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LITERATURE REVIEW

Empirical attributes and limitations of methodologies for predicting the degradability of ruminal protein

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Abstract

A. Velásquez, J. Rivero, and P.-G. Marnet. 2016. Empirical attributes and limitations of methodologies for predicting the degradability of ruminal protein. Cien. Inv. Agr. 43(2):171-189. The object of the present review is to analyze the methodologies that are commonly used to estimate protein degradability in the rumen, focusing on their attributes and limitations to offer suggestions for improving their use. This information is essential for selecting food types when formulating feed diets. A reliable prediction of the digestibility of ruminal proteins is basic information necessary for optimizing the use of nitrogenous sources because digestibility can translate, on the one hand, into higher yields of milk, milk protein, meat or wool and, on the other hand, into lower excretion of nitrogenous compounds into the environment; it also has an impact on animal health and welfare. Traditionally, the digestibility of feed proteins in the rumen has been predicted by *in vivo*, *in situ* and *in vitro* methods, but other techniques based on infrared spectroscopy have been developed, notably the NIRS and FTIR methods. All of these techniques present limitations, such as a disturbance factor or a source of error, that may result in inaccurate predictions. The *in situ* and *in vitro* methods, which use enzyme extracts of ruminal origin, and FTIR probably have the greatest advantages, but they need to be perfected through further research.

Key words: Ruminal protein degradability, methodologies, limitations.

Introduction

The efficiency of nitrogen use in ruminants is primarily affected by the degradability dynamic of the nitrogenous compounds in the ruminal ecosystem, the availability of fermentable energy in the rumen and the subsequent nitrogenous metabolism in the animal. The prediction of protein degradability in the rumen is basic information that is necessary to optimize the food resources used for ruminant nutrition (e.g., synchronization of the availability of N and energy in the rumen), and an accurate prediction also assists in ensuring animal welfare and reducing the environmental impact generated by the excretion of nitrogenous compounds into the environment (Van Duinkerken *et al*., 2005; Kaswari *et al*., 2007; Riasi *et al*., 2008; Rezaei *et*

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al., 2015). Feed protein degradation in the rumen is highly unpredictable because, on the one hand, feeds contain different nitrogenous compounds with widely varying molecular compositions and spatial structures at the histological and cell levels; on the other hand, they are subjected to an enzymatic digestion environment that is diverse and difficult to predetermine (Van Soest, 1994; Stern *et al*., 1997; Encinias *et al*., 2005; Razzaghi *et al*., 2016). Skewed information on the actual degradability of proteins in the rumen can lead to errors in ruminant feeding. Underestimating the real nitrogen contribution of the feed to the ruminal ecosystem, combined with a low availability of energy for microorganisms, would increase the amount of N excreted in urine and feces. Under such conditions, the ruminal epithelium would generate N homeostasis between the ruminal medium and the plasma level by absorbing the excess N (principally in the form of ammonium) not fixed by the microorganisms to maintain the nitrogen equilibrium in the ruminal medium. Additionally, this absorption of ammonium would have metabolic consequences for the kidneys, liver and mammary glands (Kornegay, 1996; Van Duinkerken *et al*., 2005; Castro-Montoya *et al*., 2016). It is also important to have as accurate information as possible on the degradability of polypeptides in the rumen to establish balanced diets, thus optimizing the efficient use of N. Thus, the accuracy and representativeness of the methods used for predicting protein degradability in the rumen play a fundamental role in optimizing the use of nitrogenous resources through the formulation of diets for productive ruminants (Givens *et al*., 2000; Benninghoff *et al.,* 2015). The methodologies traditionally used for predicting the dynamics of proteolysis in the rumen have undergone little technical modification; they have contributed to animal nutrition without any major examination or criticism and have been accepted as unquestionable references for the quality of the information provided. However, to a greater or lesser degree, there are a series of limitations or disturbance factors inherent to the technical aspects of these methodologies that could result in skewed estimates of the real digestibility of the proteins in the ruminal ecosystem (Broderick, 1987; Russell *et al*., 1992; Sniffen *et al*., 1992; Yan and Agnew, 2004; Encinias *et al*., 2005; Zagorakis *et al*., 2015). These limitations can lead, among other things, to the inefficient use of protein resources during ruminant feeding and economic losses for the producer. Therefore, there is a need to perfect these methodologies, or find new ones, to improve the prediction of the kinetics of feed proteolysis in the rumen. The object of the present study was to analyze the predictive effectiveness of the commonly used methodologies to estimate the degradability of proteins in the rumen to facilitate their improvement and establish guidelines for the future creation or investigation of alternative methods.

