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An In Vitro Technique to Estimate Digestibility of Amino Acids in Dairy Cattle Feeds

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An In Vitro Technique to Estimate Digestibility of Amino Acids in Dairy Cattle Feeds

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ABSTRACT

AN IN VITRO TECHNIQUE TO ESTIMATE DIGESTIBILITY OF AMINO ACIDS IN DAIRY CATTLE

by

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University of New Hampshire, Sept 2011

Three soybean meal (SBM), 3 SoyPlus® (SP), 5 dried distillers' grains with solubles (DDGS), and 5 fishmeal (FM) were obtained from FeedAC, Inc. (Homer, NY) and along with 5 cornsilage hybrid samples (CS), were used in an experiment to calculate total tract digestibility of crude protein (CP) and amino acids (AA). An in vitro procedure was used that incorporated the modified three step procedure (mTSP) developed by Calsamiglia and Stern (1994) and then modified by Gargallo et al. (2006) and the protease incubation procedure developed by McNiven et al. (2001) which replaces the in situ rumen incubation step in the mTSP. In the validation of the procedure correlation coefficients were produced from comparisons of experiments done previously in the same lab with the same feeds (Boucher et al. 2009 a,b&d, Fredin unpublished). Protein feeds had varied but promising results where as the CS sample results were too variable to be used to measure digestibility.

CHAPTER I

REVIEW OF THE LITERATURE

INTRODUCTION

Agricultural feedstuffs vary widely in their protein and amino acid content, composition, and also in the rate of degradation ruminally and absorption intestinally. Proteins and more importantly the amino acids which compose the proteins are the foundation for protein synthesis, which is vital to the maintenance, growth, reproduction and lactation of dairy cows (National Research Council (NRC) 2001). According to the *Nutrient Requirements of Swine* (National Research Council, 1998) and the *Nutrient Requirements of Poultry* (National Research Council, 1994) the amino acids that comprise protein are the actual variables that need to be satisfied in correct amounts and proportions to meet the needs of each physiological state. This notion that an ideal amino acid profile exists for each state is assumed to be true in dairy cattle as well, according to the *Nutrient Requirements of Dairy Cattle* (National Research Council, 2001). Amino acids (**AA**) are classified to be either essential (**EAA**) or non-essential (**NEAA**). Non-essential AA (**NEAA**) can be synthesized in the animal from metabolites of intermediary metabolism and amino groups from surplus amino acids at rates and amounts to satisfy the needs for that animal. Essential AA (**EAA**) are those that cannot be synthesized or synthesized in sufficient amounts within the animal to meet the animal's biological requirements (NRC 2001). In non-ruminant animals to meet a specific requirement they need to increase their consumption of that particular AA to reach the correct amounts

(Lapierre et al. 2005). In ruminant animals, the process of balancing the AA profile at the small intestine for absorption has more factors. Feedstuffs ingested by dairy cattle must past through the rumen where a large amounts of microorganisms reside, resulting in partial digestion of dietary ingredients such as proteins, fats, and starches used for growing and sustaining the microorganisms. This digestion causes feed to change from its original form and composition making predicting the feed's nutrient value more difficult (Lapierre et al. 2005). Secondly, proteins and free AA are quickly degraded in the rumen, this fact is why simple addition of free AA to the ruminant diet will not necessarily increase the supply of AA moving to the small intestine for absorption (Mangan 1972). The relationship between the rumen microorganisms and the cow creates a circumstance where ruminants have two sets of protein requirements; rumen degradable protein (**RDP**) and rumen undegradable protein (**RUP**) which both need to be satisfied in adequate amounts for optimal ruminal efficiency and to obtain the desired animal productivity (NRC 2001).

Rumen degradable protein is the primary protein requirement that needs to be satisfied in a dairy cow's diet. Rumen degradable protein's importance originates from its role in meeting the N requirements of rumen microorganisms by providing peptides, free amino acids, and ammonia. The goal is to provide these nutrients in adequate amounts to allow for optimal growth and proliferation of microorganisms. This allows microorganisms to pass to the abomasum and the small intestine where they can be digested and absorbed as microbial crude protein (**MCP**). Together MCP, RUP, and to a lesser extent endogenous crude protein (**ECP**), from saliva, sloughed epithelial cells, and the remains of lysed ruminal microorganisms comprise metabolizable protein (**MP**) and

is defined as the true protein that is digested post-ruminally and the component AA absorbed by the intestine (NRC, 2001)

The AA composition of the MCP flowing to the small intestine for digestion and absorption is considered to be constant along with the AA composition of ECP (NRC 2001). Yet the composition of the AA content in feedstuffs is variable depending on feed type and processing (Aldrich et al. 1997). To balance a dairy cow diet for AA and to be effective, supplements must compliment the forage and energy feed's AA profile that is being passed on to the small intestine from MCP and ECP. When a more complete AA profile that meets the requirements for a dairy cow is absorbed as MP, the performance and production of the animal will increase, because now it is able to use the limiting AA in the MP more efficiently.

National Research Council

The National Research Council (2001) library of estimates for RUP digestibilities was developed using published ruminal degradation of individual feeds using in situ methods of protein degradation in the literature. The default RUP digestibility coefficients in the NRC (2001) library were summarized using 54 studies where RUP digestibility of separate feedstuffs was calculated. Out of these studies 48 used the traditional mobile bag technique (**MBT**) with recovery of the bags from the feces and the in vitro procedure of Calsamiglia and Stern (1995) was used for the remaining 6 studies.

Ruminant protein digestibility measurements

In Situ Method

The development of in vitro procedures that can determine ruminal and intestinal digestibility of individual AA in processed or unprocessed feeds is important to

determining the types and quantities that need to be fed to optimize their use. For any of these methods to be validated in vivo measurements of intestinal AA digestibility would have to be gathered for comparison.

According to the NRC (2001) ruminal protein degradation is described most often by first order mass action models which are important considering the CP fraction of feedstuffs consists of multiple fractions. An in situ method using Dacron, polyester or nylon bags, is used widely as a method to estimate ruminal nutrient degradation. This procedure suspends polyester nylon, or Dacron bags filled with a quantity of feed sample in the rumen of a cannulated cow to be removed at pre-determined time intervals usually at 0,2,4,8,16,48,72 hours this allows the measurement of CP disappearance from the bags. This procedure is advantageous in many ways as it is relatively simple and low cost compared to other methods (Stern et al. 1997). It is also superior to other procedures because it involves the actual digestive processes that take place within the rumen and not a simulation of the rumen environment outside of the animal.

In any Dacron bag procedure there are some assumptions that have to be made, such as, all soluble protein is completely and instantaneously degraded in the rumen, and all protein that disappears at zero time is assumed to be soluble protein (Stern et al. 1997). These assumptions are not always true; rates of degradation for the soluble fraction could be slower than for the insoluble fraction (Mahadevan et al. 1980) and some protein can leave the bag due to the feedstuffs having a particle size smaller than the pore size of the bag (Stern et al. 1997). This procedure comes with many factors that need to be controlled; porosity of the bag, ratio of sample to surface area, particle size of the sample, method of bag placement in the rumen, physical nature of feed sample, diet of the animal

being used and the degree of bacterial attachment to feed residues. Grinding protein feeds can increase degradation, this increase can vary due to the type of feedstuff being ground since not all feeds have the same physical properties. Examples such as corn gluten meal is a glutinous material that can stick together when it becomes wet, this can lead to a decrease in surface area exposure of the feed to the digestive solution causing an artificial decrease in estimating protein digestibility (Stern et al. 1997).

The degree of bacterial attachment to feed residues can become a problem, because if they are not accounted for, the calculated passage of MCP and AA to the small intestine could be underestimated (Whitehouse et al, 1994). For accurate calculations of rumen digestion from any sample the removal of particle associated bacteria (**PAB**) must be assumed (Whitehouse et al, 1994). It is difficult to remove PAB however and a reliable removal method to correct for microbial contamination is needed (NRC, 2001). Physical treatments such as homogenization, pummellation, chilling and repeated washing have been used in the past, along with chemical treatments such as neutral detergent solution and methylcellulose to attempt to remove PAB from rumen feed samples.

Whitehouse et al. (1994) used physical and chemical treatments in combination to improve the removal of PAB and other microorganisms from feedstuffs being experimented on for digestibility of RUP AA and total AA (**TAA**). Different techniques both chemical and physical were evaluated over the course of 2 experiments. In experiment 1, 6 treatments were evaluated, and all treatments used a combination of a chemical and physical method of PAB detachment, and were evaluated by direct counting using epifluorescent microscopy or by indirect determination based on diaminopimelic acid (**DAPA**). Treatment 6 which consisted of saline-washed ruminal

digesta suspended in 600 ml of 4°C saline containing .1% Tween 80 and 1.0% methanol added to the solution for 24 h at 4°C then homogenized for 15 seconds had the greatest removal of PAB (70%). The second experiment was conducted to evaluate some of the more promising results from experiment 1. Five treatments were used using different combinations of chemical and physical PAB removal techniques. Treatments 3 (Tween 80, pH 2, Methanol (**MeOH**)), 4 (Tween 80, pH 2, MeOH, tert-butyl alcohol (**t-BuOH**)), which were both suspended in their solutions for 24 h at 4°C then homogenized for 15s and exhibited good PAB detachment ability (71.8% and 82.4% respectively ($P < 0.05$)). Treatment 5, involved incubating the samples in methylcellulose at 37°C for 30 min then suspending in a solution of saline, Tween 80, and MeOH at a pH of 2 for 24 h and homogenizing for 15s, resulted in 79.4% removal of PAB.

Mass et al. (1999) used the hypothesis of Sniffen et al. (1992) that neutral detergent insoluble N (**NDIN**) is the primary undegraded intake protein fraction of feed stuffs, to propose that refluxing rumen undegraded residue (**RUR**) in ND solution that did not contain sodium sulfite would remove PAB from the RUR. Solka floc was used for the incubation to provide a low N fiber source as to test the ability of ND solution to remove attached microbes. The Solka floc contained 0.10% N and 0.12% purines with the in situ residue containing a mean value of 0.47% N and 0.48% purines after being rinsed in water and 0.10% N with 0.11% purines after ND extraction. These results support that the Solka floc RUR had microorganisms attached to it after being rinsed with water and that they were removed during the reflux in ND solution.

Martinez et al. (2009) evaluated 3 procedures for detaching particle-associated microbes from forage and concentrates that had been incubated in Rusitec fermenters.

Method 1 took the RUR and incubated it in a saline solution containing 0.1% methylcellulose at 38°C for 15 min in a continuous-shaking water bath, method 2 the RUR was mixed with saline solution and homogenized with a Stomacher for 5 min at 230 rpm, and method 3 the RUR was immediately frozen for 72 h and then thawed at 4°C for 24 h. The detachment percentage of each procedure for forage was 65%, 72%, and 69% respectively, and for concentrates was 49%, 57%, and 53% respectively.

The largest cause of variation in determining ruminal protein digestibility was from variations among laboratory procedures used for sample preparation, processing, and type of hardware used such as filters and bag type (Stern et al. 1997). Stern et al. (1997) recommended that more emphasis go into calibration and standardization of laboratory procedures for CP analysis and measuring N solubility. Wilkerson et al. (1995) conducted a series of experiments to evaluate the variation in estimates of RUP in forages using 8 laboratories. They concluded that technician training contributes to the variation of results, and that proper standardized training would result in a decrease in that variation to a more acceptable level within and among different laboratories.

Enzymatic Method

Enzymatic techniques have also been a method of determining rumen protein digestibility of feed stuffs. In vitro enzymatic techniques have a complete disconnect from the animal model which makes them advantageous in many ways (Stern et al. 1997). The ability to control most if not all aspects of an in vitro procedure should allow for less variation among results, combined with the ability to have an exact methodology allows for the procedure to be standardized. Using prepared enzymes also allows rumen protein digestibility studies to be done without having to put surgical implants into cows

and then maintain them throughout the cow's life or experiment duration, thus removing the severe limits on the ability to routinely and rapidly get digestibility results of large numbers of samples (Mahadevan et al. 1987). Unfortunately the strength of the results can be limited due to such things as incomplete enzymatic activity compared to the animal's actual enzyme compliment (Stern et al. 1997). A common in vitro procedure is to incubate feeds in collected rumen fluid, which allows for the rate of ammonia accumulation to be determined. The drawback to this procedure comes from the varying rates of the reutilization of ammonia for microbial synthesis and absorption across the rumen wall which will reduce estimates of degradation and degradation rates for specific feeds. Quantifying protein degradation end-products will not take into account ruminal rate of passage for feed proteins which is also a determinant of ruminal protein escape. These will lead to inaccuracies when using degradation end-products to calculate feed protein degradation (Mahadevan et al. 1987, Broderick 1978). Broderick (1978) added inhibitors of amino acids and ammonia utilization in the incubation mixture. Hydrazine sulfate was added at 1.0 mM to an incubation medium consisting of strained ruminal liquor and McDougall's buffer, it was found to effectively inhibit the reutilization of added AA and ammonia. This may allow protein degradation to be estimated by the accumulation of these end-products. The specificity of the inhibition created by adding these inhibitors of AA deaminases have not yet been established (Broderick 1978).

Krishnamoorthy et al. (1983) attempted to simulate rumen proteolysis in vitro, they choose a protease enzyme concentration (0.66 units/ml) to provide proteolytic activity that closely resembles that of extracted rumen fluid. The samples where subjected to simulated rumen proteolysis for 18 to 48h to resemble mean retention times

in the rumen of grain and roughages, respectively. Residual CP at the end of the incubation period was considered to be an estimate of undegraded CP. The estimated values of rumen proteolysis in vitro and those done in vivo were in close agreement ($r = .78$; $P < .01$). These researchers realized that because of the lack of in vivo estimates in the literature the validity of this in vitro simulated rumen proteolysis procedure to estimate undegraded CP cannot be fully assessed. Yet they state that the technique can be recommended to evaluate feedstuffs on a relative basis and appears to provide results reasonably accurate with those of in vivo procedures.

Poos-Floyd et al. (1985) conducted an experiment to evaluate 5 methods of in vitro enzymatic ruminal protein digestibility to evaluate their ability to estimate ruminal protein degradation. The 5 enzymes tested were a bacterial protease (S. griseus), three plant proteases (papain, ficin and bromelain), and a neutral fungal protease. All of the correlations were high ranging from .73-.93 with ficin and neutral fungal protease having the highest correlation with in vivo ruminal protein degradation.

In the interpretation of ruminal protein digestibility experiments, models are used to describe the results. The most common model to describe these results divides feed CP into three fractions A, B and C. Fraction A consists of the percentage of CP that is non-protein N (**NPN**) plus a small amount of protein that is able to leave the bag immediately because of either high solubility or having a very small particle size as to pass through the pores of the bag being used. Fraction C is the percentage of feed CP that is completely undigested, which is represented by the feed left in the bag after a specific end point. Fraction B includes the rest of the CP consisting of protein that is potentially degraded and protein that has its degradation affected by rate of passage.

The Cornell Net Carbohydrate and Protein System (**CNCPS**) also evaluates ruminal protein degradation. This sub-model breaks down into five fractions (A, B_1, B_2, B_3, C). Fraction A is Trichloroacetic acid (**TCA**) soluble N. Protein that is unavailable or bound to the cell wall is considered to be fraction C. Fraction B_3 is neutral detergent insoluble N minus fraction C and fraction B_1 is TCA precipitable protein from the buffer soluble protein minus NPN. Fraction B_2 is protein described as having an intermediate degradation rate and is the remaining protein after all other fractions have been subtracted away. Estimates using this model were correlated with values in the NRC 1989 and had an r = .93.

Methods for Measuring Intestinal Protein Digestion

Mobile Bag Technique

Methods using a total collection approach basing results around protein disappearance with animals cannulated in the duodenum and terminal ileum have been used (de Boer et al. 1987). Improvements where made with the development of the mobile bag technique (**MBT**) to collect in vivo protein digestibility values of small allow insertion of the nylon bags into the digestive tract, containing 1g of ground feed, this amount and pore size was chosen to allow digestive enzymes and bacteria to enter the bag. Sauer (1983) conducted 3 experiments: 1. inserting the bags into the stomach of a pig and allowing them to move whole through the entire lower digestive tract, 2. placing the bags in the stomach then removing them 2.5 hours later and placing them in the duodenum via cannula, and 3. the bags were placed in a pre-digestion solution 0.01 N HCl and pepsin (1g/L), to simulate protein digestion in the stomach then placed into the small intestine via duodenal cannula. The bags would then be passed in the feces and

collected 38-48 hours later, freeze dried and analyzed for crude protein. The author observed that inserting the bags directly into the stomach resulted in problems when passing through the pyloric sphincter. The bags were not able to pass smoothly through and the digestibility of those bags was found to be over 90%. Sauer et al. (1983) decided to abandon this method. The results of the other two methods were more variable then the conventional control experiments, but he believed that the MBT showed promise in being able to determine the digestibility of feedstuffs in smaller quantities, quicker, and with less labor.

The differences in ruminant digestion compared to non-ruminant digestion require modifications to be made in the MBT with the migration of the MBT to be used in ruminants. The factors that ruminant animals introduce which make estimating intestinal AA disappearance difficult from an single, specific feed sample are microbial degradation of dietary protein in the rumen, ruminal synthesis of microbial protein, and recycling of N into the rumen. The approach taken was to implement a rumen incubation step into the protocol which would incubate the feed samples in situ before any gastric or intestinal digestion takes place. This would ensure that the sample used would closely resemble ruminally fermented feed which has been reported to have different digestibility values and AA profiles then their intact counterparts (Benchar et al., 1994; Erasmus et al., 1993).

