DEVELOPMENT OF AN IN VITRO INTESTINAL DIGESTIBILITY ASSAY FOR RUMINANT FEEDS

D. A. Ross, M. Gutierrez-Botero, and M. E. Van Amburgh
Department of Animal Science
Cornell University

INTRODUCTION

Current cattle diet formulation models rely on library estimates of intestinal digestibility of proteins and carbohydrates to predict metabolizable energy (ME) and protein (MP) supply (NRC, 2001; Fox et al., 2004; Tylutki et al., 2008). As models become more accurate and precise in the prediction of nutrient supply and evaluation of requirements and nutrient balance, greater scrutiny will be placed on inputs currently relegated to static library values. Although CP is not a functional dietary nutrient for cattle, many diets are still formulated on this metric, creating confusion due to inadequate information provided by the value, especially with regard to MP supply and amino acid availability. As diets are formulated to be closer to MP requirements and subsequently lower in CP, accurate estimates of intestinal digestibility (ID) of protein and amino acids are increasingly important to ensure an adequate supply of those nutrients. Application of outdated feed library values to all feeding conditions can lead to under- and over-estimations of MP and amino acid supply, resulting in variation from expected production. This paper describes the re-development of an in-vitro intestinal digestion (IVID) assay for protein containing feeds used in ruminant nutrition, including intact commercially available feeds designed to resist rumen degradation. The methods used were developed to provide adequate sample size, minimize sample loss, and to allow for standardization of enzyme activity and concentration. The assay contains positive and negative controls to evaluate standardization among and within laboratories.

The feed library of the Cornell Net Carbohydrate and Protein System (CNCPS) (Tylutki et al., 2008; Higgs et al., 2012) has static values for intestinal protein digestibility values for various protein fractions, and acid detergent insoluble protein (ADIP) is used to define the unavailable protein. The committee that developed the 2001 Dairy NRC adjusted available MP from feed by assigning a digestibility of 5% to the ADIP fraction based on data indicating that some amino acids could be liberated and absorbed from this fraction (NRC, 2001). The results from the assay described in this paper can be compared to both the ADIP and the adjusted ADIP value from the NRC calculation as an unavailable protein fraction.

Methods to estimate in vivo digestibility

Tilley and Terry (1963) developed a two-step *IV* procedure, combining ruminal digestion with acid pepsin to determine digestibility of forage crops. Calsamiglia and Stern (1995) used the *in situ* (IS) bag technique as a first step and proceeded with two additional IV steps in centrifuge tubes. Dried IS residue was subjected to a hydrochloric

acid (HCl) and pepsin pre-digestion prior to pancreatin digestion, similar to the procedure of Akeson and Stahmann (1964). This three-step procedure (TSP) was validated and optimized against duodenal samples obtained from an *in vivo* intestinal digestion study using the same protein sources. Results from the pancreatin digestion assay were highly correlated ($n = 34$; $r = 0.91$, $P < 0.001$) to estimates of in vivo intestinal protein digestion (Calsamiglia and Stern, 1995). In that assay, digestion was terminated by addition of trichloroacetic acid (TCA) and protein content determined on the soluble fraction. Thus, determining digestibility of individual amino acids was impossible.

A modified three step procedure (mTSP) was developed by Gargallo et al. (2006). In this procedure, the post-rumen digestion steps from the original TSP were performed in an IV system (Daisy incubator; Ankom Technology, Macedon, NY) by placing bagged IS residue in the buffer-enzyme solutions to estimate intestinal digestion while eliminating use of TCA. Although this approach is attractive for the ease of use and simplicity of the bag, at least two issues arise from use of this technique. First, any soluble components that are not ruminal degradable are lost prior to the intestinal digestibility step and second, the bag acts as a barrier to microbial movement, resulting in longer than expected fermentation lag times (Goeser, et al., 2013).

Recently, Boucher et al., (2009a, b, c) utilized the precision-fed cecectomized rooster bioassay, deemed to be an appropriate technique to estimate small intestinal (SI) digestion of amino acids in cattle by Titgemeyer et al. (1990), and an immobilized digestive enzyme system to determine intestinal true digestibility of rumen undegraded protein. However, despite the sensitivity of this approach, it is not easy to apply to a commercial feed analyses system for routine analyses.

A review by Stern et al. (1997) calculated intestinally absorbable dietary protein from specific feeds as ruminally undegraded protein multiplied by intestinal protein digestion and stated: ”It was evident that an *IV* technique to estimate protein digestion should include enzymes with activity and specificity similar to those found in the digestive tract of the animal” and ”be correlated with intestinal digestion and not total tract digestion”.