Complexity of proteolysis in the ruminal ecosystem

The kinetics of protein degradation in the ruminal ecosystem are affected by complex interactions between biotic and abiotic components resulting in biological conditions that are difficult to simulate with great accuracy or precision; there is great variability among observations made using *in vitro*, *in situ* or *in vivo* methods (Stern *et al*., 1994; Broderick *et al*., 2004; Cone *et al*., 2004; Habib *et al*., 2013; Hao *et al*., 2016). The most important biological components are related to the biodiversity and ecological behavior of the microorganisms in the rumen, principally their bioenergetic, enzymatic and hydrolytic processes and they synthesis of microbial proteins. Basically, these components involve the fermentation of carbohydrates (structural and non-structural), lipolysis-biohydrogenation, proteolytic activity and N capture, which are normally associated with reproduction and cell growth. They also constitute an important biological element that influences the effectiveness of the adherence and colonization mechanisms of the particles digested by the microorganisms.

Nitrogen recycling in the rumen and at intermediate levels in ruminants, the kinetics of particle escape (ruminal retention time) and feed consumption and feeding frequency are incidental factors affecting protein degradation dynamics in the rumen (Sniffen *et al*., 1992; Broderick *et al*, 2004; Costa *et al*., 2016). During the digestion of organic material in the rumen, the hydrolytic interaction of microbial consortia through the synthesis and excretion of hydrolytic enzymes, enzyme-substrate affinity and hydrolysis kinetics are of great importance in determining the magnitude of protein digestion. There is an ecological phenomenon known as *syntrophy* that accounts for the simultaneous proteolytic, amylolytic or fibrolytic enzyme activity of some microorganisms, such as the bacteria *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*, whose digestive activity is based on the degradation of both proteins and cellulose. Likewise, *Succinimonas amylolytica, Bacteroides amylophilus, Selenomonas ruminantium* and *Streptococcus bovis* simultaneously present proteolytic and amylolytic activity (Van Soest, 1994; Kornegay, 1996). During the digestion of structural (cell wall) and non-structural carbohydrates (e.g., starch), various species of microorganism make use of this phenomenon to break into the feed cells and digest both the free and compartmentalized cytoplasm proteins in cell organelles. They can also hydrolyze the polypeptides located among the lignocellulosic and hemicellulosic molecular complexes of the cell walls. The action of bacterial amylases and protozoa permit access to proteins, which are physically and chemically compromised with starchy structures (Chamberlain and Choung, 1995; Bull, 2001; Hristov *et al.,* 2008; Zhao *et al*., 2016).

The characteristics of polypeptides also determine their degree of susceptibility to the action of proteases. The solubility and the structural properties of proteins, such as the presence and number of sulfur bridges, the ionic charges at the surface and the degree of folding and hydrophobicity, are factors that determine the magnitude and effectiveness of the proteolytic attack by the ruminal microorganisms (Peltekova and Broderick, 1996). Proteolysis occurs in the form of nucleophile attacks on the peptide bonds, which may be endoproteolytic or exoproteolytic, by proteases, and the process normally occurs on the outside of cells that are intimately associated with the bacterial wall (e.g., adsorption of the proteins in cellulosomes) or occurs freely in other situations, with proteases being excreted into the ruminal medium. Nevertheless, bacteria exist which, depending on their species and strain, the trophic conditions of the moment, their molecular weight and the biochemical characteristics of the polypeptides to be digested, cause intracellular hydrolysis of the polypeptides (Asplund, 1994; Swanepoel *et al*., 2016). The protozoa typically swallow proteins, microparticles and microorganisms whole (e.g., bacteria, protozoa, and fungi), implying that, in most cases, protein digestion by these microorganisms is intracellular.