Kirkpatrick and Kennelly (1984) modified the original in vivo technique used in pigs for the use in cattle. They used the same 50 μm pore size in their nylon bags and combined elements from experiment 2 and experiment 3 of Sauer et al. (1983) to resolve the challenge that the ruminant animal introduces. The bags were suspended in the

rumen for 15 h then incubated in pepsin – HCl solution for 3h (1g pepsin/L 0.01 N – HCl) then placed on ice to stop any enzymatic action. The bags were inserted into the duodenum and recovered in either the terminal ileum if a cannula was present or more commonly in the feces, 16-20 hours later. The bags are then washed to remove endogenous and any other contaminating proteins, then dried and analyzed for protein and dry matter. Kirkpatrick and Kennelly (1984) found that the mobile bag technique for ruminants consistently underestimated the digestibilities but still beleived the technique showed promise for an approach for rapid determination of protein digestibilities in feedstuffs. This technique or variations on this technique have become the most popular method of running the MBT even though no standardized protocol exists when using the MBT (Boucher et al. 2009a).

The MBT has many areas of possible variation such as porosity of bag material, sample to surface area ratio, animal and diet effects, retention time, site of bag recovery and microbial contamination (Stern et al. 1997). Varvikko and Vanhatalo (1990) ran a study to asses the MBT and what the influence of free surface area and pore size of a synthetic fiber bag would have on the intestinal digestion estimates. The results of this study support that surface area significantly affects disappearance of feed components from the bags more than pore size. Free surface area affected ryegrass and barley N disappearance the most, ($P < 0.01$ and $P < 0.001$ respectively). They also observed a trend where bags with free surface areas of 5% or more consistently affected the disappearance of feedstuffs, even with smaller pore sizes. This lead them to conclude that with a ratio of surface area : pore size between the 2:1 – 33:41/μm free surface area is the major determinant of the degradation of feeds in the MBT.

Studies have given evidence that not all feeds are affected by a rumen incubation to the same extent, for example de Boer et al. (1987) incubated numerous feeds (Soy bean meal (**SBM**), Canola meal (**CM**), Corn gluten meal (**CGM**), Fish meal (**FM**), Alfalfa hay (**AH**) and Meat and Bone meal (**MBM**)) in the rumen for different time points (0, 2, 4, 8, 12, 24h) and measured rumen, intestine, total tract, and available undegraded dietary protein. They found that rumen CP disappearance increased with rumen incubation time for all feed stuffs, but the incubation of bags in the rumen before insertion into the duodenum increased intestinal CP disappearance for only MBM and AH. Meat and bone meal had a total tract protein disappearance of 25.5% at 0 hour where all other feeds had a percent disappearance >86.8% which demonstrates the importance of rumen incubation with MBM over the other feeds. Rooke (1984) incubated FM, rapeseed meal (RSM) and MBM in the rumen of a cow for 8 or 24 h in nylon bags to evaluate the effects of rumen incubation on protein and AA digestibility. The true N digestibilities of all three protein sources were reduced compared to intact when fed to rats as the only protein source after rumen incubation (FM 0.90 to 0.86; MBM 0.81 to 0.55; and RSM 0.76 to 0.67).

Variation can also occur with the use of a gastric stomach digestion phase incorporated into the MBT. During MBT's development it was shown that bags must pass through the gastric stomach to have the most accurate total tract protein and AA digestibility results. Due to problems with bags passing through the pyloric sphincter causing an artificially long passage time (Sauer et al. 1983) an in vitro substitute of incubating feeds in an HCl – Pepsin solution was developed. Cherian et al. (1988) conducted an experience to ascertain the effect of pH, pepsin activity, and duration of a

pre-incubation period prior to the MBT in swine. A pH of 2.0 resulted in the highest digestibility coefficients for each feedstuff tested at a pepsin concentration of 377 IU/liter and an incubation time of 2.5 h (SBM 90.8, MBM 81.0, CM 76.7). As pepsin activity increased from 189 IU/liter to 377 IU/liter, at a pH of 2.0 and an incubation time of 2.5 h the protein digestibility of SBM and CM also increased 89.9 to 91.5 and 76.7 to 78.4 ($P < .01$) respectively, yet MBM did not. Cherian et al. (1988) hypothesized that these results indicate that a higher concentration of pepsin may be needed to maximize the hydrolysis of plant protein compared to animal protein. Incubation time also had an effect, as it increased protein digestibility also increased (SBM 78.8-93.7; MBM 56.2-79.3; CM 65.2-77.8). At 0h, (no pre-incubation), the digestibilities were drastically lower in comparison to other treatments which indicate that pre-incubation is important to accurately estimate protein digestibility regardless of the method used. These results, however, have not always held true with swine or cattle MBT results. Graham et al. (1985) did not see any effects of what when using a pre-incubation with a pepsin-HCL solution in a variety of feeds with swine. This notion was also in agreement with many MBT studies done in cattle, (Vanhatalo et al., 1995; Rooke 1985) both emphasized that there is no need for a pre-incubation of rumen undegradable feed to accurately estimate intestinal disappearance of feed N.

Retention time of bags according to Stern et al. (1997) varies greatly within and among experiments yet they have found that it does not matter and the differences caused by retention time are minimal. In the MBT the modification of the collection region, either from a cannula in the terminal ileum or in the feces will have the greatest effects on retention time. The bags since moving through the very proteolytic environment of the

small intestine should be removed from a cannula in the terminal ileum, but for a more practical and convenient method, collection of the bags in the feces can be done (Stern et al. 1997). Two assumptions must be made when collecting bags from the feces; 1. feed residues are not contaminated with microbial protein from intestinal fermentation in the large intestine and 2. protein leaving the ileum is not further digested by microbes in the large intestine. With N remaining in the feed at very low concentrations after digestion, contamination from microbial sources could be a greater concern (Stern et al. 1997).

Even though the MBT requires the use of at least a rumen and possibly an illeal cannulated animal the procedure is still relatively easy and provides a more physiological approach to estimating protein and AA digestion then other methods, and despite all the possible sources of variation, the MBT has been successful in the determination of the digestibility of protein and AA in numerous studies and across numerous feed types.

Haugen et al. (2006) used the MBT to determine the digestibility of RUP of smooth bromegrass and birdsfoot trefoil over a 2 month period in June and July. Two ruminally and duodenally cannulated steers were used to incubate feeds prior to incubation in a pepsin and HCl solution at 37°C for 3h to simulate abomasal digestion. Bags were inserted into the duodenum 2 h after feeding at a rate of 1 bag every 10 min for a total of 8 bags per steer daily and were recovered in the feces and frozen until all bags were collected. To reduce microbial contamination the bags were machine washed and bulk-refluxed in NDF solution and dried in a forced-air oven at 60°C for 48h, then analyzed for N content. Rumen undegraded protein (%DM) of smooth bromegrass in June and July were 1.82 and 1.71 respectively ($P = 0.11$) and the RUP (%DM) for birdsfoot trefoil increased from 1.30 in June to 1.94 in July ($P < 0.01$). The MBT was

able to detect a forage x date interaction ($P < 0.01$) for RUP, indigestible dietary protein (**IDP**), and digestibility of RUP between smooth bromegrass and birdsfoot trefoil harvested in June compared to July supporting the use of the MBT when evaluations forages.

Corn silage (CS) is another feed that is used throughout the northeast. Trinácty et al. (2003) conducted a RUP and whole tract protein digestibility study using a ruminal in situ incubation and the MBT. Three dry cows fed a ration containing 89% forage : 11% concentrate with ruminal and duodenal cannulas were incubated during the experiment. Nylon bags with a pore size of 42 μm and containing 1.5g of ground sample were incubated in the rumen for 16h prior to being subjected to the MBT. The post ruminal digestibility of RUP was reported to be 81% and the apparent total tract digestibility of RUP was 91%.

The MBT is versatile in the amounts and kinds of feeds that can be evaluated. It offers a physiological method to researchers for determining the digestibility of those feeds. Unfortunately for the most accurate results duodenal and ileal cannuals are needed which, are expensive to insert and difficult to maintain, and because of this, new methods either in situ or in vitro, have to be considered.

Cecetomized Rooster Assay

Most methods of duodenal AA flow are measured using an indigestible marker which can be measured in digesta collected at the terminal ileal cannula, or using the MBT, which for the most accurate results should have the bags collected at the terminal ileum. Unfortunately, cannulas placed in the ileum come with challenges such as

reductions of feed intake due to infection or other complications. The procedure is also expensive and labor intensive to maintain (Titgemeyer et al. 1989).

The cecetomized rooster assay is an alternative method to estimate small intestinal digestibility of the RUP fraction of feedstuffs in cattle. This is done with the use of cecectomized birds, roosters in most cases, this requires removing the ceca of the bird which in turn removes most of the fermentative capacity of the avian gastrointestinal tract (Titgmeyer et al. 1990). During this procedure the RUP fraction of a feed is collected by incubating feed in situ in the rumen of a cow for a set time period then the RUR is collected and crop-intubated into the cecetomized roosters. This is done by fasting the roosters for a time period usually 24 hours (Titgmeyer et al 1990) then intubating the feed using a funnel into the crop and a plunger to push feed completely through. Over the next 48-58 h the rooster's total excreta is collected and lyophilized for analysis of CP and AA content.

In the Titgmeyer et al. (1990) precision-fed rooster assays results of 5 different diets were compared to a more traditional digestibility study where the AA in duodenal digesta were measured with reference to chromic oxide. The results of the precision-fed rooster assay were in agreement with those obtained from the chromic oxide study in steers, the un-weighted treatment means across 13 AA were 68.3, 66.1, 75.3, 68.5, and 67.9% and for the rooster assay: 72.5, 69.9, 71.5, and 70.3% for basal, SBM, CGM, BM, and FM, respectively. These results correlated well $r^2 = .938$ ($P<.05$) indicating that intestinal AA digestibility by both rooster assay and cattle using chromic oxide can be used to acquire similar treatment responses. Griffin et al. (1993) used the precision fed rooster assay to evaluate intestinal digestibility of RUP and RUP-AA in raw soybeans,

SBM, and extruded SBM. The feeds were incubated in the rumens of calves for 16h before to use in the rooster assay. The study showed that calves fed the SBM diet retained 70% more N then calves fed the heat damaged soy bean product, which suggests poor intestinal availability of the AA that escape ruminal degradation, which was also predicted by the rooster assay. Griffin et al. (1993) concluded that the precision-fed rooster assay may be useful in predicting true AA values of numerous protein sources. Aldrich et al. (1997) also conducted a study where the objectives were to predict the effects of roasting and extrusion temperature of whole soybeans on intestinal protein digestibility in cattle. This was done using a two-stage in vitro or in situ ruminal incubation/precision-fed and the rooster assay. Trypsin inhibitor (**TI**) activity in the raw and extruded ground SB was quantified, and as extrusion temperature increased the authors found that TI activity decreased, this is a likely reason for the increase in RUP-AA digestibility in the extruded samples The increased digestibility of AA in the roosters corresponding with the measured reduction in trypsin inhibitor activities found in the different SB treatments supports the use of the rooster assay as an appropriate model for intestinal digestibility in the ruminant.

Heat Treatment

Feed protein when protected from degradation in the rumen has the ability to by-pass the rumen and move into the lower digestive tract to be digested. If high quality feed proteins could be altered to do this, they could be utilized more efficiently for milk production (Faldet et al. 1990, Kung et al. 1987, Sahlu et al. 1984). One of the major components of determination of protein degradation in the rumen is the solubility of the protein. Heat treatment can be a method to lower protein solubility (Ahara et al. 1978,

Schingoethe and Ahara 1979) making the protein, or more importantly individual AA such as lysine and methonine, available for digestion and absorption in the small intestine with the goal of increasing milk production in lactating cows (Ahara et al. 1978). Heat treating feeds prior to feeding has the greatest potential to reduce protein solubility in the rumen, safely and economically (Faldet et al. 1990).

Faldet et al. (1991) conducted a study using 46 multiparous Holstein cows fed diets of 50% forage and 50% concentrate, and supplemented with 3 different soy products; SBM, raw soybeans, and heat-treated soybeans. Feeding the heat-treated soybeans supported more milk (4.5 kg/d) 3.5% fat corrected milk (FCM) (4.0 kg/d) and milk protein (.09kg/d) than SBM or raw soybeans. Schingoethe et al. (1987) conducted a study where the objective was to compare the lactational responses of cows to diets that contained one of three protein supplements (SBM, heat-treated soybean meal and extruded blend of soybeans and soybean meal). Seventy-three high producing early lactation Holstein cows feed TMR diets containing 30% corn silage, 15% alfalfa hay, and 55% of the respective concentrate were used to evaluate these protein sources. It was concluded that milk production was increased when their diet was supplemented with heat-treated SBM over commercial SBM, 34.5 kg/d to 32.2kg/d ($P<.05$) respectively.

Aldrich et al. (1997) used the precision-fed rooster assay described by Parsons (1985) to predict the effects of different extrusion temperatures on the intestinal protein digestibility of soybeans in cattle. Ground raw soybeans and soybeans extruded at 116, 138, or 160°C were placed in polyester bags (75g) (20 x 30 cm pore size) and suspended in ventral rumen of steers for 16 h. The four treatments that were incubated in situ and two un-incubated treatments (ground raw SB and 160°C extruded SB) were crop-

intubated in the roosters (20g) and total excreta were collected for 48 h, freeze dried, and ground with mortar and pestle. To make sure endogenous AA excretion did not cause variations in the estimates there was also a 48-h collection done on feed deprived birds which allowed for the calculations of endogenous AA excretion. This allowed those values to be subtracted from the AA in excreta of the treatment birds to correct for endogenous AA losses (Aldrich et al. 1997). The true digestibilities of non-essential, essential, and total AA were all increased in un-incubated samples from raw ground to extruded at 160°C. The RUP-AA digestibilities were also improved with extrusion of the in vitro incubated SB samples with the greatest improvement for incubated samples were seen at the 116°C temperature. Aldrich et al. (1997) concluded that the precision-fed roosters are a valid model for the evaluation of small intestinal digestibility of protein in ruminants. Using adequate heat treatment to reduce TI activity seems to be as stated before the factor that is allowing the increase in RUP-AA digestibility, but as seen in this experiment increasing the extrusion temperature does not exponentially increase the RUP-AA digestibility. The proper temperature and method of heating and with the eventual inclusion of varying feed varieties used in this method of feed manipulation indicates that further investigations must be done.

As stated previously, roasting whole soybeans increased milk production, fat-corrected milk production and milk protein with no reduction in milk fat production (Faldet and Satter, 1991). This improved production has been attributed to the increase of protein escaping from the rumen and moving on to the small intestine were it can be broken down into its individual AA and adsorbed. In a study by Faldet et al. (1991) soybeans were heated in a forced hot air oven at 120°C and 130°C at 60 and 180 min and

also at 140°, 150° and 160° for 10, 30, 60, 90, and 120 min. Two measurements were done 1) was to estimate the rate and extent of protein degradation in the rumen using an in vitro method and 2) was to determine the nutritional availability of lysine. Lysine is one of the co-limiting AA in a dairy cow diet and is the AA most vulnerable to heat damage through the Maillard reaction. The Maillard reaction is the major non-enzymatic sequence of reactions that cause protein to become nutritionally unavailable when heated (Faldet et al. 1991), this makes measuring lysine fairly accurate in indicating the amino acid supply that is passing to the small intestine for possible absorption. As heat was increased the RUP increased, and the rate of protein degradation decreased along with total lysine content. Faldet et al. (1991) calculated that to achieve maximal post ruminal lysine content, a loss of 15-22% of chemically determined available lysine was necessary. The authors concluded that the effect of heat treatment is not only a function of temperature but also of time and temperatures between 140°C and 160°C with incubation times of 30 and 120 min being optimal for protecting SBM protein for supplementation in dairy cow diets.

In Vitro Techniques for Estimating Digestibility of RUP and RUP-AA

Estimating digestibility of RUP and RUP-AA using in-situ and in-vivo techniques requires cannulas to be surgically inserted into the rumen and small intestine. These procedures are expensive in initial cost and are very time consuming to maintain long term (Calsamiglia and Stern 1995). The development of in-vitro techniques could provide a way to determine intestinal digestibility of RUP and RUP-AA of feeds without the need of intestinal cannula. Calsamiglia and Stern (1995) have stated guidelines to any future in-vitro process with the goal to estimate RUP and RUP-AA digestibility, the

technique should 1) closely simulate physiological conditions of ruminants, including potential effects of ruminal fermentation; 2) be rapid, reliable, and inexpensive; 3) be applicable to a wide variety of protein supplements, and; 4)accurately reflect differences in protein digestions.

The 3 Step Procedure

The 3 step procedure created by Calsamiglia and Stern (1995) is one of the most commonly used in-vitro methods for estimating the digestibility of RUP and RUP-AA. The process consists of three distinct steps; 1) Ruminal incubation; 2) Pepsin-HCl incubation; and 3) Pancreatin incubation. Intact feeds are first pre-incubated in the rumen of a cow through a rumen cannula in Dacron bags for a time period of 16 h which was selected as the best estimate of the average time a feeds spends in the rumen of the cow (Calsamiglia and Stern 1995). The bags are then removed and rinsed in tap water until runoff is clear. The rumen residue collected is then incubated in a .1 N HCl solution at a pH 1.9 containing 1 g of pepsin/L for one hour at 38°C. The pH is neutralized with .5 mL of 1 N NaOH and 13.5 mL of a buffer-pancreatin solution containing .5 M phosphate solution, pH 7.8, with 3 g of pancreatin/L. These samples are then incubated at 38°C for 24 h in a shaking water bath. After the incubations are complete 3 mL of 100% trichloroacetic acid (TCA) is added to stop enzymatic action. The samples are then centrifuged at 10,000 x g for 15 min for the measurement of TCA-insoluble N. With these results RUP digestibility can be calculated by taking the TCA-insoluble N and dividing it by the total amount of N in the rumen residue.

