sample dried in a forced-air oven at 64 °C for 48 h. Remaining bags were placed in a pepsin solution [1.6 g of pepsin (Sigma P-7000) in 800 mL of a 0.1 N HCl (for 30 bags)]. Bags were incubated for 1 h at 39 °C in the shaking incubator. Then 40 mL of 1 N NaOH and 1 L of the pancreatin solution was added. The pancreatin solution was prepared by dissolving 68 g of KH_2PO_4 and 6 g of pancreatin (Sigma, P-7545) in 1 L of distilled water (the pH was adjusted to 7.8 with adding strong NaOH). Bags were incubated for 24 h at 39 °C in the shaking incubator. After incubation, liquid from each bottle was decanted and bags were washed 6 times with distilled water. Bags were dried in a forced-air oven at 64°C for 48 h. Nitrogen

concentration of each bag was determined using the Kjeldahl method (Kjeltec 2300 Autoanalyzer, FossTecator AB, Hoganas, Sweden).

Three-step procedure: This part of the experiment was conducted using the procedure of Calsamiglia and Stern (1995). Sample from ruminal residue after 12 h incubation (as described in mobile bag technique) was taken for N analysis using kjeldahl method (Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden). Sample of ruminal un-degradable CP was weighed into a 50 mL polypropylene centrifuge tube (each sample contained 15 mg of N). Two blank tubes were also prepared to correct the N contribution of the enzymes. Ten milliliters of pre-warmed (37°C) HCl–pepsin solution [2 g of pepsin (Merck M-785) dissolved into 1 L of 0.1N HCl] was placed in each tube. Tubes were vortexed and incubated for 1 h in a shaking incubator at 38.6°C (Parsazma, Iran). After 1 h incubation, 0.5 mL of 1 N NaOH solution was added to each tube, then, vortexed. The procedure continued by adding 13.5 mL of phosphate–pancreatin buffer (68 g of KH_2PO_4 per 1 L of distilled water, 37°C), pH was adjusted to 7.8 with strong NaOH, followed by the addition of 6 g of pancreatin (Merck, M-7130)]. Tubes were vortexed and incubated for 24 h in a shaking incubator at 38.6°C. After incubation, 3 mL of trichloroacetic acid (TCA) solution (100 g of TCA/100 mL of distilled water) was added to each tube, then vortexed. The tubes were left for 15 min and then centrifuged at 10,000×g for 15 min. A part of the supernatant (5 mL) was pipetted from each tube to determine the N concentration using Kjeldahl method (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden).

Calculation and statistical analysis: Calculations as described by Subuh *et al.* (1996) were used for ruminal, post-ruminal and total tract protein disappearance using the mobile bag technique. Data of 3-step enzymatic procedure were calculated as described by Calsamiglia and Stern (1995). Data of *in vitro* enzymatic procedure was calculated as described by McNiven *et al.* (2002). Data were analyzed using the general linear models procedure of SAS (1999) with the following statistical model of Y = overall mean + block effect of procedure + feed effect within block effect + residual error, assumed normally and independently distributed.

RESULTS AND DISCUSSION

Chemical compositions (g kg^{-1} of DM) of the various oilseed meals evaluated in the present study are shown in Table 1.

Ruminal, post-ruminal and total tract CP disappearance of various oilseed meals are shown in Table 2-4, respectively. A significant ($p<0.01$) difference was found between the procedures to measure ruminal CP disappearance (*In situ* bag = 0.67 and *in vitro* = 0.83; S.E.M = 0.021). In recent years, the *in situ* mobile bag method has frequently been used for protein evaluation in ruminant. This method provides a valuable data of protein disappearance (Hvelplud, 1985; De Boer *et al*., 1987). However, it requires ruminal access and is still time consuming for routine evaluation of feeds. McNiven *et al*. (2002) indicated that 1 h incubation in buffer solution plus 4 h incubation in protease enzyme gave a high correlation with ruminal *in situ* bag nitrogen disappearance. Danesh Mesgarana and Stern (2005) compared results of the

Table 1: Chemical composition of various oilseed meals originated from Iranian plant (g kg^{-1} of DM)