The above information suggests that the greatest concentration of proteases may be found in the periplasm of the bacteria and on the surface of their cell walls, on the cytoplasm of some of the bacteria and in the protoplasm of most of the protozoa as well as in the most intimate microhabitats around and within the feed particles to be digested. These findings are particularly important for the *in vitro* methods that are based on the extraction of ruminal enzymes to predict the degradability of proteins in the rumen (Velásquez and Pichard, 2010a).

Following protein degradation, the generated amino acids may follow different metabolic paths, which will depend in part on the concentrations of ammonium, amino acids, peptides, oligopeptides, polypeptides, carbonic skeletons and fermentable energy in the ruminal medium. These amino acids can be deaminated, producing ammonium and isoacids, or used directly in the synthesis of microbial proteins. For example, the deamination of *valine, leucine* and *isoleucine* produces the final molecules of *isobutyrate, isovalerate* and

2-methylbutyrate, respectively (as well as carbon dioxide and ammonium). From a methodological point of view, the importance of these phenomena for predicting protein degradability is that these acids and other metabolites can form substrates that promote microbial growth and reproduction, principally for many cellulolytic bacteria and other microorganisms that use these fatty acids as sources of energy and/or carbon chains. Normally, these isoacids, in conjunction with ammonium fixation, allow *de novo* synthesis of microbial amino acids (Ørskov, 1992; Van Soest, 1994; Chamberlain and Choung, 1995; Bull, 2001; Swanepoel *et al*., 2016), and the magnitude of the synthesis is mainly regulated by the activity of the enzymes *glutamine synthetase, NADP-glutamate dehydrogenase* and *NAD-alanine dehydrogenase,* whose K_m values for ammonium fixation are 1.8, 1.8 to 3.1, and 70 mM of ammonium, respectively (Asplund, 1994). It should be noted that the degree of enzyme substrate affinity (K_m) will determine, in some way, the activity levels of these enzymes as a function of the intracellular nitrogen concentration of the microorganisms and the N content in the ruminal medium. The variability in the activity of these enzymes will affect the availability and diversity of synthesized amino acids and their subsequent involvement in proteic anabolism and later effects on the levels of microbial protease excretion (Chamberlain and Choung, 1995; Bull, 2001).

All of these phenomena, which are in some way related to the enzyme activity in the rumen, must be empirically considered in any biological method that attempts to predict protein degradability in the rumen. Microbial biodiversity, including the resulting variability in the enzyme spectrum in the rumen, can be modified through substrate-induction, which can lead to changes in the extent and rates of ruminal protein degradation. This has been empirically demonstrated through protein digestibility assays both *in situ* and *in vitro*, so it is indeed possible to generate a change in the microbial biodiversity of the ruminal ecosystem by differentially stimulating the synthesis and specific secretion of microbial proteases and carbohydrases (Hungate, 1966; Velásquez and Pichard, 2010a). For example, it appears that a feeding plan rich in cell walls (NDF) would stimulate population (biomass-microbial) growth and, therefore, the cellulolytic activity of certain fibrolytic bacteria such as *Butyrivibrio fibrisolvens, Bacteroides succinogenes, Ruminococcus flavefaciens* or *Fibrobacter succinogenes*. As a consequence, the *Pyruvate-Acetyl-CoA-Acetate* or the *Pyruvate-Acetyl-CoA-Aceto-acetyl-CoA-Butyrate* pathways would be favored over the *Pyruvate-Lactate-Acrylate-Propionate* pathway. This intra- and interspecific genetic variation in the ruminal ecosystem would also have the effect of changing the volatile fatty acid (VFA) excretion profile, thus influencing the final proteolytic status of the ruminal environment (Asplund, 1994; Van Soest, 1994; Sarmadi *et al*., 2016). Therefore, for methods that use ruminants directly (*in vivo* or *in situ*) or as donors of ruminal fluid *(in vitro*), it is suggested that the animals should be offered diets that are sufficiently balanced in terms of fiber (NDF), energy, minerals and nitrogenous sources to maintain a stable ruminal steady state with greater synchronization in the bioavailability of N/energy, and at the same time, a diet should be established that is as representative as possible of the production conditions under which the animals are fed (Nocek and Grant, 1987; Kaswari *et al*., 2007; Zagorakis *et al*., 2015).