The pancreatin procedure was compared against, for the purposes of validation, 34 duodenal samples that had been freeze dried from experiments in which intestinal

digestion had been determined in vivo (Calsamiglia and Stern 1995). The regression equations of the in vivo estimates on pancreatic digestion had a coefficient of determination of 0.91 (Stern et al. 1997)

Several studies have used the TSP since its conception to estimate the digestibility of the RUP fraction (Howie et al. 1996, Kopecny et al. 1997, McNiven et al. 2002, Kleinschmit et al. 2007) yet there have been questions about how accurately it can estimate RUP – AA digestibility. Borucki-Castro et al. (2006) conducted an experiment to determine the impact of different methods of treating SBM on ruminal degradability and intestinal digestion of CP and AA. The estimates of the intestinal digestibility of AA and CP were lower in vitro then when measured in situ, and treated SBM products were greater than solvent extracted SBM which caused Borucki-Castro et al. (2006) to conclude that further development is needed for using the 3 TSP for obtaining estimates of RUP – AA digestibilities instead of the in MBT.

The Modified Three Step Procedure

Gargallo et al. (2006) took the major procedure of the original TSP designed by Calsamiglia and Stern (1995) and modified the procedure by adapting it to a Daisy^{II} Ankom incubator to reduce the cost and labor involved in the use of the TSP to determine intestinal digestion of protein. Five g of feed ground through a 2-mm screen was weighed and placed in 5x10 nylon bags (Ankom R510, pore size 50 um; Ankom, Fairpoint, NY) and suspended in the rumen for 12 hours. Through his experiments, the pre-incubation of feeds in the rumen was favorable to more accurately estimate intestinal digestibility of the RUP protein fraction when using the Daisy^{II} Ankom incubator technique. After ruminal incubation the bags were rinsed for 5 min 3 times in an

automatic washing machine (or until runoff was clear). When determining AA concentration in high-fiber, low-protein feeds it is advantageous to suspend the bags in a 0.1% methylcellulose solution and incubate in a shaking water bath at 37°C for 30 min. This procedure was found necessary to remove or detach PAB from fibrous feeds as to not have the bacteria cause incorrect levels of measured digestibility (Gargallo et al 2006). Bags are then rinsed with tap water and can be stored at -18°C until analyses when the bags are thawed and rinsed in a washing machine for 3 to 5 min (or until run off is clear. Samples are dired in a oven at 55°C for 48 h and residue is pooled and composited for determination of N content by Kjeldahl method.

The rumen residue not analyzed is then weighed into nylon bags (Ankom R510, pore size 50 um) at 0.5 to 5g each and heat sealed. The use of the R510 bag on strictly a more practical level made more sense for the overall procedure then the R57 bag which is smaller, as it allows for determination of intestinal digestion in the same bag used for the rumen incubation and the incubation of a lager sample of 5g reduces the labor involved (Gargallo et al. 2006). Up to 30 bags can be placed into a Daisy^{II} incubator bottle contains 2 L of a prewarmed 0.1 N HCl solution (pH 1.9) containing 1 g of pepsin/L (P-7000, Sigma, StLouis, MO) and incubated in constant rotation at 39°C for 1 h. According to Gargallo et al. (2006) the number of bags used in each incubation bottle does not have any effect on the outcome of the RUP digestibility. Gargallo et al. (2006) also studied if there was a way to alter the concentration or enzymatic activity of the pepsin enzyme used in the original TSP (P-7012, Sigma, St. Louis, MO) as it was of a very high enzymatic activity and also very expensive. The use of a less purified pepsin was experimented on (P-7000, Sigma) which was 22 times less in cost per unit of activity.

The results of the less purified pepsin enzyme were highly correlated to results obtained with the use of the high enzymatic activity pepsin ($P<0.001$) (Gargallo et al. 2006).

After the incubation all bottles are drained and all bags were rinsed with tap water until runoff is clear. Two L of a prewarmed pancreatin solution (0.5 M KH_2PO_4 buffer, pH 7.75, containing 50 mg/kg of thymol and 3 g/L of pancreatin; Sigma P-7545) is added to each bottle, then the bags are added to the solution for incubation in constant rotation at 39°C. Once the incubation is complete, the contents of the bottle is drained and the bags are once again rinsed until runoff is clear. The bags are drained and dried in an oven at 55°C for 48 h weights are recorded and the N content is determined by Kjeldahl method. The calculation of pepsin-pancreatin digestion of protein and AA can be done by subtracting the protein or AA left in the final sample by the amount in the original sample, these results correlated well to the original TSP ($R^2 = 0.84$, $P <0.001$) but has not yet been evaluated against any animal model. The residue remaining undegraded at the end of the procedure can be collected and analyzed for AA and CP, making the use of TCA to percipitate out the protein in the end sample unnecessary. These modifications from the work of Gargallo et al. (2006) allows RUP – AA digestibility estimates to be made using AA analysis with ion exchange chromatography.

In a study by Boucher et al. (2009) the modified TSP and other in vitro digestibility methods were evaluated for the purpose of determining if cecetomized roosters could be used as an animal model for RUP intestinal digestibility. Three SBM, 3 SoyPlus (**SP**), 5 DDGS, and 5 FM samples were used to estimate digestibility of AA in rumen-undegraded protein. This is the first study to attempt to evaluate the modified TSP with in vivo data which was obtained for all samples using the precision-fed

cecectomized rooster assay (Boucher et al. 2009). The TSP was run on the RUR and was analyzed for AA and the digestibility of RUP-AA was calculated from the disappearance of residue form the bag. The RUP-AA estimated digestibilities were highly correlated to the in vivo estimates from the cecectomized rooster assay (Total AA $R^2 = 0.93$) which goes a long way to validating the MTSP for the calculation of RUP – AA digestibilities.

McNiven Protease Incubation

The TSP (Calsamiglia and Stern, 1995) is an in vitro procedure preceded by a rumen incubation for estimating RUP digestibility. This procedure removed the need for having duodenal and terminal ileal cannulas placed in cows for the purposes of obtaining RUP digestability estimates, yet still requiring rumen cannulas for the pre-incubation of feed samples in the rumen prior to any intestinal digestibility estimates in vivo or in vitro. McNiven et al. (2002) modified the TSP to eliminate the use of live animals by an in vitro incubation to simulate rumen fermentation. This in vitro incubation was done by first weighing 1 g of feed into Ankom bags (F57, pore size 50 μm) and placing a maximum of 30 bags in a 2.4 l bottle containing 1.6 l of borate (0.0345 M)- phosphate (0.0551 M) buffer at a pH of 7.8-8.0 and incubating in a shaking waterbath at 39° C for 1h. After the incubation 400 ml of protease solution (protease type xiv from *S. griseus* (Sigma P-5147) adjusted for a concentration to make 66 units of protease/g feed in the complete solution that will be incubated in a shaking water bath at 39°C for 4 h. The feed samples after this incubation are rinsed 6 times with distilled water and then can be entered into the last 2 steps of the three step procedure to evaluate RUP digestibility.

In the study conducted by McNiven et al. (2002) feed samples were also run in vivo using the MBT for an animal model to compare digestibilities against the full in

vitro procedure. The relationship between N digestibility by the in vitro method and the MBT with rumen pre-incubation having an $R^2 = 0.951$ made McNiven et al. (2002) conclude that the in vitro method including the protease, pepsin, and pancreatin enzyme incubations results in an accurate estimate of mobile bag N digestibility (McNiven et al. 2002).

An experiment that tested feeds originating from Iranian plant varieties conducted by Mesgaran and Stern (2004) including SBM and two corn silage samples one with 16g of urea per kg of dry matter (**MS16**) and one with 24 g urea per kg of dry matter (**MS24**). The goals of the experiment were to determine ruminal and post ruminal protein disappearance of these various feed types using the in situ MBT, an in vitro enzyme incubation, which included the protease pre-incubation step developed by McNiven et al. (2002), and a three step in situ/in vitro procedure developed by Calsamiglia and Stern, (1995). Total tract protein disappearance of SBM for the in situ mobile bag, in vitro, and TSP were 0.87, 0.96, and 0.92 respectively. These values were in agreement with other observations made when using these methods in previous studies (Calsamiglia and Stern, 1995; De Boer et al., 1987; McNiven et al., 2002). The total tract protein disappearance for MS16 were 0.57, 0.60, 0.61 and for MS24 were 0.78, 0.80, and 0.79 respectively using the three techniques. Accoriding to the data in the Mesgaran and Stern (2004) study correlations between procedures were variable, when looking at total tract protein digestibility the relationship between the in situ MBT and the in vitro procedure were similar to the MBT and the three step procedure with $r^2 = 0.61$ and 0.66 respectively. These relatively low values are in disagreement with McNiven et al. (2002) who found a very high correlation ($r^2 = 0.95$) for N digestibility when comparing the MBT to their

specific in vitro procedure when using SBM, barely, and oats. Mesgaran and Stern (2004) concluded that the large variation between results could be from the fact that the present study used a wide range of feeds for evaluation causing feed characteristics to be responsible for the varying correlations. When comparing the weak correlations between methods in this study and the mediocre ability of the TSP to estimate protein disappearance of the feeds analyzed by the MBT in this study, Mesgaran and Stern (2004) recommend that modifications of these methods for estimating feed protein digestibility need to be developed.

CONCLUSIONS

Dairy cattle feedstuffs and supplements vary in their AA content and digestibility, each physiological state during a cow's life needs the proper balance of certain AA to reach optimal production and or growth. Taking into account the difficulties the rumen digestive tract introduces as feed pass through the microbial rich environment of the rumen, it is very important to know the amino acid profile of the intact feed being fed to the animal and more importantly the RUP content and total tract digestibility of the feed being fed. The ability to quickly, easily, and accurately analyze feedstuffs gives producers the ability to balance their rations effectively. The most accurate way to analyze for protein and AA is with methods that require animals fitted with rumen and intestinal cannulas, these are expensive to have surgically implanted and are expensive and labor intensive to maintain. The development of in vitro procedures are an important advancement in this field of study, as being able to remove the need of cannulated animals would save time and money in testing feed for their protein and AA digestibility.

CHAPTER II

AN EVALUATION OF AN IN VITRO TECHNIQUE TO ESTIMATE DIGESTIBILITY OF AMINO ACIDS IN FEEDSTUFFS

INTRODUCTION

Balancing protein, and the amino acids (**AA**) that comprise protein, are important to maximizing the production of lactating dairy cows. Proteins are categorized into two groups: rumen degradable protein (**RDP**) and rumen undegradable protein (**RUP**) that resists breakdown in the rumen and passes to the small intestine to then be broken down and possibly absorbed. Microbial crude protein, RUP, and endogenous crude protein (**ECP**) make up metabolizable protein (**MP**), the protein available for digestion post-ruminally to AA and absorbed in the intestine. The MP pool contains AA which are the building blocks for tissue and milk protein synthesis, along with being crucial to the proper maintenance, growth, and reproduction of a dairy cow (NRC, 2001). The AA supply and digestibility of the MCP in MP is considered constant, but the RUP fraction of MP will vary among feeds (NRC, 2001). This characteristic of RUP allows manipulation of the AA profile of MP passing to the small intestine. This can be attempted by feeding rations with high concentrations of favorable limiting AA, but to predict the exact RUP-AA profile supplied to MP is difficult to accomplish, considering the rapid degradation of protein and absorption of free AA in the microbial rich environment of the rumen. The ability to quantify protein – AA digestion to help create a complete dairy cow ration is very important. The possible reduction or the elimination of wasting protein in a diet

which would in turn reduce the amount of protein fed, coupled with the reduction of waste products such as methane and ammonia, and reduced cost of the ration all supports the quantitative importance of protein – AA in dairy rations (Stern et al. 1997).

The most widely used approach for protein digestibility studies is the mobile bag technique (**MBT**) where cannulas are placed in the rumen, duodenum and possibly the terminal ileum. Yet cannulation procedures are expensive, labor intensive and time consuming (Stern et al. 1997), and once they are surgically implanted they need to be maintained. In vitro methods to estimate protein digestibility have been developed (Calsamiglia and Stern 1994), and modified (Gargallo et al 2006) which has eliminated the need for intestinal cannulas, but still require the use of rumen cannulation. Even with this modification, the variation caused by the rumen incubation still exists and surgical cannulas are still needed to perform AA digestion studies. Therefore the goal to make a completely in vitro procedure that mimics complete rumen digestion that can be standardized and repeatable is needed.

The development of any in vitro procedure should: (1) closely simulate physiological conditions of ruminants, including potential effects of rumen fermentation; (2) be rapid, reliable, and inexpensive; (3) be applicable to a wide variety of protein supplements; and (4) accurately reflect differences in protein digestion (Calsamiglia and Stern, 1995). The TSP first developed by Calsamiglia and Stern (1995) allowed for protein digestibility estimates to be made without the use of any small intestinal cannulas. The modifications made a by Gargallo et al (2006) allowed for the three step procedure (**TSP**) to make not only protein digestibility estimates but estimates of the digestibility of individual AA. The objective of this study is to incorporate the McNiven protease

incubation (McNiven et al, 2002) into the TSP to replace the ruminal incubation, with the goal being a complete in vitro procedure that can accurately quantify individual AA digestion of dairy cattle feedstuffs.

Methods and Materials

The feed samples used in the current study were used in previous studies conducted at the University of New Hampshire. The protein feeds, SoyPlus (**SP**), soybean meal (**SBM**), dried distillers grains with solubles (**DDGS**), and fish meal (**FM**) were used by Boucher et al. (2009a,c,d) and the in vivo digestibility data obtained from the cecectomized rooster assay and the modified three step procedure (**mTSP**) will be used to evaluate the accuracy of the procedure in the current experiment. The corn silage samples were the same as used in an unpublished master's thesis experiment conducted by Fredin in 2009 also at the University of New Hampshire. The in vivo digestibility results from the mobile bag technique (**MBT**) and the in vitro results from the mTSP will be used to evaluate the accuracy of the current experiment as well.

In vivo measurements were obtained differently between the protein feeds and the corn silage samples. The MBT was used for the corn silage instead of the cecectomized rooster assay because the ground corn silage became too bulky and not enough of the sample could be fed to the roosters for accurate digestibility values.

Feed Samples

Protein Feeds

Two kg of each of the following samples 3 soy bean meal (**SBM**), 3 Soyplus® (**SP**) (West Central, Ralston, IA), 5 dried distillers grains (**DDGS**) with solubles and 5 fish meal (**FM**) [1 Anchovy (**ANVY**), 1 Catfish (**CFSH**), 2 Menhaden (**MNHN**), and 1

Pollcok (**PLCK**), were obtained from the Feed Analysis Consortium, Inc.(www.feedac.org). The protein feed samples used for this experiment and heat treatment done prior to analysis were the same for all samples that were used in the in vitro experiments of Boucher et al. (2009a,b&c).

Corn Silage

Five unprocessed corn silage (CS) samples were obtained from SALLC. Corn silage 1 was is a hybrid that was harvested in September 2007 at 35% DM which was ensiled in a 3 x 18 m concrete stave silo at an estimated packing density of 620 kg/m^2 and ensiled 360 days. The other 4 CS samples (CS 2 through 5) were chopped in September of 2008 averaging (mean ± SD) 32 ± 2% DM. These are the same samples used in a previous unpublished MS thesis study done at the University of New Hampshire by Ferdin et al. (unpublished)

McNiven modified protease incubation

Five g of each 3 SBM, 3 SP (West Central, Ralston, IA), 5 DDGS, 5 FM (1 ANVY, 1 CFSH, 2 MNHN, and 1 PLCK), and 1 gram of each corn silage sample were ground to pass a 2mm screen and weighed into nylon bags with a pore size of 50μm (Ankom R510, Ankom Technology, Macedon, NY) and then heat sealed. Thirty bags were placed into a Daisy II incubator bottle (Ankom Technology, Macedon, NY) in 1.6L of borate phosphate buffer created by dissolving 15.20g $NaH_2PO_4H_2O$ and 26.34g $Na_2B_4O_710H_2O$ into 2L dH_2O which is pre-warmed to 39°C. The pH was adjusted with HCl to a pH of 7.8-8.0, and then the bags were incubated in constant rotation at 39°C for 1 hour. With the remaining 0.4 L of borate phosphate buffer, dissolve an amount of protease (type xiv from *S.griseus* [Sigma P-5147]) to create a solution with 66 units/g of

feed in a 2.0L solution. The solution was added to the Daisy II incubator jar after the 1 h incubation and the incubation was continued in constant rotation at 39°C for 4 h. The jars were removed from the Daisy incubator after the 4 h incubation and all bags were rinsed filling the jars 3 times with cold H_2O to halt enzymatic activity. The bags were rinsed 4 times with tap water for 30 min or until rinse water is clear, then again with dH_2O.