To address these issues, the following discussion points are provided to highlight potential problems or concerns with current methods and to provide evidence for the need to develop alternative approaches.

Use of bags:

- Created a microbial barrier to feed access and microbial attachment which artificially prolongs the lag phase of digestion.
- Demonstrated loss of highly soluble components of feeds from the bag prior to digestion and loss of particles as digestion progresses. Measured losses of up to 30% of the initial sample prior to any analyses have been reported.

Use of enzymes:

- Profiles and activities are not properly described and characterized.
- The digestive process of the ruminant is a continuous flow of digesta with continuous secretion of enzymes and digestive juices (Hill, 1965).

Abomasal digestion:

- Pepsin, an endopeptidase, hydrolyzes approximately 15-20 % of dietary protein to AA and small peptides (Kutchai, 1998). Bovine pepsin has approximately ~60-70 % of the activity of porcine pepsin with hemoglobin as substrate (Lang and Kassell, 1971). Porcine pepsin is generally used in the first step of *IV* intestinal digestion assays to measure ruminant intestinal digestion (Calsamiglia and Stern, 1995; Gargallo et al., 2006).

- One mg of porcine pepsin contains 200 to 625 units with pH between 1.5 and 2.5, for optimum pepsin activity.

- Lysozymes which aid in digestion of microbes are also secreted in the digestive tract. Bovine digestive lysozyme has a lower optimum pH than chicken lysozyme (7.65 vs. 10.7, respectively) with a pH optimum 5, not 7, making it resistant to pepsin hydrolysis. Furthermore, bovine lysozymes lyse gram-negative *and* gram-positive bacteria, while chicken lysozyme acts only on gram positive bacteria (Dobson et al., 1984; Protection of plants against plant pathogens: http://www.patentstorm.us/patents/5422108/description.html; accessed Nov 1, 2010). However, bovine digestive lysozyme is commercially unavailable.

Small intestine digestion:

- Species differences exist in the activities of proteases in the pancreas. In rats, trypsin activity represents ~80 % while in ruminants it represents only 15 % and chymotrypsin makes up 43 % (Keller et al., 1958).

- The calculated activities of trypsin and chymotrypsin in intestinal contents from 5 month old calves (Gorrill et al., 1968) were 19.48 and 15.9 U/ml, respectively using p-toluene-sulfonyl-L-arginine methyl ester (TAME) and benzoyl-L- tyrosine-ethyl ester (BTEE), as substrates.

- In sheep, the activities of trypsin, chymotrypsin and carboxypeptidase A increased from the pylorous to 7 m beyond with maximum specific activities of 24, 150, and 35 μM of respective substrates (benzoyl-L-arginine-ethyl ester (BAEE), acetyl- L- tyrosine-ethyl ester (ATEE), hippuryl-DL-phenyl-lactic acid) per minute per ml digesta, and then decreased (Ben-Ghedalia et al.,1974).

- Sklan and Halevy (1985) found maximal activities of pancreatic enzymes in the proximal segments of the ovine SI at 1 m distal to the pylorous and then relatively constant ratios of enzyme levels (trypsin, chymotrypsin, elastase, carboxypeptidases A & B) to cerium-141, an unabsorbed reference, of 0.065,

0.053, 0.015, 0.05 and 0.045, respectively, 1.5 to 9 m distal to the pylorous. No other *in vivo* activities for bovine pancreatic proteolytic enzymes were measured.

- Units of enzyme activity are dependent upon substrate (a protein or ester) hydrolyzed in addition to the wavelength used. Among the studies reviewed, this data varies considerably and is not standardized.

- The current three step assays (Calsamiglia and Stern, 1995; Gargallo et al., 2006; Borucki Castro et al., 2007; Boucher et al., 2009a,b,c) use 3 g of pancreatin per L after an IV abomasal digestion with 1 g L^{-1} of porcine pepsin in 0.1 N HCl N at pH 1.9 or 2. However, the pancreatin concentration in the assay of Calsamiglia and Stern (1995) was 1.69 mg ml^{-1} based on the conditions described for the assay as published.

- Pancreatin always contains amylase and lipase but over time the proteolytic enzyme has changed from trypsin to many enzymes, including trypsin, ribonuclease and protease (specifications for P7545; (www.sigmaaldrich.com/catalog/product/sigma/p7545?lang=en. accessed, Nov 10, 2010) and specific units of enzymatic activity are not provided.