Another phenomenon to be considered in the assays is the fact that the digestion of nitrogenous compounds in the rumen is particularly important during the periods following feeding; normally, a high percentage of the polypeptides are hydrolyzed during the first 6 to 8 hours after being subjected to the digestive action of microorganisms in the ruminal ecosystem. The extent and rates of this early degradation are directly associated with the solubility and the physical-chemical availability of the proteins in the food to be digested. It should be noted that, in the case of fresh forage, special consideration must be given to the activation of the endogenous proteases in the plant, which usually

occurs after cutting or grazing. Furthermore, these endogenous enzymes may undergo induction in the rumen due to the anaerobic conditions and high temperature in the ruminal medium. Among the principal consequences of the activity of the endogenous proteases is autohydrolysis of the plant proteins, resulting in an increase in the concentration of soluble N in the medium, which would generate a kinetic moment that would overestimate microbial proteolytic activity and result in a mathematical bias in experiments used to determine lag times and hydrolysis rates (*kd*). This situation is important in assays in which fresh forage is evaluated and may produce skewed predictions of real protein degradability in the rumen (Zhu *et al*., 1999; Kaswari *et al*., 2007; Riasi *et al*., 2008).

Methodologies for predicting protein degradability in the rumen

Today, various methodologies exist for predicting the extent and rates of protein degradation of feeds in the rumen. The most traditional are based on biological techniques, and the most representative of these are *in vivo*, *in situ* and *in vitro*. Chemical methodologies are also used, and one of the most common is the chemical fractioning of the proteins in a feed. Another currently available technique for this purpose is near infrared reflectance spectroscopy (NIRS), which is based on the physical principle that atoms and molecules exhibit electromagnetic vibrations when they are excited with lasers, allowing the determination of the wavenumbers of different materials (Belanche *et al*., 2013). When light strikes a sample, some photons may be transmitted through the material while the rest are reflected or absorbed by covalent bonds (Murray, 1993). When applied in the near infrared light spectrum, the results can provide information about the main structural elements associated with living organisms because the functional groups that respond to radiation in this spectrum are CH, OH, NH and, probably, SH and C=O (Alomar and Fuchslocher, 1998). This makes it possible not only to learn the molecular structures of a feed but also to predict the degradation dynamics of its components.