HCL – Pepsin Incubation

A 0.1N HCl solution was prepared (8.36ml HCl/L of distilled water, 16.72ml for 2 L), the solution was pre-warmed to 37°C and the pH was adjusted using strong NaOH (10g of NaOH in 10ml dH_2O) to a pH of 1.9. The temperature was checked to make sure the solution was below 38°C to prevent enzyme denaturation, then 1g of pepsin (P-7000, Sigma, St. Louis, MO) /L of solution was added. The pre-warmed HCl/pepsin solution, and the rinsed bags from the protease incubation in a Daisy II incubator bottle were combined and incubated in constant rotation at 39°C for 1h. After the incubation period, the bags were removed from the bottles by filling with cold tap water to halt enzymatic activity then rinsing with tap water until the runoff is clear.

Pancreatin/Phosphate buffer incubation

A 2 L 0.5M KH_2PO_4 solution was prepared by adding 68g KH_2PO_4/L of dH_2O equaling 136g for the 2L solution. A thymol (5ml/L) solution was added by mixing 1g thymol in 20 ml of boiling water, once dissolved and cooled to room temperature it was filtered through Whatman #1 filter paper. The phosphate + thymol solution was warmed to 37°C and adjusted to a pH of 7.8 with strong NaOH solution. The temperature was checked to make sure it was under 37°C before adding 3g/L of pancreatin (Sigma P-7545) to the solution. A stir rod was used to make sure the solution was mixed well. The

bags were reintroduced into the solution containing 50 ppm thymol and 3g/L of pancreatin (Sigma P-7545) in the Daisy II incubator bottle and incubated at 39°C in constant rotation for 24h. The bags were removed from the Daisy and the bottles were drained and rinsed by filling the bottles with cold tap water to halt enzymatic activity then rinsed until runoff was clear. Bags were placed in a forced hot air oven at 55°C for 48h (VWR Scientific 1380 forced air oven, Bridgeport, NJ). The dry weights of the samples and bags were recorded, the bags were opened and contents pooled by sample for CP and AA analysis by using cation-exchange chromatography (cIEC-HPLC) coupled with prost-column ninhydrin derivatixation and quantitation (Experimental Station Chemical Laboratories, University of Missouri-Columbia, Columbia, MO).

Chemical Analysis

The residue obtain through the McNiven procedure was ground to pass a 40-μm screen for AA analysis via post column ninhydrin derivatization and quantitation (Experimental Station Chemical Laboratories, University of Missouri Columbia, Columbia, MO).

Calculations and Statistical Analysis

Amino acid digestibility was calculated for the intact feed after being subject to the McNiven procedure for each individual AA as:

Digestibility, % = [(AA in – AA out) / AA in] x100

Linear data were analyzed by fed type as a completely randomized design according to the following model

$$Y_i = \mu + P_i + E_i$$

where Y_i = the dependent variable, μ = overall mean, P_i = the fixed effect of the ith

procedure, and E_i = the random residual ~ N (0, σ^2). The REG procedure of SAS (SAS Institue, 2001) was used to examine the relationship between the AA digestibility feed protein, the digestibility of individual AA and total AA in feed protein, and the digestibility of AA and the profile (% of total) of AA in feed protein.

RESULTS AND DISSCUSSION

Regression Analysis Protein and Corn Silage Feed Samples

Results of the regression analysis of all protein feeds to examine the relationship between digestibility values obtained with the current experiment and either the mTSP or the rooster bioassay conducted by Boucher et al. (2009a,c,d) are presented in Table 13. There was a higher correlation for TAA in the relationship between the current experiment and the mTSP with a $r^2 = 0.59$ than the relationship between the current experiment and the rooster assay with a $r^2 = 0.50$ when analyzing the protein feeds. This outcome was not unexpected as the current procedure contains the last two steps of the mTSP within its procedure. The r^2 values for individual amino acids in protein feeds ranged from, 0.60 for Ala to 0.84 for Cys for the relationship between the current experiment and the mTSP and 0.36 for Tyr to 0.80 for Lys for the relationship between the current experiment and the rooster assay. These results fall slightly lower than previously published results where in vivo digestibility techniques were compared to in vitro digestibility results (Boucher et al. 2009b)

Results of the regression analysis to examine the relationship of corn silage digestibilities between values obtained with the current experiment and either the mTSP

or the MBT at two different rumen incubation times from work done by Ferdin (2009) are presented in Table 14. The correlation of TAA in corn silage was higher at 0.41 and 0.20 for the MBT at 16 and 24h rumen incubations respectively, than 0.01 and 0.16 for the mTSP at 16 and 24h rumen incubations respectively. This outcome differed from the protein feeds as the in vivo MBT at the 16h rumen incubation instead of the in vitro procedure had the highest overall TAA correlation to the current experiment with a r^2 of 0.41. Correlation coeffients varied between individual corn silage AA samples in all other categories more than the protein feeds with increasing variability when observing the in vitro relationship or when increasing rumen incubation time. Previous studies have looked at corn silage digestibility relationships between in vivo and in vitro procedures for the purpose of validation of an in vitro method (Ferdin 2009). Using the MBT as the in vivo standard Ferdin (2009) looked at the relationships between the MBT and either the mTSP or the Sapienza Analytica, LLC in vitro procedure. The range of r^2 values were 0.36 for Met to 0.92 for Ser for the comparison with the mTSP and 0.32 for Met to 0.84 for Lys and Glu for the comparison with the Sapienza Analytica LLC in vitro procedure, these values fell close to the comparison of the MBT and the current procedure.

The individual corn silage AA r^2 values ranged from 0.05 to 0.98 displaying that some of the individual amino acids had correlations which were more favorable; Arg, His, Leu, Lys, Glu, Pro, Phe, and Ser all had r^2 values above 0.80 all other measured AA falling below that level. The lowest correlations for the MBT at 16h were for Met, Val, and Cys, which is troubling because Met is one of the two most limiting AA and being able to accurately predict its digestibility is important (Schwab et al., 1992), and that two

of the three lowest correlations were between AA containing sulfur, which suggest that this protease procedure could have trouble accurately predicting sulfur AA digestibility. One of the critical differences between sulfur containing AA and other AA that could cause this discrepancy is that sulfur makes the AA non-polar and very hydrophobic. Methionine being the most hydrophobic AA, causes most Met residues to exist in the interior hydrophobic core of globular proteins, and Cys plays a critical role in protein structure with its ability to form inter- and intrachain disulfide bonds and is also rarely found on the outside of proteins. These characteristic could be the source of the variation between the digestibilities using the current experiment. This would cause an error in the ability to quantify the TAA digestibility of a feed and this has caused a somewhat artificially low correlation when looking at TAA in comparison to other techniques such as the MBT and the mTSP in the current experiment (Brosana et al. 2006).

Differences between the McNiven protease procedure and the rooster assay for Lys, Met, EAA, and TAA digestibility estimates are presented in Figures 1C, 1D, 2C, and 2D. Differences between the McNiven protease procedure and the mTSP with a rumen incubation for Lys, Met, EAA and TAA are present in Figures 3C, 3D, 4C, and 4D respectively. The r^2 value for Lys digestibility in the relationship between the McNiven procedure and the rooster assay and the McNiven procedure and the mTSP is 0.80 and 0.78 respectively. Even with this strong correlation, Lys was over-estimated for all samples when compared to the in vivo rooster assay as seen in Figure 1C and for most samples when compared to the mTSP as seen in Figure 3C. The trend of Lys being over-estimated when in vitro digestibilities are compared to in vivo digestibilities was also seen in the work done by Boucher et al. (2009a), where the mTSP was compared to the

rooster assay for purposes of validating the mTSP. This difference between in vivo and in vitro Lys digestibility is likely due to the Maillard reaction taking place, this can cause Lys to be present in forms that are not readily available for absorption in the small intestine (Mauron, 1990). Boucher et al. (2009a) also stated that for highly digestible samples (>90%), digestibility estimates of Met, TAA, and EAA obtained with the mTSP were very similar to or slightly higher than the same in vivo results. In the current experiment this held true in both cases, the McNiven procedure compared to mTSP and the rooster assay, these results had an even greater tendency for the values to be further over-estimated than in the work of Boucher et al. (2009a). In the current experiment under-estimation of the digestibility was only seen when the actual digestibility values were well below 90%, such as in the heated SBM and DDGS samples. As seen in Figure 1D and 3D the heated SBM and heated DDGS sample for Met digestibility were under-estimated compared to the other samples.

Differences between the McNiven procedure compared to the MBT at the 16h rumen incubation time for Lys, Met, EAA, and TAA are presented in figures 7C, 7D, 8C, and 8D respectively. As with the protein feeds the corn silages were also overestimated in vitro compared to the in vivo standards across most samples. Lys once again was completely overestimated on all samples as seen in figure 7C, and this could also be caused by the Maillard reaction (Mauron, 1990) as was hypothesized for the protein feed samples in the current experiment. Ferdin (2009) saw the same over estimation in Lys, EAA, and TAA digestibility when comparing total tract digestibilities of the in vitro mTSP and total tract digestibility using the in vivo MBT across most individual samples.

When observing figures 1C, 1D, 2C, and 2D a trend for FM samples to be more

overestimated than the soy products or the DDGS was seen. Removing these values was done to see if an increase in r^2 values would result. After removing the FM digestibility values, an increase in all r^2 values was seen across all AA except for tryptophan, these results are presented in Table 15. Correlation coefficients of TAA and EAA increased from 0.50 to 0.67 and 0.66 to 0.82 respectively and values for Lys and Met increased from 0.80 to 0.85 and 0.52 to 0.74 respectively.

One explanation for this could be the higher concentration of Lys in the AA profile of fish meal compared to the heated and unheated soy products and DDGS. The soy products and DDGS had an average of 6.77 and 3.38 % Lys out of TAA respectively in unheated samples compared to an average of 9.00 % Lys out of TAA in unheated FM samples. Lysine being very digestible in the current experiment, when using the McNiven protease incubation, had an average digestibility of 97% across all FM samples. This could have caused an increase to the overall digestibility of each fish meal sample which inflated the digestibilities which caused the depression in r^2 values.

Essential AA and TAA in either comparison compounded with the concern of over-estimating Lys, even with a heated sample, brought into question the ability of the McNiven procedure to accurately rank feeds based on digestibility. As of now the current data available regarding in vitro procedures to estimate individual AA in protein feed samples is very limited. The McNiven procedure in its current form has variability and some specific short comings like accurate sulfur AA estimation, but has the potential to be a viable way to estimate the digestibility of individual AA in protein feeds and corn silages. More research in this area can always be done since this procedure does have room to improve to become accurate, and be validated by benchmark in vivo procedures.

CONCLUSIONS

The McNiven procedure would allow for the digestibility of individual AA to be quantified for a variety of dairy cattle feedstuffs without the need for rumen or duodenal cannula. Validation of the McNiven protease procedure was attempted by using protein feeds previously used in research by Boucher et al. (2009) and corn silage samples used by Ferdin (2009). This was done to make a direct correlation between the current experiment and the results reported by Boucher et al. (2009) and Ferdin (2009) for the in vivo rooster assay, the in vitro mTSP and the in vivo MBT results. The correlations between the current experiment and that of Boucher et al. (2009) and Ferdin (2009), were promising, yet there was variability depending on feed sample and individual AA. There was greater variability in the protein feed samples when looking at animal protein such as FM then plant protein from soy products and DDGS. This was confirmed when removing FM from the regression analysis improved results. Variability also existed in the corn silage samples between in vitro and in vivo procedures and between both rumen incubation times. The McNiven protease procedure also exhibited difficulty when quantifying sulfur AA in corn silage samples in contrast to the other quantified AA.

The McNiven protease procedure has the ability to quantify individual AA, it does however have short comings, it is overall more accurate when experimenting with plant protein feeds over animal protein feeds such as fish meal where a trend to overestimate the digestibility of those samples was observed. When using this method to quantify AA in corn silage the procedure has difficulty accurately estimating sulfur amino acids and these are two areas where future research should focus. As this area of research moves farther away from in vivo procedures and more towards completely in

vitro procedures due to the difficulty of acquiring and maintaining cannulated cows for the traditional MBT, results are becoming less accurate. Boucher (2009b) used a rooster bioassay as the in vivo model which in vitro procedures were compared to and then the mTSP removed any in vivo small intestinal digestibility step from the procedure. Now with the addition of the McNiven protease incubation the rumen incubation step is gone, and as these in vivo aspects of the procedure were removed the correlations and accuracy of results were reduced. Yet the ability of the current experiment to quantify individual AA is still adequate to be a beneficial tool for dairy producers and dairy nutritionist to use, and the accuracy and repeatability will continue to improve with continued research.

References

Ahrar, M., and D. L. Schingoethe. 1979. Heat-treated soybean meal as a protein supplement for lactating cows. J. Dairy. Sci. 62:932

Aldrich, C. G., N.R. Mechen., C.M. Parsons., H. S. Hussein., S. Ingram., and J. R. Clodfelter. 1997. Assessment of postruminal amino acid digestibility of roasted and extruded whole soybeans with the precision- fed rooster assay. J. Anim. Sci. 75: 3046-3051.

Benchaar. C., R. Moncoulon, C. Bayourthe and M. Vernay. 1994. Amino acid absorption in dairy cows effects of a supply of raw or extruded white lupin seeds on protein digestion. J. Anim. Sci. 72:492-501.

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

Boucher S. E., S. Calsamiglia., C. M. Parsons., H. H. Stein., M. D. Stern., P. S. Erickson., P. L. Utterback, C. G. and C. G. Schwab. Intestinal digestibility of amino acids in rumen undegradable protein estimated using a precision-fed cecectomized rooster bioassay: I. Soybean meal and SoyPlus. 84:1610-1620. (a)

Boucher S. E., S. Calsamiglia., C. M. Parsons., H. H. Stein., M. D. Stern., P. S. Erickson., P. L. Utterback, C. G. and C. G. Schwab. Intestinal digestibility of amino acid in rumen-undegraded protein estimated using a precision-fed cecectomized rooster bioassay: Distillers dried grains with solubles and fish meal. 92:6056-6067. (b)

Boucher, S. E., S. Calsamiglia., C. M. Parsons., M. D. Stern., M. Ruiz – Moreno., M. Vazquez-Anon, and C. G. Schwab. 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. (c)

Broderick, G. A. 1978. In vitro procedures for estimating rates of ruminal degradation and proportions of protein escaping the rumen undegraded. J. Nutr. 108: 181-190.

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

CAST. 2002. Animal diet modification to decrease the potential for nitrogen and phosphorous pollution. 21:1-16.

Chase, L., and M. Van Amburgh. 2002. Ammonia emissions: the next regulatory battleground. Northeast Dairy Business. Aug.:23.

Cherian, G., W. C. Sauer, and P. A. Thacker. 1998. Effect of predigestion factors on the apparent digestibility of protein for swine determined by the mobile bag technique. J. Anim. Sci. 66:1963-1988.

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.

Erasmus, L. J. 1993. Ruminal degradation of protein of various feedstuffs and its affect on post ruminal amino acid flow in high producing dairy cows. Ph. D. Diss. Univ. Pretoria, South Africa.

Faldet, A. M., L.D. Satter., and G. A. Broderick. 1991. Determining optimal heat treatment of soybeans by measuring available lysine chemically and biologically with rats to maximize protein utilization by ruminants. J. Nutr. 0022-3166/92.

Faldet, M. A., L, D. Satter, and G. A. Broderick, 1992. Determining optimal heat treatment of soybeans by measuring gabailable lysine chemically and biologically with rats to maximize protein utilization by ruminants. J. Nutr. 122:151-160.

Ferdin, Shane. 2009. *Evaluation of in vitro methods to determine the digestibility of amino acids in rumen undegraded corn silage*. Master's thesis, University of New Hampshire.

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. 84:2163-2167.

Graham, H., Åman, P. Newman, R.K. and Newman, C. W., 1985. Use of nylon-bag technique for pig feed digestibility studies. Br. J. Nutr., 54:719-726.

Griffin, C. D. Jr., L. D. Bunting., L. S. Sticker., B. Vora. 1993. Assessment of protein quality in heat-treated soybean products using the growth responses of lambs and calves and a nylon bag-rooster assay. 71:1924-1931.

Haugen, H., L. S. K. Ivan., J. C. Macdonald., and T. J. Klopfenstein. 2006. Determination of undegradable intake protein digestibility of forages using the mobile nylon bag technique. J. Anim. Sci. 84:886-893.

Howie, S. A., S. Calsamiglia, and M. D. Stern. 1996. Variation in ruminal degradation and intestinal digestion of animal byproduct proteins. Anim. Feed Sci. Technol. 63:-1-7

Kirkpatrick, B.K., and J.J. Kennelly. 1984. Prediction of digestibility in cattle using a modified nylon bag technique. Can. J. Anim. Sci. 61:1211

Kopency

Krishnamoorthy, U., C. J. Sniffen, M. D. Stern, and P. J. Van Soest. 1983. Evaluation of a mathematical model of rumen digestion and an in vitro simulation of rumen proteolysis to estimate the rumen-undegraded nitrogen content of feedstuffs. Br. J. Nutr. 50:555-568.

Kung, L., Jr., and J. T. Huber. 1983. performance of high producing cows in early lactation fed protein of varying amounts, sources and degradability. J. Dairy. Sci. 66:227

Lapierre, H., D. Pacheco., R. Berthiaume., D.R. Ouellet, C.G. Schwab., P. Dubreuil., G. Holtrop, and G.E. Lobley. 2005. What is the true supply of amino acids for a dairy cow? J. Anim. Sci. 89(E. Suppl.): E1-E14.