Feed	Crude protein	Neutral detergent fiber	Acid detergent fiber	Ether extracted	Ash
Cotton seed meal high oil (105 g kg^{-1} of DM)	247	420	320	105	50
Cotton seed meal low oil (65 g kg^{-1} of DM)	282	450	360	65	46
Soybean meal	492	158	100	35	73
Rapeseed meal	403	280	190	50	71
Sunflower meal	291	380	300	75	65

Table 2: Ruminal protein disappearance values of the various oilseed meals originated from Iranian plant using *in situ* bag and *in vitro* procedures

	Procedure *In situ* bag or			Feed effect		Procedure effect	
Feeds	3-step[a]	*in vitro*[b]	Mean[c]	S.E.M	P	S.E.M	P
Cottonseed meal high oil (105 g kg^{-1} of DM)	0.45	0.72	0.58	0.034	0.01	0.021	0.01
Cottonseed meal low oil (65 g kg^{-1} of DM)	0.54	0.83	0.68				
Soybean meal	0.61	0.88	0.74				
Rapeseed meal	0.83	0.80	0.81				
Sunflower meal	0.90	0.93	0.91				

[a]Protein disappearance from nylon bags used in the mobile nylon bag and 3-step techniques after 12 h incubation in the rumen of steers, [b]Protein disappearance from nylon bags after 1 h incubation in borate-phosphate buffer followed by 4 h incubation protease solution, [c]When the difference between means in greater than two times the S.E.M. it is considered as significant ($p<0.01$)

Table 3: Post-ruminal disappearance of the ruminal-undegraded protein of various oilseed meals originated from Iranian plant using the *in situ* mobile bag, 3-step and *in vitro* procedures

	Procedure				Feed effect		Procedure effect	
Feeds	*In situ* bag[a]	3-step[b]	*in vitro*[c]	Mean[d]	S.E.M	P	S.E.M	P
Cotton seed meal high oil (105 g kg^{-1} of DM)	0.83	0.57	0.73	0.71	0.031	0.01	0.023	0.01
Cotton seed meal low oil (65 g kg^{-1} of DM)	0.71	0.51	0.54	0.59				
Soybean meal	0.85	0.60	0.98	0.81				
Rapeseed meal	0.64	0.59	0.86	0.70				
Sunflower meal	0.66	0.73	0.67	0.69				

[a]Protein disappearance from nylon bags used in the mobile nylon bag technique through the intestine, [b]Protein disappearance after 1 h incubation in pepsin solution and 24 h incubation in pancreatin solution using centrifuge tubes, [c]Protein disappearance from nylon bags after incubation in pepsin solution (1 h) followed by 24 h incubation in pancreatin solution, [d]When the difference between means in greater than two times the S.E.M. it is considered as significant ($p<0.01$)

Table 4: Total tract protein disappearance of various oilseed meals originating from Iranian plant varieties using the *in situ* bag, *in vitro* and 3- Step procedures

	Procedure				Feed effect		Procedure effect	
Feeds	*In situ* bag[a]	3-step[b]	*in vitro*[c]	Mean[d]	S.E.M	P	S.E.M	P
Cotton seed meal high oil (105 g kg^{-1} of DM)	0.91	0.77	0.92	0.87	0.030	0.01	0.011	0.01
Cotton seed meal low oil (65 g kg^{-1} of DM)	0.88	0.79	0.92	0.86				
Soybean meal	0.92	0.79	0.99	0.90				
Rapeseed meal	0.94	0.93	0.97	0.95				
Sunflower meal	0.96	0.97	0.98	0.97				

[a]Protein disappearance from nylon bags after rumen incubation and passage through the intestine, [b]Protein disappearance from nylon bags after rumen incubation and samples incubated in pepsin and pancreatin solutions using centrifuge tubes, [c]Protein disappearance from nylon bags after incubation with protease, pepsin and pancreatin solutions, [d]When the difference between means in greater than two times the S.E.M. it is considered as significant ($p<0.01$)