One of the main chemical methods is that developed by Sniffen *et al*. (1992) and Licitra *et al*. (1996), who proposed a system for the chemical fractioning of proteins that was incorporated into the Cornell Net Carbohydrate and Protein System (CNCPS, 1992) and adopted by the NRC (2001) for their version of nutrient requirements and recommended feed composition for dairy cattle. This methodology establishes the chemical analysis of the nitrogenous fractions of a feed; its main characteristics being its ease of application and the reliability of its measurements. The measurement system establishes five nitrogenous fractions corresponding to A, B_1, B_2, B_3 and C pools, where: pool A= soluble in buffer, not precipitable with trichloroacetic acid (TCA), and corresponding to non-protein N (NPN); pool B_1= soluble in buffer and precipitable with TCA; pool B_3= insoluble in neutral detergent but soluble in acid detergent (difference between NDF-N and ADF-N); pool C= ADF-N, non-degradable or unavailable fraction (associated with lignin, tannins or the Maillard reaction); and pool B_2 is calculated as the difference between total N and the other N pools. Fractions A, B_1 and B_2 are normally associated with cell content, while fraction B_3 is associated with plant cell walls. Despite the strengths of this technique, some aspects of the procedure have been criticized, mainly its methodological rigidity and the lack of representation of the biological phenomena that occur in the rumen (Stern *et al*., 1997; Mustafa *et al*., 2001). Moreover, some authors have reported large differences in the sizes of the different nitrogenous fractions between samples of a single type of feed, suggesting that local conditions and the method itself are major sources of variation in the characterization of these chemical fractions of proteins (Mullahey *et al*., 1992; Jones *et al*., 1995; Reed, 1995; Velásquez and Pichard, 2010b). Additionally, this technique does not allow for the hydrolysis rate (*kd*) to be calculated as it does not yield different degradation

levels for different kinetic periods. There is an unavoidable source of experimental error, which can skew the real value of fractions A and B_1, that is related to the capability of TCA to precipitate polypeptides. Under this methodology, fraction A, which represents the whole of the NPN, could include low-molecular-weight polypeptides not precipitated by TCA, thus overestimating this nitrogenous fraction. According to Gabriel *et al.* (2008), the cut-off molecular weight for peptide precipitation by TCA is 3 kDa, and even higher molecular weight peptides might be randomly excluded from the fraction precipitated with TCA. In the case of pool B_1, a reverse situation may occur as a result of the above; i.e., low-molecular-weight peptides might not be included because they were not precipitated by TCA. This situation may be demonstrated by isolating these fractions and determining their molecular characteristics by electrophoresis.

The primary merit of biological methods is their proximity and their more direct representation of the ruminal ecosystem. The *in vivo* method is probably the oldest and one of the best known, and the simplest form of this technique allows the digestible protein (DP) to be estimated by subtracting the protein found in the feces from the total amount of protein consumed. Dividing this result by the total protein consumed and multiplying by 100 gives the apparent digestibility coefficient. The main limitation of this method is the uncertain and highly variable origin of the N found in the feces because it may be of endogenous origin (NMF), i.e., coming from ruminal microorganisms, and/or an undigested part from these two sources. It has been estimated that a ruminant excretes approximately 0.5-0.6 g of NMF in its feces for every 100 g of dry matter (DM) consumed. This figure represents close to 3.8 g of the crude protein (CP), so in a feed with low CP levels, the apparent digestibility coefficient will be strongly influenced by this effect. Therefore, there is a major limitation in the basic conception of this technique, and if we seek to estimate digestibility under grazing conditions, the situation becomes even more complex given the operational difficulties of the technique, firstly, in measuring the DM consumed and, secondly, in measuring the quantity of feces excreted. The result of these limitations is that the *in vivo* measurement of protein digestibility is inappropriate for routine use, and moreover, these measurements generally present low repeatability. Another important limitation of some *in vivo* protocols is the requirement for the ruminal and duodenal fistulation of the animal, which is problematic both for welfare reasons and because the samples taken by cannula are sometimes not representative and microbial contamination is inevitable during handling (Seymour *et al.,* 1992). The *in situ* protein degradation method was developed to avoid the sources of error inherent in the *in vivo* method as it consists of the intra-ruminal incubation of dacron bags containing feed (Mehrez and Ørskov, 1977; Ørskov, 1992; Shannak *et al.,* 2000; Pawelek *et al*., 2008; Zhang *et al.,* 2015; Tayyab *et al*., 2016). This technique is one of the most traditional and representative for studying the proteolysis dynamics in the rumen. Its principal merit is the incubation protocol, which is directly applied in the ruminal ecosystem and is the protocol that is most frequently used today thus providing an important reference for the validation of alternative methods. It has been criticized, however, for the presence of various disturbing effects, the most important of which are microbial and N-compound contamination in the residue bags, the escape of particles from the bags into the ruminal medium, and the rarefication of the environment inside the bags (Mathis *et al*., 2001; Cone *et al*., 2004). One possibility for correcting particle loss from the bags into the ruminal medium is the method developed by Velásquez and Pichard (2010b) and modified from Weisbjerg *et al*. (1996), which states that the corrected b fraction (bc) = b + P (b (1-(P + SN))$^{-1}$), where b is the nitrogenous fraction enzymatically degraded *in situ* (corrected, in turn, for microbial contamination); P is the particle loss from the bag into the ruminal medium; and SN is the soluble nitrogen. The particle loss is