Mangan, J. L.1972. Quantitative studies on nitrogen metabolism in the bovine rumen. The rate of proteolysis of casein and ovalbumin and the release and metabolism of free amino acids. Br J. Nutr. 27:261-283.

Mahadevan, S., J. D. Erfle, and F. D. Sauer. 1980. Degradation of soluble and insoluble proteins by Bacteroides amylophilus protease and by rumen microorganisms. J. Anim. Sci. 48:947-953

Mahadevan, S., F. D. Sauer, and J. D. Erfle, 1987. Preparation of protease from mixed rumen microorganisms and its use for the invitro determination of the degradability of true protein in feedstuffs. Can. J. Anim. Sci. 67:55-64.

Martinez, C. M., Y.-H. Chung, V. A. Ishler, K.W. Bailey, and G. A. Varga. 2009. Effects of rumen-protected choline and dry propylene glycol on feed intake and blood parameters from Holstein dairy cows in early lactation. J Dairy Sci. 92: 2729-2736.

Mass, R.A., G.ap. Lardy, R.J. Grant, and T.J. Klopfenstein. 1999. In situ neutral detergent insoluble nitrogen as a method for measuring forage protein degradability. J. Anim. Sci. 77:1565-1571.

Mauron J. 1990. Influence of processing on protein quality. J Nutr Sci Vitaminol 36:S57-S69.

McNiven, M. A., E. Prestlokken., L.T. Mydland., and A.W. Mitchell. 2001. Laboratory procedure to determine protein digestibility of heat-treated feedstuffs for dairy cattle. J. Anim. Feed Sci. Tech. 96:1-13.

Mesgaran, M. D., and M. D. Stern. 2004. Ruminal and post-ruminal protein disappearance of various feeds originating from Iranian plant varieties determined by the in situ mobile bag technique and alternative methods. Anim. Feed Sci. Technol. 118:31-46.

Mielke, C. D. and D. J. Schingoethe. 1981. Heat treated soybeans for lactating cows. J. Dairy. Sci. 64:1579.

Moughan, P.J., and S.M. Rutherfurd. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press. Washington, DC.

Parsons. C. M. 1985. Short note: Influence of caecectomy on digestibility of amino acids by roosters fed distillers' dried grains with solubles, *J. Agric. Sci.* **104** (1985), pp. 469–472.

Poos-Floyd, M., T. Klopfenstein, and R. A. Britton. 1985. Evaluation of laboratory techniques for predicting ruminal protein degradation . J. Dairy Sci. 68:829-839

Rooke, J. A. 1985. The nutritive value of feed proteins and feed protein residues resistant to degradation by rumen microorganisms. J. Sci. Food Agric. 36:629-637.

Sahlu, T., D. J. Schingoethe, and A. K. Clark. 1984. Lactational and chemical evaluation of soybean meal heat-treated by two methods. J. Dairy. Sci. 62:1725

Sauer, W.C., H. Jorgensen, and R. Berzins. 1983. A modified nylon bag technique for determining apparent digestibilities of protein in feedstuffs of pigs. Can. J. Anim. Sci. 63:233

Schwab, C. G., L. D. Satter, and A. B. Clay. 1976. Responses of lactating dairy cows to abomasal infusion of amino acids. J. Dairy Sci. 59:1254-1270.

Sniffen, C. J., J. D. O'Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992 A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J Anim. Sci. 70:3562-3577.

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

Trinácty, J., P. Homolka, L. Zeman, M. Richter. 2003. Whole tract and post ruminal digestibility determined by in situ ruminal, intestinal mobile nylon bag and whole tract nylon capsule methods. Anim. Feed Sci. Technol. 106:59-67.

Titgemeyer, E, C., N. R. Merchen, and L. L. Berger. 1989. Evaluation of soybean meal, corn gluten meal, blood meal and fish meal as sources of nitrogen and amino acids disappearing from the small intestine of steers. J. Anim. Sci. 67:262.

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. 73:690-6993

Vanhatalo, A., I. Aronen, and T. Varvikko. 1995. Intestinal nitrogen digestibility of heat-moisture treated rapeseed meals as assessed by the mobile-bag method in cows. Anim. Feed. Sci. Technol. 55:139-152

Varvikko, T., and A. Vanhatalo, 1990. The effect of differing types of cloth and of contamination by non-feed nitrogen on intestinal digestion estimates using porus synthetic fiber bags in a cow. Br. J. Nutr. 63:221-229.

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

Wilkerson, VA., T. J. Klopfenstein, and W. W. Stroup. 1995. A collaborative study of in situ forage protein degradation. J. Anim. Sci. 73:583-588.

Table 1. Chemical composition of intact SoyPlus® and soybean meal

	Sample[1] Intact feed					
Item[2], (%of DM)	HSP	HSBM	SP2	SP3	SBM2	SBM3
CP	50.70	54.10	48.60	49.30	52.90	54.70
ADF	22.80	17.50	12.40	11.30	7.20	6.50
NDF	41.40	46.90	22.00	24.60	11.90	9.10
Lignin	12.50	9.40	1.40	2.30	1.30	1.10
ADICP[2]	11.70	10.80	1.30	1.80	1.20	2.40
NDICP[3]	27.50	35.30	9.70	12.90	6.40	6.10
Fat	6.80	1.90	7.70	6.70	1.80	1.90
NFC[4]	22.00	25.20	25.00	25.90	32.30	33.10
Starch	1.00	0.80	1.20	0.90	1.00	1.10
Ash	6.61	7.32	6.37	6.35	7.50	7.29
Ca	0.29	0.37	0.31	0.31	0.33	0.29
P	0.67	0.78	0.69	0.68	0.72	0.74

[1]H indicates the sample was subjected to additional heat treatment; SP = SoyPlus®; SBM = soybean meal.
[2]ADICP = acid detergent insoluble CP.
[3]NDICP = neutral detergent insoluble CP.
[4]NFC = non-fiber carbohydrates.

Table 2. Amino acid profile (% of total AA) of intact SoyPlus® and soybean meal

	Sample[1,2] Intact Feed					
AA[3]	HSP	HSBM	SP2	SP3	SBM2	SBM3
Arg	6.00	6.70	7.70	7.50	7.70	7.80
His	2.80	2.90	2.90	2.90	2.90	2.90
Ile	5.40	5.40	5.20	5.10	5.10	5.10
Leu	9.20	9.00	8.50	8.50	8.40	8.40
Lys	3.70	4.00	6.70	6.20	7.00	7.20
Met	1.60	1.70	1.50	1.60	1.50	1.70
Phe	5.90	5.70	5.50	5.50	5.50	5.40
Thr	4.50	4.50	4.20	4.30	4.10	4.20
Trp	1.00	1.00	1.50	1.40	1.40	1.40
Val	5.70	5.60	5.40	5.50	5.50	5.50
BCAA	20.30	19.90	19.00	18.90	19.00	19.00
EAA	45.90	46.40	49.10	48.30	49.10	49.50
Ala	5.10	5.00	4.70	4.70	4.70	4.90
Asp	12.90	12.70	12.10	12.20	12.10	12.00
Cys	1.40	1.70	1.50	1.60	1.60	1.50
Glu	20.40	20.00	19.00	19.30	19.30	18.80
Pro	5.50	5.40	5.10	5.00	5.00	5.10
Ser	4.90	5.00	4.80	4.60	4.60	4.60
Tyr	4.00	3.90	3.80	3.60	3.60	3.70
NEAA	54.10	53.60	50.90	51.70	51.00	50.50

[1]H indicates the sample was subjected to additional heat treatment; 150°C for 90 min

[2]SP = SoyPlus® ; SBM = soybean meal

[3]BCAA = branch chain amino, EAA = essential amino acids, NEAA = nonessential amino acids

Table 3. Digestibility (%) of amino acids in samples of intact SoyPlus® and soybean meal determined using the McNiven protease incubation in conjunction with steps of the modified three step procedure

	Sample[1]						
AA[2]	HSP	HSBM	SP2	SP3	SBM2	SBM3	SE
Arg	81.9[b]	66.9[c]	98.9[a]	99.5[a]	98.5[a]	98.6[a]	0.667
His	77.8[b]	62.4[c]	97.9[a]	98.7[a]	97.3[a]	97.6[a]	0.798
Ile	73.0[b]	59.8[c]	98.3[a]	99.2[a]	97.5[a]	97.6[a]	0.890
Leu	73.4[b]	60.3[c]	98.1[a]	99.1[a]	97.0[a]	97.2[a]	0.807
Lys	77.2[b]	61.2[c]	98.3[a]	99.0[a]	97.8[a]	97.8[a]	0.957
Met	75.1[b]	51.5[c]	98.3[a]	99.4[a]	96.9[a]	97.4[a]	0.637
Phe	72.2[b]	61.7[c]	98.2[a]	99.1[a]	97.2[a]	97.3[a]	0.934
Thr	74.6[b]	57.3[c]	98.2[a]	99.1[a]	97.3[a]	97.6[a]	0.736
Trp	67.9[b]	52.6[c]	98.9[a]	99.0[a]	98.8[a]	98.8[a]	1.189
Val	73.1[ab]	58.7[b]	97.9[a]	98.9[a]	96.8[a]	97.0[a]	4.158
BCAA	75.1[b]	60.5[c]	98.3[a]	99.1[a]	97.5[a]	97.6[a]	0.910
EAA	73.3[b]	58.2[c]	98.1[a]	99.0[a]	97.3[a]	97.5[a]	0.800
Ala	74.4[b]	64.5[c]	98.6[a]	99.3[a]	98.0[a]	98.1[a]	0.713
Asp	78.4[b]	48.7[c]	98.0[a]	99.1[a]	96.4[a]	96.7[a]	0.915
Cys	75.3[b]	68.6[c]	98.8[a]	99.4[a]	98.2[a]	98.4[a]	0.676
Glu	72.2[b]	62.0[c]	97.7[a]	98.6[a]	97.0[a]	97.3[a]	0.767
Pro	76.3[b]	62.1[c]	97.9[a]	98.6[a]	97.4[a]	97.7[a]	0.682
Ser	91.9[b]	61.0[c]	98.4[a]	98.9[a]	98.0[ab]	98.1[a]	1.025
Tyr	76.0[b]	64.3[c]	98.4[a]	99.1[a]	97.8[a]	98.0[a]	0.703
NEAA	87.8[b]	81.3[c]	99.4[a]	99.7[a]	99.1[a]	99.2[a]	0.491
TAA	73.2[b]	59.7[c]	98.1[a]	99.1[a]	97.1[a]	97.2[a]	1.245

[a-c] Least square means within the same row without a common superscript differ ($P < 0.05$).

[1]HSP = heated SoyPlus®; HSM = heated soybean meal; SP = Soy Plus®; SBM = soybean meal.

[2]BCAA = branch chain amino acids; EAA = essential amino acids; NEAA = nonessential amino acids; TAA = total amino acid

Table 4. Chemical composition of samples of dried distillers' grains with solubles (intact feed)

	Sample[1,2]				
Item, (% of DM)	HDDGS	DDGS2	DDGS3	DDGS4	DDGS5
CP	32.00	29.30	32.00	30.50	29.00
ADF	24.10	16.50	16.10	16.10	16.70
NDF	38.60	37.90	30.90	31.00	30.80
Lignin	9.40	3.80	4.60	3.40	4.10
ADICP[3]	11.80	6.50	4.80	5.80	4.80
NDICP[4]	16.80	9.80	8.30	9.70	9.70
Fat	11.80	12.40	13.10	11.40	10.70
NFC	29.00	25.30	28.10	31.20	33.30
Starch	3.50	4.00	4.40	7.80	5.60
Ash	5.36	4.99	4.22	5.69	5.89
Ca	0.04	0.07	0.04	0.17	0.18
P	0.78	0.89	0.77	0.80	0.81

[1]H indicates the sample was subjected to additional heat treatment; 140°C for 60 min.
[2]DDGS = dried distillers' grains with solubles
[3]ADICP = acid detergent insoluble CP.
[4]NDICP = neutral detergent insoluble CP.

Table 5. Amino acid profile (%) of dried distillers' grain with solubles in intact feed samples.

	Sample[1,2] Intact Feed				
AA[3]	HDDGS	DDGS2	DDGS3	DDGS4	DDGS5
Arg	4.90	4.10	5.30	4.80	4.70
His	3.10	2.80	3.10	3.00	3.00
Ile	4.90	4.30	4.70	4.50	4.50
Leu	14.20	13.50	13.40	13.70	13.80
Lys	2.10	2.90	4.20	3.30	3.10
Met	2.20	2.30	2.30	2.20	2.20
Phe	5.90	5.50	5.70	5.70	5.70
Thr	4.20	4.10	4.40	4.20	4.20
Trp	0.00	1.00	0.80	0.60	0.60
Val	6.30	5.70	5.80	6.00	5.70
BCAA	25.50	23.50	23.90	23.80	24.10
EAA	47.70	45.70	49.50	47.50	47.50
Ala	8.30	8.00	7.90	8.00	8.10
Asp	7.40	7.10	7.30	7.30	7.10
Cys	2.20	2.10	2.10	2.20	2.50
Glu	17.10	19.30	15.70	17.20	17.00
Pro	8.60	8.70	8.20	8.50	8.60
Ser	4.60	5.00	5.10	5.00	5.00
Tyr	4.10	4.10	4.40	4.30	4.30
TNEAA	52.30	54.40	50.50	52.50	52.50

[1]H indicates the sample was subjected to additional heat treatment

[2]DDGS = dried distillers' grains with solubles.

[3]BCAA = branch chain amino acids, EAA = essential amino acids, NEAA = nonessential amino acids

Table 6. Digestibility (%) of amino acids in samples of dried distillers grains with solubles for intact feeds determined using the McNiven protease incubation in conjunction with steps of the modified three step procedure.

	Sample[1]					
AA[2]	HDDGS	DDGS2	DDGS3	DDGS4	DDGS5	SE
Arg	42.1[b]	93.3[a]	95.6[a]	95.1[a]	94.3[a]	0.667
His	48.6[b]	92.6[a]	94.2[a]	94.1[a]	93.2[a]	0.798
Ile	48.3[b]	92.8[a]	95.2[a]	95.1[a]	94.1[a]	0.890
Leu	51.9[b]	94.6[a]	95.6[a]	96.1[a]	95.4[a]	0.808
Lys	41.3[b]	90.7[a]	94.1[a]	92.7[a]	92.2[a]	0.956
Met	43.7[b]	93.5[a]	95.9[a]	95.6[a]	94.9[a]	0.637
Phe	47.2[b]	93.6[a]	95.1[a]	95.3[a]	94.5[a]	0.934
Thr	42.2[b]	90.5[a]	93.1[a]	92.8[a]	92.0[a]	0.735
Trp	18.9[b]	88.8[a]	92.8[a]	91.8[a]	91.6[a]	1.188
Val	43.2[b]	77.5[a]	93.9[a]	93.7[a]	92.6[a]	4.158
BCAA	49.1[b]	90.2[a]	95.1[a]	95.4[a]	94.5[a]	0.910
EAA	46.8[b]	91.2[a]	94.8[a]	94.8[a]	94.0[a]	0.800
Ala	41.5[b]	92.2[a]	95.0[a]	94.7[a]	93.5[a]	0.713
Asp	50.5[b]	94.1[a]	95.4[a]	95.5[a]	94.9[a]	0.915
Cys	46.2[b]	91.5	93.8	93.9	93.6	0.675
Glu	46.5[b]	95.3[a]	95.4[a]	96.1[a]	95.2[a]	0.767
Pro	50.9[b]	93.5[a]	94.3[a]	94.4[a]	93.8[a]	0.682
Ser	46.8[b]	94.0[a]	95.4[a]	95.6[a]	95.0[a]	1.025
Tyr	49.4[b]	94.9[a]	96.0[a]	96.2[a]	95.6[a]	0.703
NEAA	47.4[b]	94.1[a]	95.1[a]	95.4[a]	94.6[a]	0.491
TAA	73.6[b]	97.5[a]	98.3[a]	97.8[a]	98.1[a]	1.244

[a,b] Least square means within the same row without a common superscript differ ($P < 0.05$).

[1]HSP = heated SoyPlus®; HSM = heated soybean meal; SP = Soy Plus®; SBM = soybean meal.

[2]BCAA = branch chain amino acids; EAA = essential amino acids; NEAA = nonessential amino acids; TAA = total amino acid

Table 7. Chemical composition of intact Anchovy, Catfish, Menhaden, and Pollock fish meal samples.

	Sample[1] Intact Feed				
Item, (% of DM)	ANVY	CFSH	MNHN1	MNHN2	PLCK
CP	74.10	69.50	73.60	73.70	75.60
ADICP[2]	0.80	5.50	0.60	0.60	0.80
NDICP[3]	12.40	32.80	19.40	20.40	19.20
Fat	11.90	12.30	12.70	12.40	10.40
Ash	18.71	22.66	19.57	20.21	19.00
Ca	4.33	8.32	5.18	4.95	6.48
P	2.93	3.98	3.18	2.99	3.10

[1]ANVY = Anchovy fishmeal; CFSH = Catfish meal; MNHN = Menhaden fishmeal; PLCK = Pollock fishmeal.
[2]ADICP = acid detergen insoluble CP.
[3]NDICP = neutraldetergen insoluble CP.