- Further, lipase activity is essentially nonexistent in bovine pancreatic juice (Keller, 1958) but is high in saliva. Calsamiglia and Stern (1995) attributed the increase in digestion of their proteins over those obtained using the multi-enzyme system of Hsu et al. (1977) to the presence of amylase and lipase in pancreatin.

Thus, the enzymes used in the assay for the abomasal and intestinal digestion step and their respective activities were based on the data described and were adopted and run in parallel with pancreatin.

ASSAY DEVELOPMENT

A description of the assay development follows in a sequential manner with statements about sources of variation and decisions made to optimize the assay while minimizing or eliminating irrelevant sample loss.

General procedures:

- Unless specified otherwise, all analyses were conducted on duplicate samples.

- Dry matter was determined at 105ºC in a forced-air oven overnight.

- Nitrogen (N) content of original feeds and residues was measured by block digestion and steam distillation with automatic titration (Application Note, AN300; AOAC Official method 2001.11; Foss, 2003; Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden; AOAC 2001.11).

Exposure to rumen microbes:

This step in the assay was evaluated in three stages to evaluate variation and sample loss.

- Three bag materials with different pore sizes (15 µm, mesh; 25 µm, fiber (Ankom) and 50 µm, *in situ* (Ankom)) were evaluated for *in vitro* intestinal digestion following *in vitro* vs. *in situ* fermentation (Ross, et al., 2010). After many attempts at developing conditions that minimized loss of material prior to assay or during the assay, it was difficult to distinguish digestion from bag loss, thus the use of any bags was abandoned.

- From this point forward 16-h fermentation was performed via IV methods in Erlenmeyer flasks.

- Plastic centrifuge tubes were evaluated as a fermentation vessel and found to be unfavorable for rumen bacterial growth and sample size had to be reduced to work appropriately in 50 mL tubes.

- Glass Erlenmeyer flasks provided the greatest digestibility values, and had lower variability and superior repeatability compared to plastic centrifuge tubes. For this reason, flasks were chosen as the vessel for the fermentation step. Commercial protein sources (0.5 g) were included in their un-ground form, while forages, byproducts and non-commercial protein sources were ground through a 2 mm screen in a Wiley Mill (Thomas Scientific, Swedesboro, NJ).

Enzymatic hydrolysis

- Pepsin: Porcine pepsin used but added at 60 % of previous methods in pH 2 HCl (~0.013 M) to contain ~282 U ml^{-1} in flask.

- Intestinal (ID) enzymes: Initially, enzymes and activities described by Ben-Ghedalia et al. (1974) were used in the enzyme mix until carboxypeptidase A became unavailable. Different combinations of elastase and carboxypeptidase Y in addition to trypsin and chymotrypsin were then evaluated without duplication of intestinal digestion. Amylase and lipase were added along with trypsin and chymotrypsin (50 and 4; 24 and 20 U ml^{-1}, respectively) which yielded digestion approximately similar to levels observed with carboxypepetidases A & B. Pancreatin at a level similar to Calsamiglia and Stern (1995; 1.72 mg ml^{-1}, difference due to initial dilution so maintained throughout) was also analyzed concurrently with the mixture of individual enzymes.

- Assay termination for both IV fermentation and enzymatic digestion was accomplished by quantitative filtration under vacuum though 9 cm glass microfiber filter (pore size of 1.5 µm; Whatman 934-AH; GE Healthcare Bio-Sciences Corp., Piscataway, NY) using hot water to transfer. Hot water was necessary to help dissolve away viscous residues from the in vitro step.

DISCUSSION

To manufacture feeds that escape ruminal degradation, companies have reduced particles to a size that will flow with rumen fluid and liquid passage rate thus, the IV residues are not always captured on small pore filter paper, despite small pore size (1.5 µm). Consequently, when known water soluble components are present that can pass prior to being fermented or filtered, the filtration eluent has to be captured, the N analyzed and then the freeze dried eluent has to be added back to the filtered residue. This process became necessary when we recognized that several samples provided for analysis had components that solubilized immediately upon addition to water and when immediately filtered could not be recovered on the filter paper. Alternatively, the entire IV fermentation mix can be freeze dried, analyzed for N and corrected for microbial contamination using corn silage ND residue with and without rumen fluid treated in similar fashion. This process makes the rumen escape protein (RUP) estimation a little more ambiguous but it is the only way to capture the soluble component which has been shown to provide MP amino acids to the animal (Reynal et al., 2007; Volden et al. 2002).