ruminal CP disappearance obtained from *in situ* mobile bag procedure and *in vitro* enzymatic method. They reported that there was not a significant difference between procedures. However, the authors found a low correlation ($r^2 = 0.38$) between *in situ* mobile bag and *in vitro* enzymatic procedure for estimating ruminal CP disappearance of various feeds. Variable ruminal CP disappearance results of the *in vitro* enzymatic procedures, in the present study, with the *in situ* mobile bag, might be related to different bag materials and the method of bag incubation in the rumen (Jirl *et al*., 1996). Results of post-ruminal CP disappearance of rumen undegradable feed protein indicated a significant ($p<0.01$) difference between the procedures applied in this experiment (*In situ* bag = 0.74, 3-step = 0.60 and *in vitro* = 0.75; S.E.M = 0.023). Post-ruminal protein disappearance measured with *in vitro* procedure was higher than mobile *in situ* bag and 3-step techniques (Table 3). Three step method had lower post-ruminal protein disappearance compared with *in situ* mobile bag (0.60 and 0.74, respectively). Similarly, Jahani-Azizabadi *et al.* (2007) reported a significant difference between MNB and 3-step procedure to measure post-ruminal CP disappearance. The results of present study did not confirm the finding of Stern *et al.* (1997) and Danesh Mesgarana and Stern (2005). Hvelpund (1985) calculated that 50 and 27% of SBM and RSM protein that leaving the ileum was digested in the large intestine. In addition, bag pore size, animal, diet, large intestinal fermentation and bacterial contamination may contribute to variation (Hvelpund, 1985; Jirl *et al*., 1996; Vogit *et al*., 1985). Stern *et al.* (1997) compared results of the 3-step procedure with *in vivo* intestinal protein digestion and found a high correlation ($r^2 = 0.91$) between the procedures.

There was a significant ($p<0.01$) effect of feeds on ruminal, post-ruminal and total tract CP disappearance. Mean *in situ* mobile bag and *in vitro* ruminal CP disappearance of CSMH, CSML, SBM, RSM and SFM was 0.58, 0.68, 0.74, 0.81 and 0.91, respectively. The CSMH had a lower ruminal CP disappearance, while the SFM had the highest value ($p<0.01$). The CSMH had lower ruminal CP disappearance compared with CSML (0.58 vs. 0.68, respectively). This might be the result of oil content of CSMH. Released unsaturated fatty acids during microbial hydrolyzed of triglycerides in the rumen, might have negative effect on ruminal degradation (Henderson, 1973; Maczulak *et al*., 1981). Similarly, Danesh Mesgaran *et al.* (2007) reported that ruminal CP disappearance of CSM reduced with increase oil content. Mean *in situ* mobile nylon bag, 3-step and *in vitro* procedures of post-ruminal CP disappearance of ruminal undegradable feed protein were 0.71, 0.59, 0.81, 0.70 and 0.69, for CSMH, CSML,

SBM, RSM and SFM, respectively. The procedures had a significant effect ($p<0.01$) on the total tract protein disappearance of various feeds evaluated in the present study (MNB = 0.92, 3-step = 0.85 and *in vitro* = 0.95, S.E.M = 0.011). The results of the present study confirmed the findings of Jahani-Azizabadi *et al.* (2007), who compared results of MNB with 3-step of total tract CP disappearance. In the present study total tract CP disappearance of oilseed meals measured with *in vitro* enzymatic procedure was higher than 3-step and MNB procedures (*in vitro* = 0.95, 3-step = 0.85 and MNB = 0.92). These results did not confirm the observation of Danesh Mesgarana and Stern (2005), who reported that there was not significant difference between the procedures when total tract protein disappearance was consumed.

Total tract CP disappearance of SFM and RSM was higher ($p<0.01$) than SBM, CSMH and CSML (0.97 and 0.95 vs. to 0.90, 0.86 and 0.87, S.E.M = 0.030, respectively). It was also observed that total tract protein disappearance of CSML was lower than the other oilseed meals.

CONCLUSION

Accurate estimation of ruminal and post-ruminal protein disappearance of a feed is essential for optimization of the animal performance. Recently, several *in vitro* and *in situ*/*in vitro* methods have been developed to determine the ruminal and post-ruminal protein disappearance of ruminant feeds. In the present study, none of the *in vitro* enzymatic technique and 3-step procedure evaluated resulted in protein disappearance in rumen and pos-rumen data that had a consistent relationship with those generated by the *in situ* techniques. Therefore, modification of these methods for estimating the ruminal and post-ruminal feed protein disappearance need to be developed using wide rang of feedstuffs.

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