Table 8. Amino acid profile (% of total AA) of intact Anchovy, Catfish, Menhaden, and Pollock fishmeal samples

	Sample[1] Intact Field				
Amino acid[2]	ANVY	CFSH	MNHN1	MNHN2	PLCK
Arg	6.70	8.30	7.20	7.10	7.20
His	3.50	2.50	3.30	2.80	2.60
Ile	5.20	4.50	4.80	4.90	5.00
Leu	8.90	7.70	8.40	8.50	8.80
Lys	9.40	8.00	9.30	9.10	9.10
Met	3.30	2.70	3.20	3.30	3.50
Phe	4.80	4.30	4.60	4.70	4.60
Thr	4.90	4.50	4.80	4.80	5.00
Trp	1.30	0.60	1.10	1.20	1.20
Val	6.00	5.70	5.70	5.70	5.90
BCAA	20.00	17.90	18.90	19.10	19.70
EAA	53.80	48.70	52.30	52.00	52.80
Ala	7.40	9.20	7.70	7.50	6.60
Asp	10.50	10.20	10.50	10.50	10.70
Cys	1.20	1.00	0.90	0.90	1.30
Glu	14.50	15.40	15.00	15.00	15.00
Pro	4.50	8.30	5.60	5.50	4.50
Ser	4.30	4.20	4.40	4.40	5.10
Tyr	3.80	3.00	3.50	3.60	4.20
NEAA	46.20	51.30	47.70	48.00	47.20

[1]ANVY = Anchovy fishmeal; CFSH = Catfish meal; MNHN = Menhaden fishmeal; PLCK =Pollock fishmeal

[2]BCAA = branch chain amino acids, EAA = essential amino acids, NEAA = nonessential amino acids.

Table 9. Digestibility (%) of amino acids in samples of Anchovy, Catfish, Menhaden, and Pollock fishmeals of intact feeds determined by using the McNiven protease incubation in conjunction with steps of the modified three step procedure

	Sample[1]				
AA[2]	CFSH	PLCK	ANVY	MNHN1	MNHN2
Arg	88.3	94.6	94.7	95.9	91.4
His	95.3	95.7	97.7	98.1	96.0
Ile	94.0	97.3	98.0	98.6	97.5
Leu	94.3	97.3	98.1	98.5	97.3
Lys	93.9	97.4	98.2	98.4	96.8
Met	93.2	93.1	95.3	97.0	94.2
Phe	93.7	95.9	96.6	97.7	95.3
Thr	92.9	96.3	97.2	97.6	95.5
Trp	98.7	98.0	99.2	99.1	98.9
Val	93.3	96.9	97.7	97.9	96.3
BCAA	93.9	97.2	97.9	98.4	97.0
EAA	93.3	96.3	97.2	97.8	95.7
Ala	92.6	95.9	96.7	97.5	95.1
Asp	87.9	95.6	94.8	95.3	88.5
Cys	96.0	95.2	96.0	98.2	97.5
Glu	91.6	96.0	96.8	97.4	94.7
Pro	79.7	94.7	92.5	92.7	82.0
Ser	91.2	92.6	95.6	96.7	94.5
Tyr	94.7	95.3	96.7	98.1	97.0
NEAA	90.3	95.3	95.6	96.6	93.0
TAA	96.1	98.8	98.3	98.7	98.5

[1]HSP = heated SoyPlus®; HSM = heated soybean meal; SP = Soy Plus®; SBM = soybean meal.

[2]BCAA = branch chain amino acids; EAA = essential amino acids; NEAA = nonessential amino acids; TAA = total amino acid

Table 10. Chemical composition of 5 intact corn silage samples

	Sample[1]				
	CS 1	CS 2	CS 3	CS 4	CS 5
DM	41.1	32.6	32,5	40.3	40.7
Item, (% of DM)					
CP	7.9	4.7	4.5	4.8	6.1
Total AA[2]	4.5	3.4	3.1	3.9	4.7
ADF	24.3	27.6	28.8	25.4	21.7
NDF	36.6	45.8	55.6	52.9	37.1
Lignin	4	3	2.8	3	2.6
ADICP[3]	1	0.4	0.7	0.6	0.5
NDICP[4]	2.3	1	1.1	1.4	0.7
Fat	3.2	3.2	3.2	3	3.1
NFC[5]	46.2	41.9	31.5	35.2	50.9
Starch	32.3	31.5	18.1	36.8	43
Ash	6.4	4.7	5.5	4.3	3.2
Ca	1	0.4	0.5	0.4	0.3
P	0.2	0.2	0.2	0.2	0.2

[1]CS = Corn silage

[2]AA = amino acids.

[3]ADICP = acid detergent insoluble CP.

[4]NDICP = neutral detergent insoluble CP.

[5]NFC = non-fiber carbohydrates; NFC = 100 – [CP + (NDF-NDICP) + fat + ash].

Table 11. Amino acid profile (% of total AA) of 5 corn silage samples

	Sample[1]				
AA[2]	CS 1	CS 2	CS 3	CS 4	CS 5
Arg	3.1	3.1	2.8	3.3	2.9
His	2.1	2.5	2.4	3.1	2.7
Ile	4.8	4.7	4.8	4.4	4.5
Leu	9.8	11.3	11.1	11.1	12.3
Lys	4.3	4.7	5.5	5.3	4.3
Met	1.9	1.9	2.1	2.5	2.2
Phe	4.1	5	5.2	5	5.2
Thr	5.2	4.7	4.5	4.4	4.3
Val	7.4	6.6	6.9	6.4	6.3
BCAA	21.9	22.5	22.8	21.9	23.1
EAA	42.6	44.4	45.3	45.4	44.6
Ala	13.6	10	9.7	9.1	9.2
Asp	7.1	8.4	9	8.6	8.1
Cys	1.7	1.9	2.1	2.2	2
Glu	14.5	15	13.8	14.7	16.1
Gly	6.4	5.6	5.9	5.5	5.2
Pro	7.6	8.4	7.6	8	8.5
Ser	4.1	4.1	4.2	3.9	3.8
Tyr	2.4	2.2	2.4	2.5	2.5
NEAA	57.4	55.6	54.7	54.6	55.4

[1]CS = Corn silage, AA = amino acid.

[2]BCAA = branch chain AA
EAA = essential AA
EAA = essential AA
NEAA = nonessential AA
TAA = Total AA

Table 12. Digestibility (%) of amino acids in samples of 5 Corn Silage samples of intact feeds determined by using the McNiven protease incubation in conjunction with steps of the modified three step procedure

	Sample[1]				
AA[2]	CS1	CS2	CS3	CS4	CS5
Arg	81.2	76.8	74.5	85.2	85.4
His	88.4		85.4	92.0	93.7
Ile	89.6	84.5	85.4	88.9	92.4
Leu	90.6	89.7	88.8	92.2	94.5
Lys	88.4	81.4	84.1	88.3	90.0
Met	92.4	90.1	83.0	84.5	91.3
Phe	85.7	82.6	83.0	87.6	90.0
Thr	87.3	84.5	84.3	88.9	90.0
Trp	65.4	53.6	49.0	55.5	62.0
Val	85.4	86.7	87.2	90.3	91.9
BCAA	88.6	87.7	87.6	91.0	93.4
EAA	87.2	73.3	84.1	88.9	91.2
Ala	86.1	82.8	82.3	87.0	90.5
Asp	93.9	88.4	87.2	90.6	93.5
Cys	80.1	76.8	74.5	83.3	83.1
Glu	91.4	88.4	87.2	91.6	94.7
Pro	85.9	82.8	81.5	86.2	92.0
Ser	85.7	82.1	78.7	87.8	88.8
Tyr	89.5	80.1	78.1	85.1	89.6
NEAA	89.6	85.2	83.7	88.7	92.2
TAA	99.7	91.1	93.2	97.1	96.3
CP	85.5	79.2	76.9	79.8	87.5

[1]HSP = heated SoyPlus®; HSM = heated soybean meal; SP = Soy Plus®; SBM = soybean meal.

[2]BCAA = branch chain amino acids; EAA = essential amino acids; NEAA = nonessential amino acids; TAA = total amino acid

Table 13. Correlation coefficients (R^2) of protein feeds for AA digestibility of residue samples created from the McNiven protease incubation in conjunction with steps of the modified three step procedure and residue collected from a rumen incubation in conjunction with either the cecectomized rooster assay or modified three step procedure.

	R^2		R^2	
Item[1]	Cececomized Rooster	$P>F^2$	mTSP	$P>F^2$
Arginine	0.75	<0.01	0.79	<0.01
Histidine	0.53	<0.01	0.76	<0.01
Isoleucine	0.68	<0.01	0.73	<0.01
Leucine	0.55	<0.01	0.71	<0.01
Lysine	0.80	<0.01	0.78	<0.01
Methionine	0.52	<0.01	0.67	<0.01
Phenylalanine	0.62	<0.01	0.75	<0.01
Threonine	0.72	<0.01	0.82	<0.01
Tryptophan	0.36	0.0180	0.76	<0.01
Valine	0.68	<0.01	0.74	<0.01
Branch Chain AA	0.65	<0.01	0.75	<0.01
Essential AA	0.66	<0.01	0.76	<0.01
Alanine	0.52	<0.01	0.60	<0.01
Aspartic Acid	0.56	<0.01	0.71	<0.01
Cysteine	0.45	<0.01	0.84	<0.01
Glutamic Acid	0.59	<0.01	0.71	<0.01
Proline	0.55	<0.01	0.67	<0.01
Serine	0.40	<0.01	0.66	<0.01
Tyrosine	0.68	<0.01	0.78	<0.01
Nonessential AA	0.50	<0.01	0.65	<0.01
Total AA	0.50	<0.01	0.59	<0.01

[1]AA = amino acids.

[2]Probability of significant linear relationship; n = 16.

Table 14. Correlation coefficients (R^2) of corn silage for AA digestibility of residue samples created from the McNiven protease incubation in conjunction with steps of the modified three step procedure and residue collected by the mobile bag technique with collection in the feces and the modified three step procedure incubated in the rumen for either 16 or 24 hours.

	MBT				mTSP			
	R^2		R^2		R^2		R^2	
Item[1]	CS - 16h	$P>F^2$	CS - 24h	$P>F^2$	CS - 16h	$P>F^2$	CS - 24	$P>F^2$
Arg	0.86	0.02	0.68	0.09	0.66	0.10	0.02	0.81
His	0.98	0.01	0.998	0.00	0.80	0.10	0.08	0.71
Ile	0.82	0.03	0.75	0.06	0.18	0.47	0.003	0.93
Leu	0.98	0.00	0.88	0.02	0.47	0.20	0.15	0.51
Lys	0.87	0.02	0.70	0.08	0.01	0.89	0.0002	0.98
Met	0.05	0.71	0.25	0.39	0.002	0.95	0.13	0.55
Phe	0.81	0.04	0.52	0.17	0.11	0.58	0.08	0.65
Thr	0.73	0.07	0.61	0.12	0.26	0.38	0.004	0.92
Trp								
Val	0.40	0.26	0.51	0.17	0.72	0.07	0.41	0.24
BCAA	0.87	0.02	0.87	0.02	0.51	0.18	0.14	0.53
EAA	0.43	0.23	0.27	0.37	0.005	0.91	0.02	0.81
Ala	0.60	0.13	0.59	0.13	0.38	0.27	0.01	0.86
Asp	0.65	0.10	0.40	0.25	0.05	0.71	0.33	0.31
Cys	0.55	0.15	0.79	0.05	0.86	0.02	0.01	0.88
Glu	0.96	0.00	0.93	0.01	0.48	0.19	0.002	0.94
Pro	0.92	0.01	0.80	0.04	0.26	0.38	0.02	0.84
Ser	0.91	0.01	0.82	0.03	0.86	0.02	0.72	0.07
Tyr	0.75	0.06	0.59	0.13	0.09	0.62	0.27	0.37
NEAA	0.99	0.00	0.92	0.01	0.09	0.62	0.005	0.91
TAA	0.41	0.25	0.20	0.46	0.01	0.88	0.16	0.51

[1]BCAA = branch chain amino acid, EAA = essential amino acid, NEAA = non-essential amino acid, TAA = total amino acid

[2]Probability of linear relationship; n = 5

Table 15. Correlation coefficients (R^2) of protein feeds for AA digestibility of residue samples created from the McNiven protease incubation in conjunction with steps of the modified three step procedure and residue collected from a rumen incubation in conjunction with the cecectomized rooster assay with Fish meal samples omitted.

	R^2		R^2	
Item[1]	Cececomized Rooster	$P>F^2$	Cecectomized Rooster with out FM	$P>F^2$
Arginine	0.75	<0.01	0.85	<0.01
Histidine	0.53	<0.01	0.80	<0.01
Isoleucine	0.68	<0.01	0.82	<0.01
Leucine	0.55	<0.01	0.79	<0.01
Lysine	0.80	<0.01	0.85	<0.01
Methionine	0.52	<0.01	0.74	<0.01
Phenylalanine	0.62	<0.01	0.83	<0.01
Threonine	0.72	<0.01	0.81	<0.01
Tryptophan	0.36	0.018	0.34	0.07
Valine	0.68	<0.01	0.80	<0.01
Branch Chain AA	0.65	<0.01	0.80	<0.01
Essential AA	0.66	<0.01	0.82	<0.01
Alanine	0.52	<0.01	0.77	<0.01
Aspartic Acid	0.56	<0.01	0.65	<0.01
Cysteine	0.45	<0.01	0.74	<0.01
Glutamic Acid	0.59	<0.01	0.77	<0.01
Proline	0.55	<0.01	0.66	<0.01
Serine	0.40	<0.01	0.53	0.01
Tyrosine	0.68	<0.01	0.81	<0.01
Nonessential AA	0.50	<0.01	0.60	<0.01
Total AA	0.50	<0.01	0.67	<0.01

[1]AA = amino acids.

[2]Probability of significant linear relationship; n = 11.

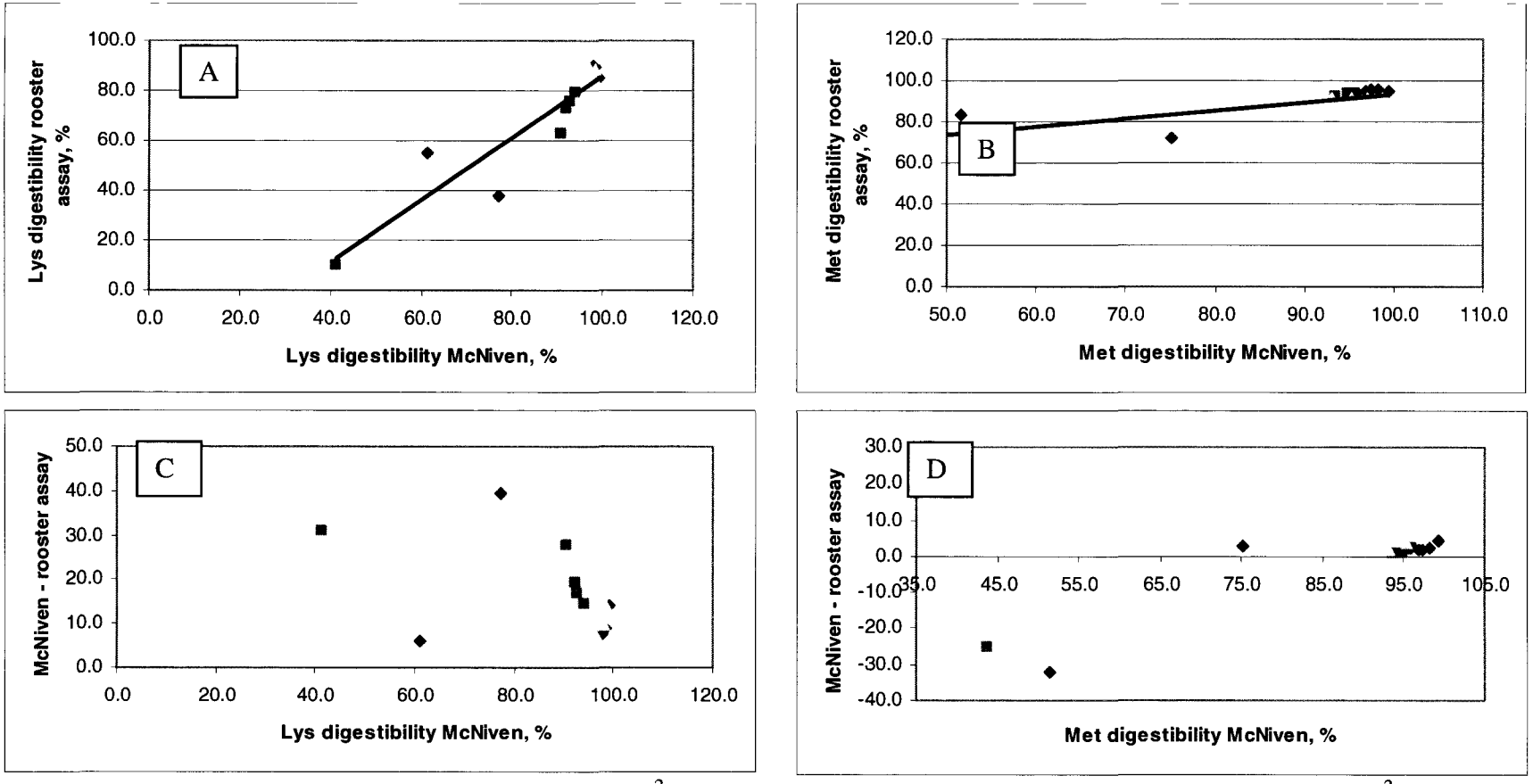

Figure 1. Comparison of Lys (A; Y =1.2464x – 38.554; $R^2 = 0.80$, $P < 0.01$, n = 16) and Met (B; Y = 0.3828x +54.575; $R^2 = 0.52$, $P < 0.01$, n = 16) digestibility of soy product (♦; n = 6), dried distillers' grains with solubles (■; n = 5), and fishmeal (▲; n = 5) from the McNiven protease incubation with steps of mTSP, and the cecectomized rooster assay, and plots of Lys (C) and Met (D) total tract digestibility measured using the McNiven protease incubation with steps of the mTSP minus digestibility from cecectomized roosters assay.