Correction of in vitro residues for microbial contamination

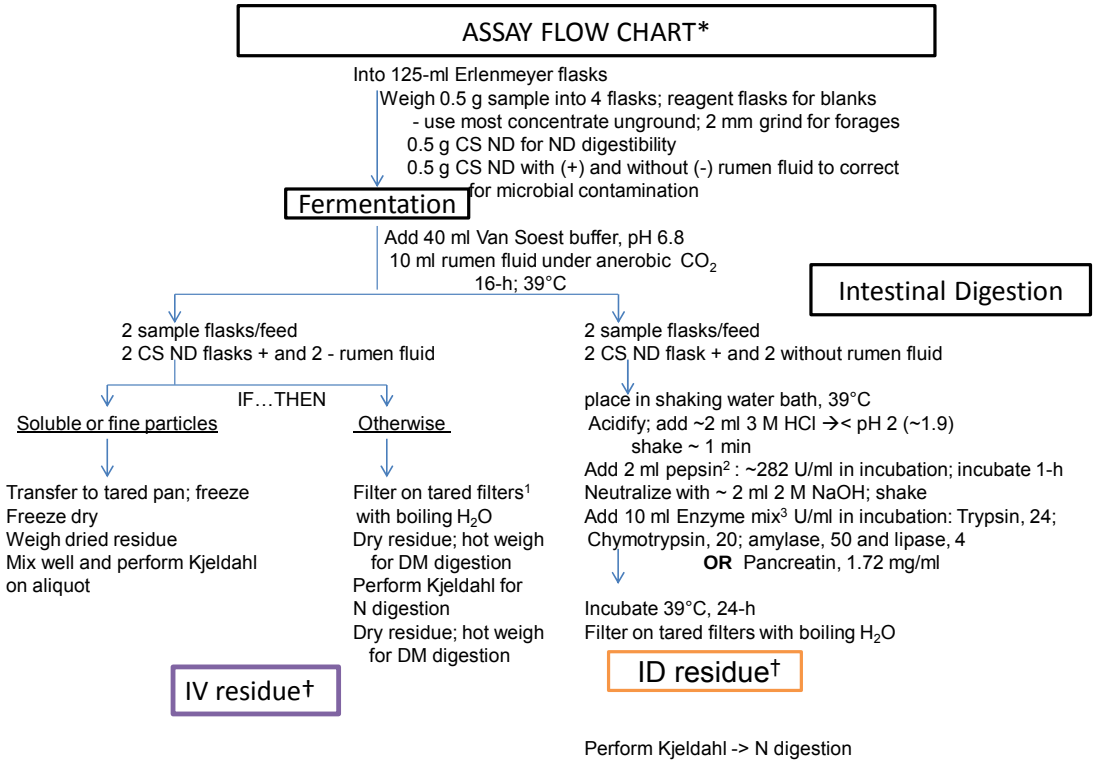
The original objective of the assay was to estimate ID, not RUP, however, it became apparent that in either case, microbial contamination should be accounted for if possible to ensure less bias in the ID determination. Therefore, a series of approaches were evaluated to provide a quantifiable and repeatable indication of microbial contamination:

- ^{15}N was used to label bacteria for estimation of contamination
- Washing with methylcellulose to remove attached bacteria
- Measuring purines as a label for contamination

After much work, none of the previously mentioned approaches were consistent and repeatable among all samples, thus an alternative was needed. The alternative was to develop a substrate that was low in N content, provided adequate substrate for microbial growth, was available to a commercial laboratory, and produced repeatable results. The substrate eventually chosen was neutral detergent residue from corn silage. Bulk volumes of ground corn silage was washed in hot ND solution with amylase, rinsed, washed in ammonium sulfate to remove detergent, and then used both as the fermentation control sample and to estimate microbial contamination. Properly washed corn silage ND residue has very low N content, so any N that is accumulated is assumed to have come from bacteria. Corn silage ND residue can also grow a significant quantity of bacteria, making it superior to pure cellulose, easy to recover, and easy to measure. Given this, the corrections that are made with the corn silage ND residue are as follows:

- The difference in N content between CS ND residue with and without rumen fluid (after IV incubation), on per g DM basis provides a robust estimate of microbial contamination and is used for both the “RUP” and ID steps.
- For ‘RUP’ estimate, CS ND is included to correct for NDIN digestibility.

Figure 1. Assay flow chart with enzyme activity and specificity descriptions.