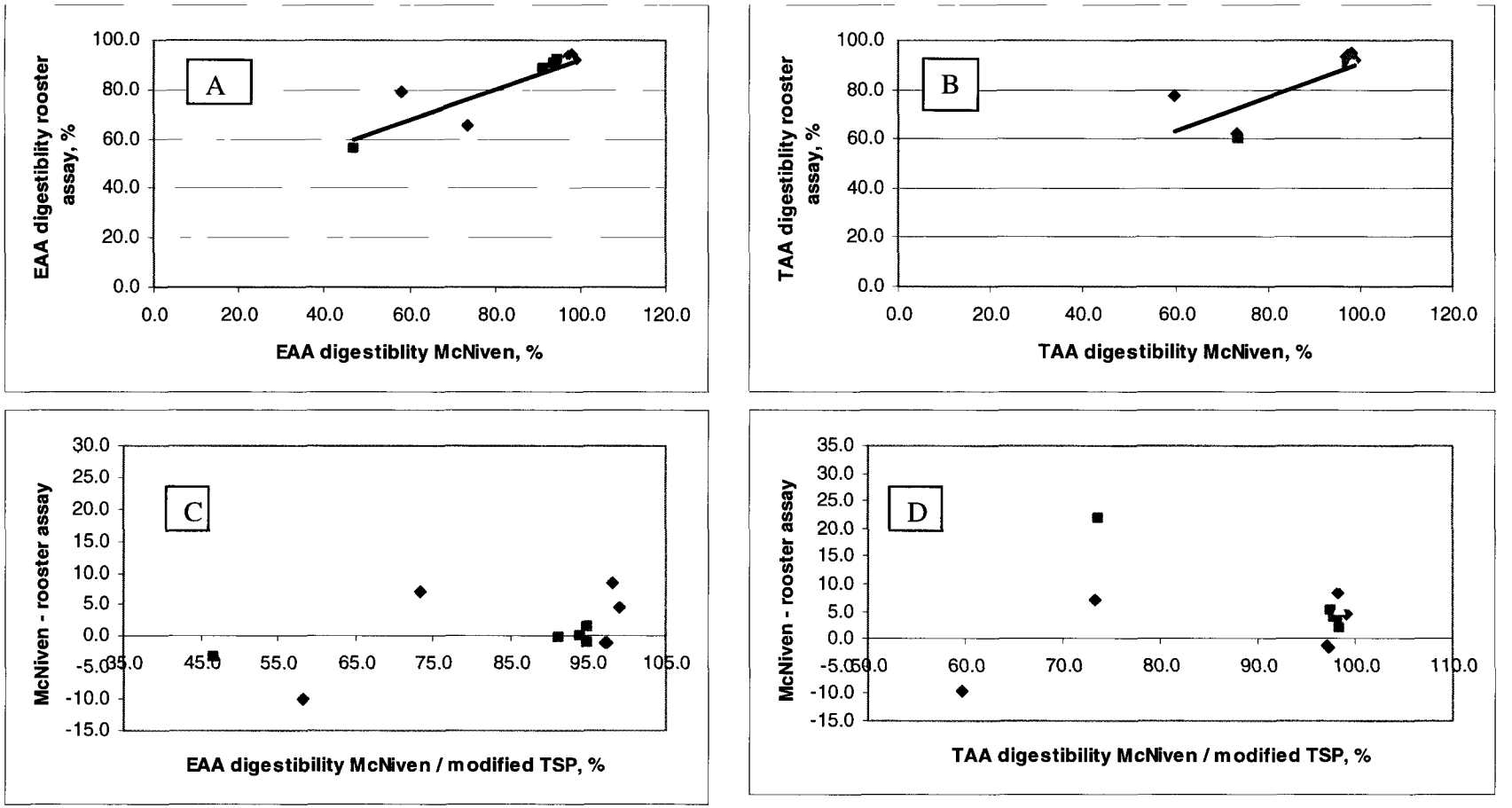

Figure 2. Comparison of essential amino acids (A; Y = 0.6016x + 31.773; R^2 = 0.66, P < 0.01, n = 16) and total amino acids (B; Y = 0.68x + 22.325; R^2 = 0.50, P < 0.01, n = 16) digestibility of soy products (♦; n = 6), dried distillers' grains with solubles (■: n = 5), and fishmeal (▲; n = 5) from the McNiven protease incubation with steps of the mTSP, and in cecectomized roosters, and plots of EAA (C) and TAA (D) total tract digestibility measured using the McNiven procedure in conjunction with the modified TSP minus digestibility from cecectomized roosters.

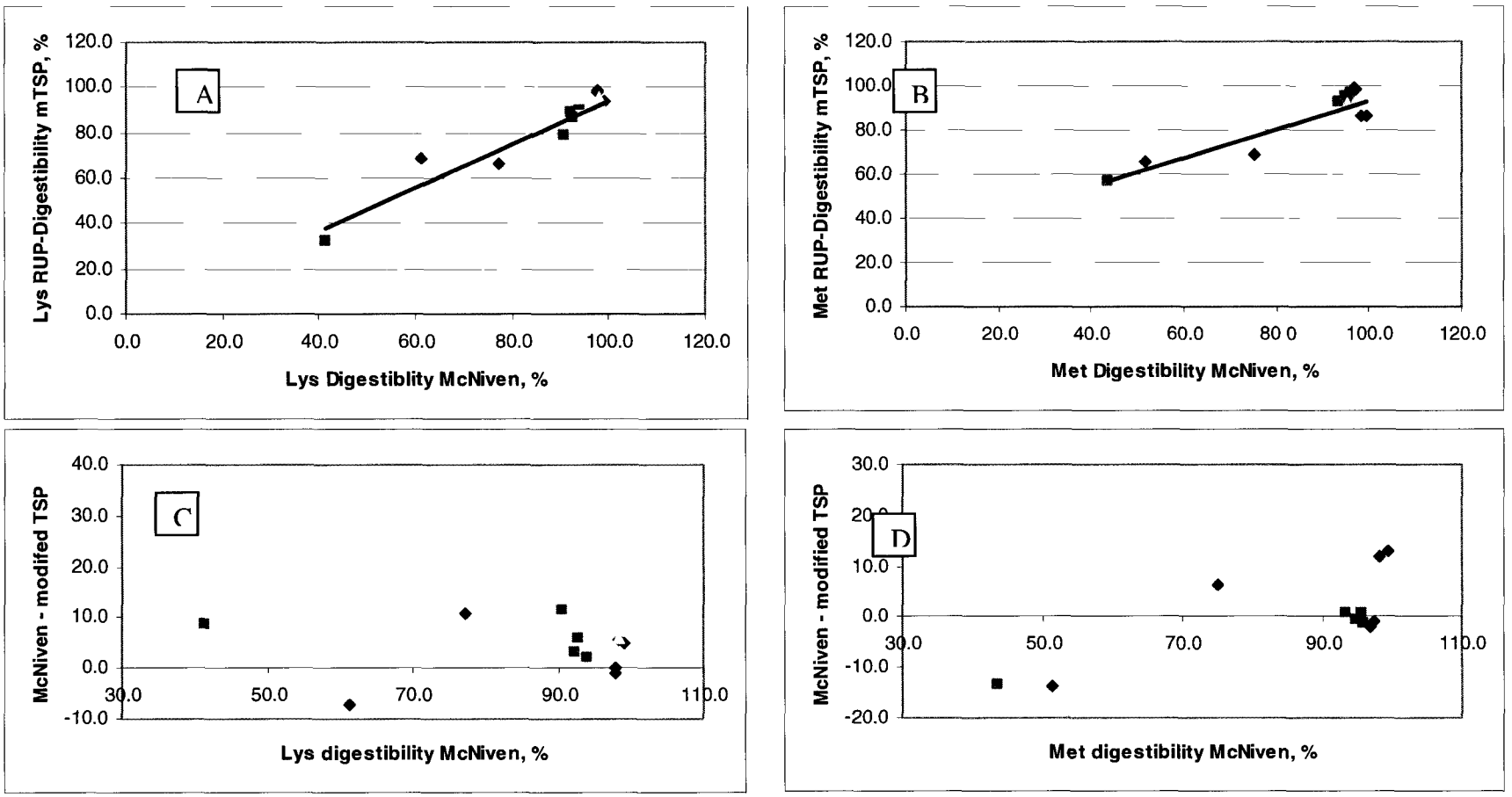

Figure 3. Comparison of Lys (A; Y = 0.9613x – 1.9653; $R^2 = 0.78$, $P < 0.01$, n = 16) and Met (B; Y = 0.6436x + 28.446; $R^2 = 0.67$, $P < 0.01$ n = 16) digestibility of soy product (♦; n = 6), dried distillers' grains with solubles (■; n = 5), and fishmeal (▲; n = 5) from the McNiven protease incubation with steps of the modified TSP, and rumen undegraded residue via the modified TSP, and plots of RUP-Lys (C) and RUP-Met (D) digestibility measured using the McNiven protease incubation with steps of the mTSP, minus digestibility obtained with the in vitro mTSP.

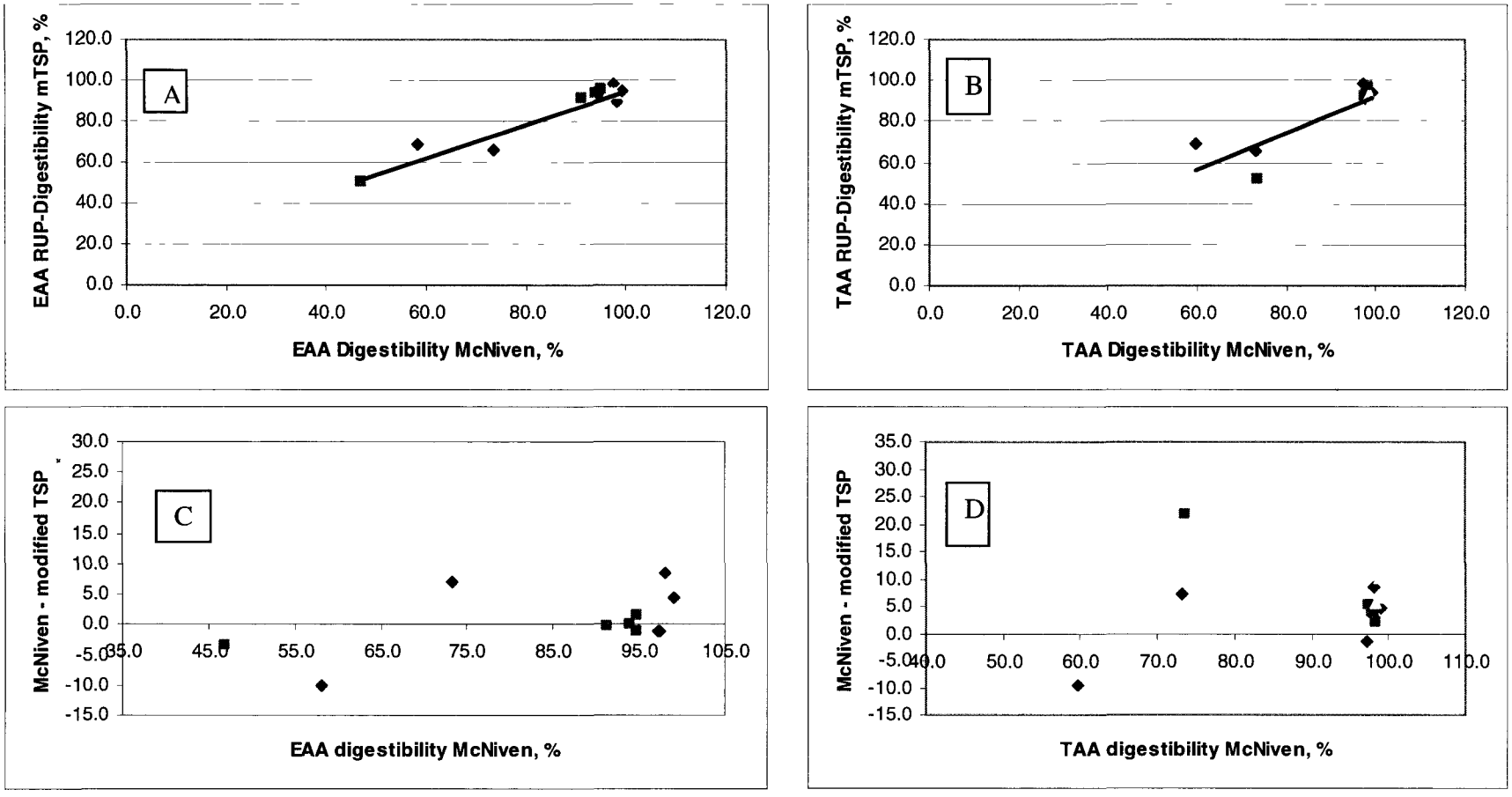

Figure 4. Comparison of EAA (A; Y = 0.8081x +13.918; $R^2 = 0.76$, $P < 0.01$, n = 16) and TAA (B; Y = 0.8993x + 2.4968; $R^2 = 0.59$, $P < 0.01$ n = 16) digestibility of soy product (♦; n = 6), dried distillers' grains with solubles (■; n = 5), and fishmeal (▲; n = 5) from the McNiven protease incubation and steps of the mTSP, and rumen undegraded residue from the in vitro mTSP, and plots of EAA (C) and TAA (D) total tract digestibility measured using the McNiven protease incubation with steps of the mTSP, minus digestibility obtained with the in vitro mTSP.

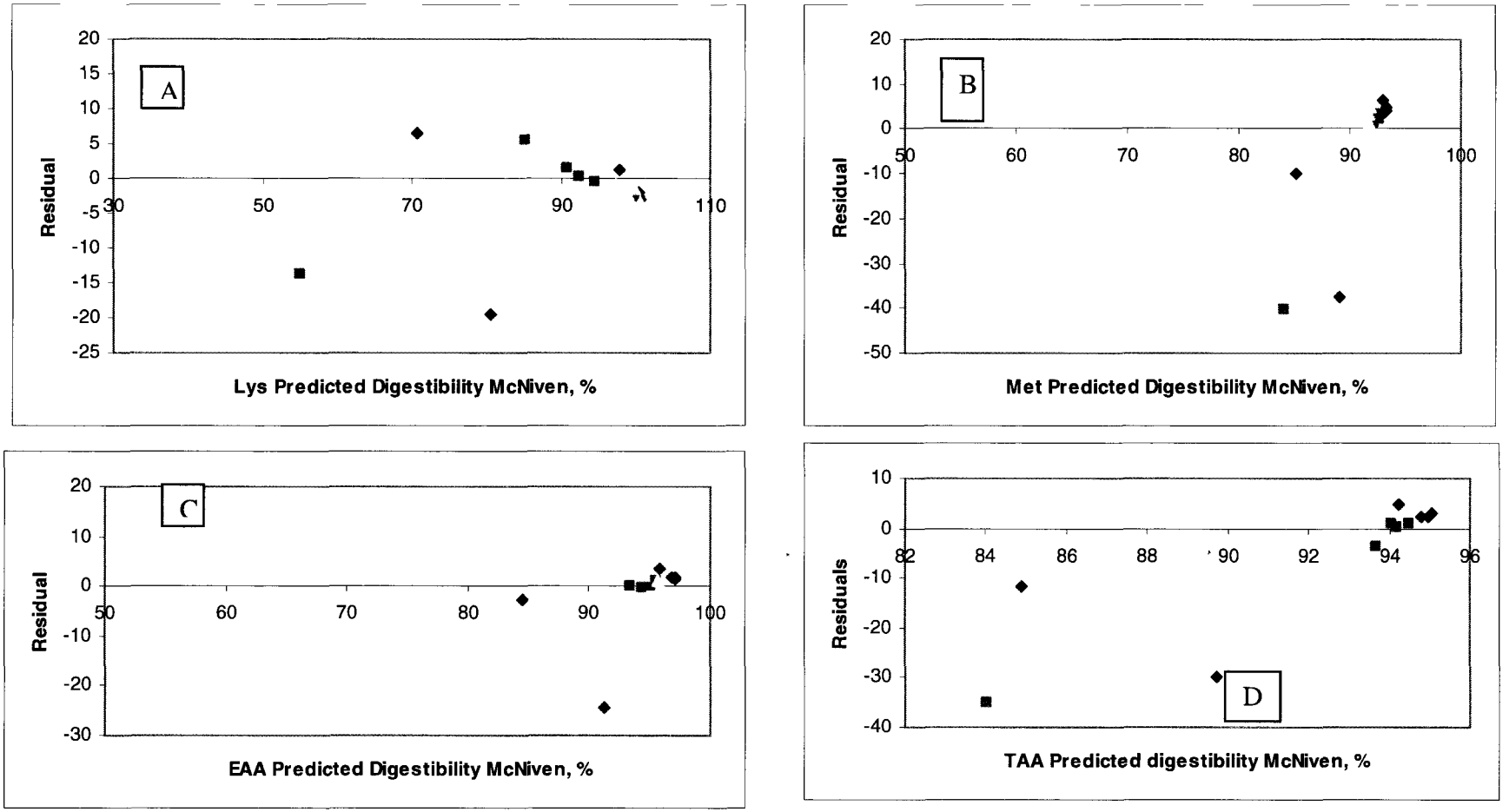

Figure 5. Residual plots of total tract digestibility of Lys (A), Met (B), EAA (C, and TAA (D) measured in vivo using the cecectomized rooster assay and Lys, Met, EAA, and TAA predicted from analysis of Lys, Met, essential AA, and total AA of soy products (♦; n = 6), dried distillers' grains with solubles (■; n = 5), and fishmeal (▲; n = 5) samples determined via the McNiven protease incubation and steps of the mTSP.

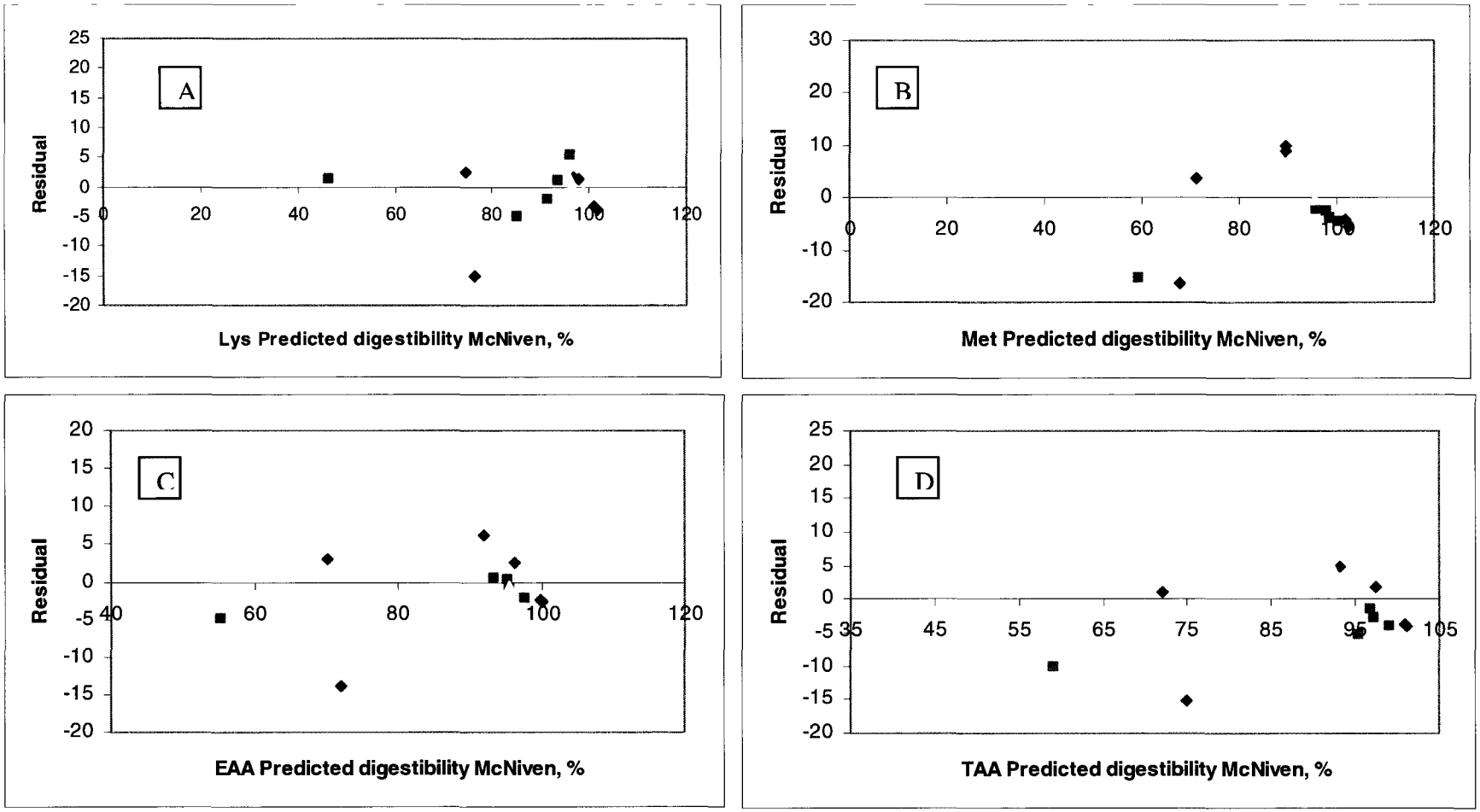

Figure 6. Residual plots of total tract digestibility of Lys (A), Met (B), EAA (C, and TAA (D) measured using the in vitro modified TSP and Lys, Met, EAA, and TAA predicted from analysis of Lys, Met, essential AA, and total AA of soy products (♦; n = 6), dried distillers' grains with solubles (■; n = 5), and fishmeal (▲; n = 5) samples determined via the McNiven protease incubation with steps of the mTSP.

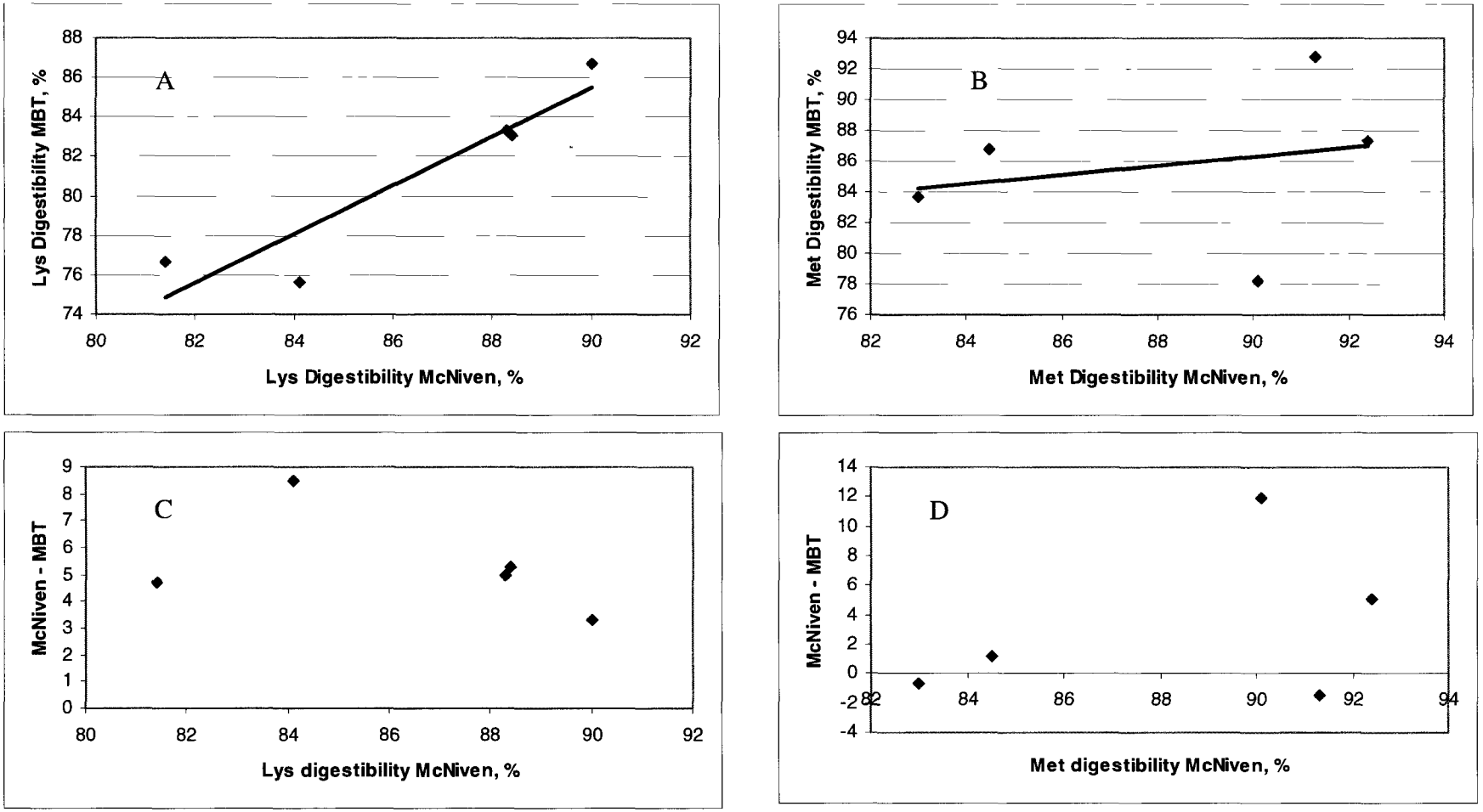

Figure 7. Comparison of Lys (A; Y = 1.2388x – 25.999; R^2 = 0.87) and Met (B; Y = 0.2905x + 60.12; R^2 = 0.05) digestibility of corn silage(♦; n = 5), from the McNiven protease incubation and steps of the mTSP, and in the mobile bag technique with collection form the feces after a 16h incubation in the rumen, and plots of Lys (C) and Met (D) digestibility measured using the McNiven protease incubation with steps of mTSP, minus the mobile bag technique with a 16h rumen incubation.

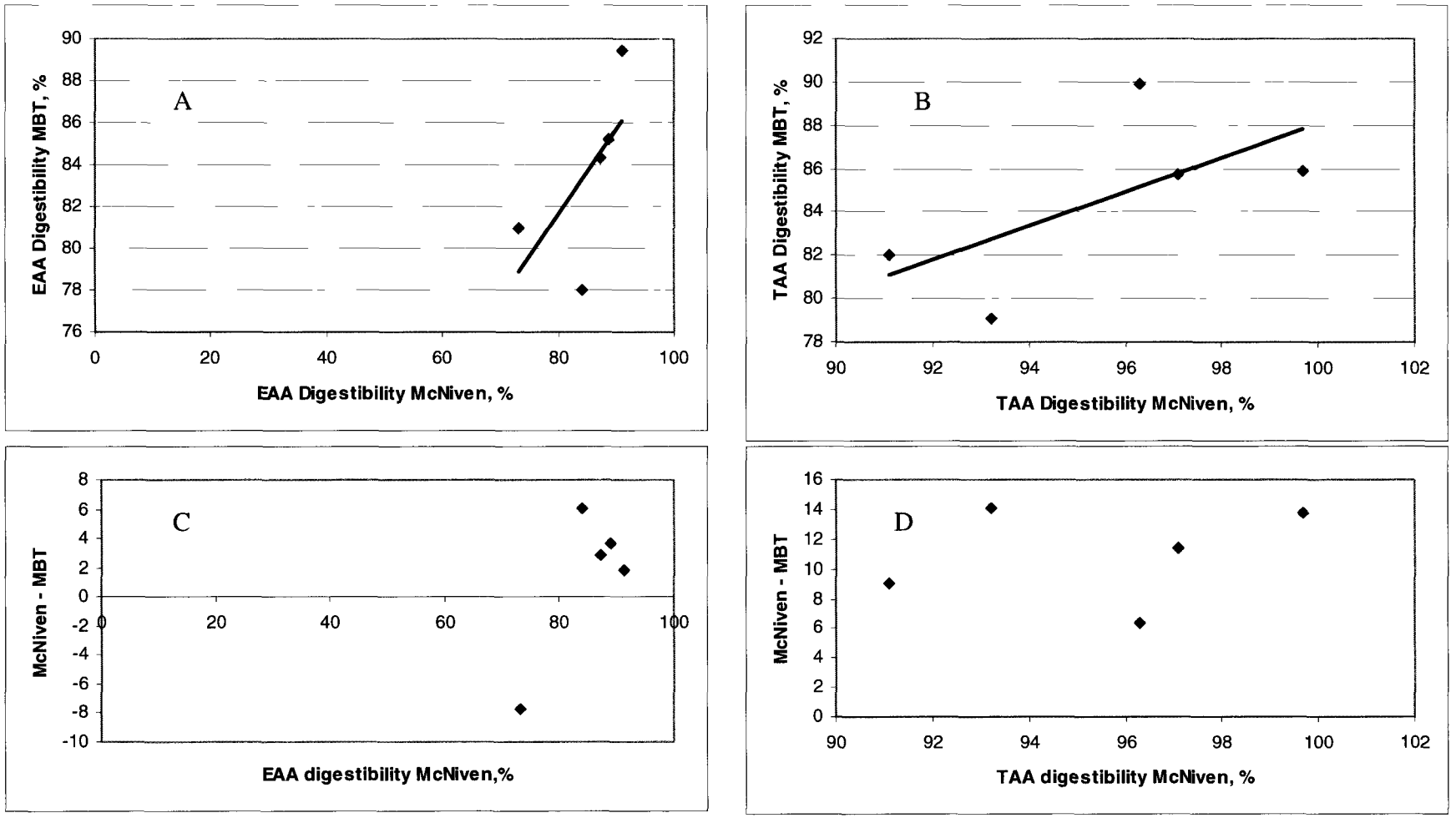

Figure 8. Comparison of EAA (A; Y = 0.7813x +9.9168; R^2 = 0.43) and TAA (B; Y = 0.7813x + 9.9168; R^2 = 0.41) digestibility of corn silage(♦; n = 5), from the McNiven protease incubation and steps of the mTSP, and in the mobile bag technique with collection form the feces after a 16h incubation in the rumen, and plots of EAA (C) and TAA (D) digestibility measured using the McNiven protease incubation with steps of the mTSP, minus the mobile bag technique with a 16h rumen incubation.

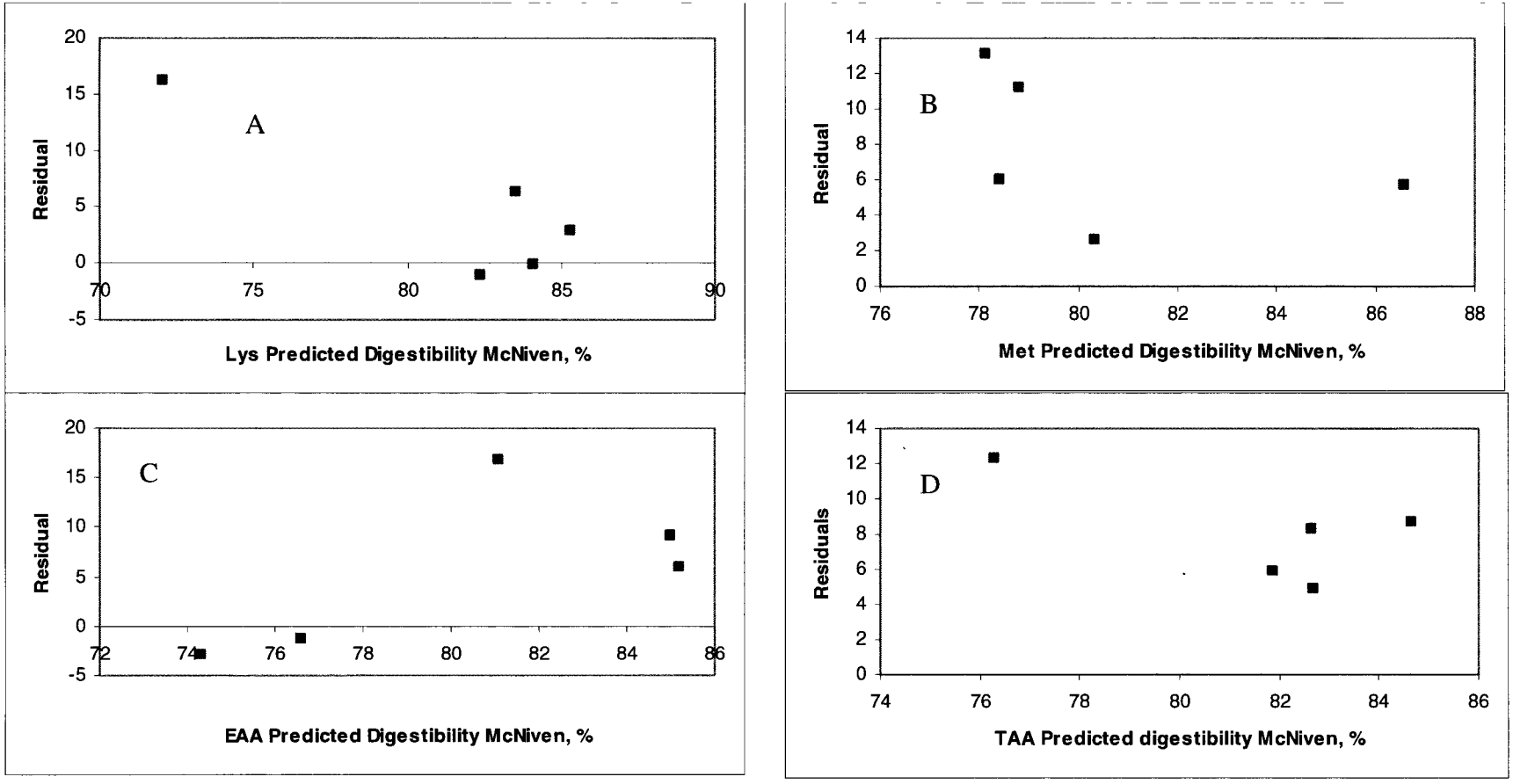

Figure 9. Residual plots of total tract digestibility of Lys (A), Met (B), EAA (C, and TAA (D) measured in vivo using the mobile bag technique and Lys, Met, EAA, and TAA predicted from analysis of Lys, Met, essential AA, and total AA corn silage samples determined via the McNiven protease incubation with steps of mTSP.