*NOTE: Quantitatively transfer all residues

†Corrections
- IV residue = 'RUP'
  - original - assay blank and microbial contamination [((cs nd + rumen fluid)/g, DM)-((cs nd + rumen fluid)/g, DM)]*wt, DM)
  - new - assay blank and microbial contamination using the above and adjusting for feed NDIN content by CS NDIN digested
- ID residue = undigested N
  - original – assay blank
  - new – assay blank and microbial contamination using cs nd +/- rumen fluid carried through entire procedure

[1]Filters: 90 mm; Whatman 934AH, 1.5µm

[2]Pepsin in pH 2 HCl: 16.6 mg/ml

[3]Enzyme mix and Pancreatin prepared daily in 1.8 M KH_2PO_4. Enzyme mix prepared to contain the following U in 10 ml: Trypsin, 1680; chymotrypsin, 1400; amylase, 7050, and lipase, 280. If using pancreatin, prepared to contain 120.4 mg in 10 ml.

Enzyme activity definitions

Pepsin	ΔA_{280nm} of 0.001 per min at pH 2.0,37°C measured as TCA-soluble products using hemoglobin.
Trypsin	ΔA_{253nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-arginine ethyl ester (BAEE).
Chymotrypsin	ΔA_{256nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-tyrosine ethyl ester (BTEE).
Amylase	One unit will liberate 1.0 mg maltose in 3 min at pH 6.9, 37°C.
Lipase	One unit releases 1 uEq of acid from olive oil per min.

Use of positive and negative controls to evaluate IV and intestinal digestibility:

Positive and negative controls for both fermentation and intestinal digestibility steps were included. To evaluate the fermentation phase, NDF digestion of corn silage ND residue sample was run concurrently. A heat damaged blood meal with near zero ruminal and intestinal digestibility was included throughout as a negative control. A feed with similar digestibility as samples, i.e., a soy product or blood meal, was also included. A blood meal with known high intestinal digestibility was included as a positive control for the ID assay.

Comparison of modified TSP with new Cornell assay

Digestibility of two bloodmeals (from Boucher et al., 2011) were evaluated using the new method with the enzyme mix and pancreatin (Table 1) and compared with the modified TSP. Rumen N digestibility of BM4 was 18 % higher using bags but 6 % lower for BM5. The implication from this comparison is that material was solubilized or lost from the bag prior to being analyzed which provided higher rumen degradability in the TSP. Total N digestibility for BM5 was similar between both procedures and the enzyme mix and pancreatin. However pancreatin digestion of BM4 in the modified TSP was lower than either ID digestion using the Cornell procedure. Using the Cornell method BM4 had higher intestinal digestion. These differences in intestinal digestibility are partially an artifact of the calculations for intestinal digestibility. If a greater amount of feed protein escapes rumen fermentation, with identical unavailable protein values, the intestinal digestibility is mathematically higher for the feed protein with the greater rumen escape.

Table 1. Comparison of the percent N digested in two blood meals using the modified three step procedure (from Boucher et al., 2011) with Cornell procedure.

	Modified TSP*		Cornell		
	Rumen	Pancreatin	Rumen	Enzyme Mix	Pancreatin
	----% N digested----		-------% N digested----------		
BM4	19.9	89	1.0	96.6	97.1
BM5	42.3	94	48.7	97.4	97.0

*Boucher

Comparison of intestinal digestion with the acid detergent insoluble protein

Within the current structure of many contemporary nutrition models, acid detergent insoluble nitrogen (ADIN) represents the unavailable N component of feed (NRC, 2001; Tylutki et al., 2008) however, the NRC for Dairy Cattle (2001) provides for 5% digestibility of the ADIN fraction. The implication is that the ADIN fraction is not completely unavailable to the animal. Accordingly, the ID assay as outlined was utilized to ascertain whether ADIN is indigestible (Table 2). The ADIN of solvent extracted soybean meal and Soy1 were very similar to undigested feed N following IV fermentation, abomasal and intestinal digestion with either the enzyme mix or pancreatin, ; however, the ADIN of heat damaged blood meal was roughly 2 % while

undegraded N from both intestinal digestion treatments was 95 %. Undegraded N of corn silage following digestion and after correction for microbial contamination was roughly 3 times higher than ADIN content.

This approach for determining the unavailable N from feeds departs from the traditional detergent partitioning system established by Van Soest and others, and implementation within nutrition models like the CNCPS will create a fraction that crosses the fractions described by detergent chemistry and has a different behavior. We believe this to be more appropriate approach for describing available protein for cattle.

Table 2. Comparison of percent feed N and acid detergent insoluble N versus undigested feed N after 16-h IV ruminal fermentation followed by 1-h abomasal digestion with pepsin in HCl and 24-h intestinal digestion using either a mix of trypsin, chymotrypsin, amylase and lipase or pancreatin (n=2).

	Feed N	ADIN	% Undigested Feed N	
	% DM	% N	Enzyme Mix*	Pancreatin
Anchovy meal	11.50	1.3	25.5	20.1
Alfalfa silage	3.80	6.1	23.2	21.9
Bakery waste	1.80	3.3	20.6	23.6
Blood meal 1	16.20	4.7	22.9[a]	8.0[b]
Blood meal 285	16.89	1.1	0.0	na
Blood meal 300	16.20	7.5	4.6	na
Blood meal 350	15.13	0.9	23.6	na
Blood meal 800	16.50	1.8	2.8	na
Canola 1	6.50	6.3	16.2	12.5
Canola 2	6.60	0.0	14.0	14.0
Citrus pulp	1.04	15.8	55.0	45.4
Corn germ	4.27	11.2	18.5	9.4
Corn gluten	3.13	16.9	28.7	18.9
Corn gluten feed	3.08	11.2	20.7	16.2
Corn silage 1	1.40	9.2	30.0	25.9
Corn silage 2	1.30	8.6	13.9	21.1
Distillers grains 1	4.90	13.1	11.7	9.5
Distillers grains 2	6.40	32.7	27.9[a]	13.6[b]
Hay silage	2.40	12.5	29.6	31.9
Solv. extract. soybean meal	7.60	6.7	7.8	7.6
Soy1	7.70	6.5	9.0	4.3
Soy2	7.30	7.9	11.1[a]	6.6[b]
Wheat midds	3.30	3.1	9.3	7.2
Heat damaged blood meal	16.10	1.8	95.0	95.0

[abc]Means with different superscripts in same row differ ($P < 0.05$) using Duncans Multiple Range test. Not all samples were statistically evaluated for this manuscript. NA – not available.

SUMMARY

An *IV* assay to estimate intestinal protein digestion for ruminants was developed using an enzyme mix of trypsin, chymotrypsin, lipase and amylase at activities found in cattle and sheep digesta to replace pancreatin. The assay was developed to reduce sample loss and variation among samples by eliminating the use of bags, employing Erlenmeyer flasks and utilizing small pore size filter papers to improve recoveries of undegraded feed N. The procedure was modified when necessary to account for soluble components of feeds and the calculations were described. The assay is a modification of published methods and the enzyme levels are standardized. The procedure allows for comparison with assay conditions of other published assays and acid detergent insoluble N and further, it allows for recovery of residue for analyses of amino acids. Concurrent samples of corn silage ND with and without rumen fluid are assayed to correct for microbial contamination. The protease and lipase activities are similar between the two intestinal enzyme treatments, but the amylase activity in the enzyme mix is double that of porcine pancreatin. The assay provides an opportunity to evaluate intestinal digestibility of protein and amino acids in ruminant feeds and is designed in a manner that would allow adoption by commercial laboratories.

REFERENCES

Akeson, W. R. and M. A Stahmann,. 1964. A pepsin pancreatin digest index of protein quality evaluation. J Nutr 83:257-261.

Antoniewicz, A. M., A. M Van Vuuren, C. J.van der Koelen, and I. Kosmala, 1992. Intestinal digestibility of rumen undegraded protein of formaldehyde-treated feedstuffs measured by mobile bag and IV technique. An Feed Sci Tech 39:111-124.

AOAC International. 2003. Nitrogen. AOAC Official Methods of Analysis. Arlington, VA.

Ben-Ghedalia, D., H.Tagari, , and A.Bondi, 1974. Protein digestion in the intestine of sheep. Br J Nutr 31:128-142.

Borucki Castro, S. I., L. E., Phillip, H. Lapierre, P. W., Jardon, and R. Berthiaume,. 2007. Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. J Dairy Sci. 90:810-822.

Boucher, S. E., Calsamiglia, S., Parsons, C. M., Stein, H. H., Stern, M. D., Erickson, P. S., Utterback, P. L., and Schwab, C. G. 2009. Intestinal digestibility of amino acids in rumen undegradable protein estimated using a precisio-fed cecectomized rooster bioassay: I. Soybean meal and SoyPlus. J Dairy Sci. 92:4489-4498.

Boucher, S. E., Calsamiglia, S., Parsons, C. M., Stein, H. H., Stern, M. D., Erickson, P. S., Utterback, P. L., and Schwab, C. G. 2009. Intestinal digestibility of aino acids in rumen-undegraded protein estimated using a precision-fed cecectomized rooster bioassay: II. distillers dried grains with solubles and fish meal. J Dairy Sci. 92:6056-6067.

Boucher, S. E., S. Calsamiglia, C. M. Parsons, M. D. Stern, M. Ruiz Moreno, , M. Vázquez-Añón , and C. G. Schwab. 2009. *In vitro* digestibility of individual amino acids in rumen-undegraded protein: The modified three-step procedure and the immobilized digestive enzyme assay. J Dairy Sci. 92:3939-3950.

Boucher, S. E., S. Calsamiglia, C. M. Parsons, M. D. Stern, C. G. Schwab, K. W. Cotanch, J. W. Darrah and J. K. Bernard. 2011. Method evaluation for determining digestibility of rumen undegraded amino acids in blood meal. J Dairy Sci. 94:388. E-Suppl. 1.

Broderick, G. A., Wallace, R. J., and N, McKain,. 1988. Uptake of small neutral peptides by mixed rumen microorganisms *in vitro*. J. Sci. Food Agric. 42:109-118.

Calsamiglia, S. and Stern, M. D. 1995. A three-step *in vitro* procedure for estimating intestinal digestion of protein in ruminants. J Anim. Sci. 73:1459-1465.

Chu, S. S. T. and Y.Nakagawa. 1982. Comparison of pepsins isolated from porcine, bovine and *Penicillium jathiuellum*. Comp Biochem Physiol 72B:625-629.

Clark, J. H., T. HKlusmeyer and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J Dairy Sci. 75:2304-2323.

de Boer, G., J. J. Murphy and J. J. Kennelly. 1987. Mobile nylon bag for estimating intestinal availability of rumen undegradable protein. J Dairy Sci. 70:977-982.

Doane, P. H., P. Schofield and A. N. Pell. 1997. Neutral detergent fiber disappearance and gas and volatile fatty acid production during the *in vitro* fermentation of six forages. J Anim. Sci. 75:3342-3352.

Dobson, D. E., E. M. Prager and A. C., Wilson. 1984. Stomach lysozymes of ruminants. I. distribution and catalytic properties. J. Biological Chem. 259:11607-11616.

Fauconneau, G. and M. C. Michel. 1970. 37. The role of the gastrointestinal tract in the regulation of protein metabolism. In: H. N. Munro (Ed.) Mammalian Protein Metabolism. pp. 481-518. Academic Press, Inc., New York.

Fox, D. G., L. O. Tedeschi, T. P. Tylutki, J. B. Russell, M. E. Van Amburgh, L. E. Chase, A. N. Pell and T. R. Overton. 2004. The Cornell net carbohydrate and protein system model for evaluating herd nutrition and nutrient excretion. Animal Feed Science Tech 112:29-78.

Gargallo, S., S. Calsamiglia and A. Ferret. 2006. Technical note: A modified three-step *in vitro* procedure to determine intestinal digestion of proteins. J Anim Sci 84:1-5.

Goering, H. K., C. H. Gordon, R. W. Hemken, D. R. Waldo, P. J. Van Soest and L. W. Smith. 1972. Analytical estimates of nitrogen digestibility in heat damaged forages. J Dairy Sci 55:1275-1280.

Goeser J., C. Heuer, and L. Meyer. 2013. In vitro NDF digestion parameters differ when using a forage fiber bag. J. Dairy Sci. 96:553. E-Suppl. 1

Gorrill, A. D. L., D. J. Schingoethe and J. W. Thomas. 1968. Proteolytic activity and *in vitro* enzyme stability in small intestinal contents from ruminants and nonruminants at different ages. J Nutr 96:342-348.

Higgs, R. J., L. E. Chase, D. A. Ross and M. E. Van Amburgh. 2012. Evaluating and refining the CNCPS feed library using commercial laboratory feed databases. Cornell Nutr. Conf. Pp. 146-156. Syracuse, NY.

Hill, K. J. 1965. Abomasal secretory function in the sheep. In: R. W. Dougherty, R. S. Allen, and A. D. McGillard (Eds.) Physiology of Digestion in the Ruminant. pp. 221-230. Buttersworths, Washington.

Hsu, H. W., D. L. Vavak, L. D. Satterlee and G. A. Miller. 1977. A multienzyme technique for estimating protein digestibility. J Food Sci 42:1269-1273.

Johnson, T. O., G. E. Mitchell, R. E. Tucker and G. T. Schelling. 1974. Pancreatic lipase secretion by sheep. J. Animal Sci 39:947-951.

Julien, R., P. Canioni, J. Rathelot, L. Sarda, and T. H. Plummer, Jr. 1972. Studies on bovine pancreatic lipase and colipase. Biochim Biophys Acta 280:215-224.

Keller, P. J., E. Cohen and H. Neurath. 1958. The proteins of bovine pancreatic juice. J. Biological Chem. 233:344-349.

Kreikemeier, K. K., D. L. Harmon, J. P. Peters, K. L. Gross, C. K. Armendariz and C. R. Krehbiel. 1990. Influence of dietary forage and feed intake on carbohydrase activities and small intestinal morphology of calves. J Animal Sci. 68:2916-2929.

Kutchai, H. C. 1998. Chapter 39. Digestion and Absorption. In: R. M. Berne and M. N. Levy (Ed.). Physiology, 4th ed. Pp. 651. Mpsby. Inc. St. Louis.

Lang, H. M. and B. Kassell. 1971. Bovine pepsinogens and pepsins. III. composition and specificity of the pepsins. Biochem 12:2296-2301.

Luchini, N. D., G. A. Broderick and D. K. Combs. 1996. Characterization of the proteolytic activity of commercial proteases and strained ruminal fluid. J Animal Sci 74:685-692.

Makkar, H. P. S. and K. Becker. 1999. Technical report: Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. Br J Nutr 81:107-112.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. National Academy of Science. Washington, DC.

Pittman, K. A., S. Lakshmanan and M. P. Bryant. 1967. Oligopeptide uptake by Bacteroides ruminicola. J. Bacteriology 93:1499-1508.

Recktenwald, E. B. 2010. Urea-N recycling and its utilization by ruminal microbial populations in lactating dairy cattle . Ph.D. Cornell University.

Ross, D. A., M. M. McCullouch, and M. E. Van Amburgh. 2010. A comparison of methods to evaluate in vitro intestinal digestibility. J Dairy Sci. 93:164. E-suppl. 1.

Sayre, K.D. and P. J. Van Soest. 1972. Comparison of types of fermentation vessels for an in vitro artificial rumen procedure. J. Dairy Sci. 55:1496-1498.

Sir ElKhatim, M. M. and A. M. Osman. 1983. Effect of concentrate feeding on the bovine intestinal and pancreatic carbohydrases, evidence of induced increae in their activities. Comp Biochem Physiol 74A:275-281.

Sklan, D. and O. Halevy. 1985. Digestion and absorption of protein along ovine gastrointestinal tract. J Dairy Sci 68:1676-1681.

Steg, A., W. M. Van Straalen, V. A. Hindle, W. A. Wensink, F. M. H. Dooper and R. L. M. Schils. 1994. Rumen degradation and intestinal digestion of grass and clover at two maturity levels during the season in dairy cows. Grass Forage Sci 49:378-390.

Stern, M. D., A. Bach and S. Calsamiglia. 1997. Alternative techniques for measuring nutrient digestion in ruminants. J Animal Sci 75:2256-2276.

Tilley, J. M. A. and R. A. Terry. 1963. A two-stage technique for the *in vitro* digestion of forage crops. J British Grassland Soc. 18:104.

Titgemeyer, E., N. Merchen, Y. Han., C. Parsons and D. Baker. 1990. Assessment of intestinal amino acid availability in cattle by use of the precision-fed cecectomized rooster assay. J Dairy Sci 73:690-693.

Tylutki, T. P., D. G. Fox, V. M. Durbal, L. O.Tedeschi, J. B. Russell, M. E. Van Amburgh, T. R. Overton, L. E. Chase and A. N. Pell. 2008. Cornell net carbohydrate and protein system: a model for precision feeding of dairy cattle. An Feed Sci Tech 143:174-202.

Van Soest, P. J., J. B. Robertson and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583-3597.

Van Straalen, W. M., J. J. Odinga and W. Mostert. 1997. Digestion of feed amino acids in the rumen and small intestine of dairy cows measured with nylon-bag techniques. Br J Nutr. 77:83-97.

Whitehouse, N. L., V. M. Olson, C. G. Schwab, W. R. Chesbro, K. D. Cunningham, and T. Lydos. 1994. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. J Animal Sci. 72:1335-1343.

Yang, C.-M. J. and J. B. Russell. 1992. Resistance of proline-containing peptides to ruminal degradation in vitro. Appl. Environ. Microbio. 58: 3954-3958.