determined by the difference between the total material escaping from the bag after washing and the fraction of soluble N at time zero, so the total *in situ* protein degradation is equal to the sum of pool A + pool B_1+ pool bc. This correction is an attempt to estimate the digestibility of the lost particles (P) if they had remained inside the bag and been digested *in situ*, and the estimate is performed by adding fraction b to the expression P (b (1-(P + SN))$^{-1}$, where b (1- (P + SN))$^{-1}$ provides an estimate of the potential digestibility of the lost particles. On many occasions when the *in situ* technique is used to estimate protein digestibility, no correction is made for particle loss, causing – as may be appreciated from the above analysis – a serious skew in the measurement of the rumen digestibility potential of the proteins in a feed. Furthermore, when this *in situ* method is used, no correction is usually made for the microbial contamination present in the residues *in sacco*. In fact, during the colonization and adherence of the bacteria on the microparticles to be digested, a physical-chemical union is formed between these components that is hard to break and may persist in the undigested residues (after washing the bags upon their removal from the intra-ruminal medium). So, if the presence of microbial N in the bag residues is not corrected, the number of N atoms present in the undigested fraction of the feed will be overestimated. It should be noted that the microbial contamination can be corrected by various methods, which are mainly based on microbial markers, but the protocols are complex and not free of difficulties. Among these is the use of *2,6 diaminopimelic acid* (DAPA), an amino acid that is only found in bacterial walls. One of the limitations is the high variation in the N:DAPA ratio, which leads to a quantitative bias in the estimation of the total N of microbial origin. Another alternative is the use of *D-alanine*, the stereoisomer of the amino acid *L-alanine* that is only present in bacteria (enantiomer of *L-alanine*), but this option also results in some variability in the N:*D-alanine* ratio. In the case of protozoa, *aminoethylphosphonic* acid (AEPA) can be used, but this method may present some sources of error, such as the presence of the marker in other microorganisms and the remains of feed particles. The N:AEPA ratio also varies between protozoa species and genera. Then, there is the possibility of using the isotopes of some chemical elements as microbial markers, such as N^{15}, S^{35} or P^{32} (Robinson *et al*, 1996; D'Mello, 2000), which can be used for evaluations both *in situ* and *in vitro*. One of the reported limitations of this method is that enrichment with these isotopes is not homogeneous for among different groups of bacteria and protozoa, leading to large skews in the final measurements of microbial N. A simpler technique for correcting microbial contamination of *in situ* experiments is that used by Velásquez and Pichard (2010b), which consists of incubating bags similar to those used in the *in situ* assay and containing 500 mg of sterile cotton wool. It is hypothesized that the microorganisms will adhere to the cellulose fibers of the cotton wool (free of N) simulating the colonization of feed particles. Thus, after the bags are washed using the same experimental protocol, only the microorganisms, especially the bacteria, will remain on the fibers, so all of the N measured in this residue will be of exclusively microbial origin. However, even if attempts are made to correct the principal limitations, uncontrollable skewness will probably persist with the *in situ* technique. For example, correction for particle loss may show some variability for the same feed because the kinetics of particle escape from the bag into the ruminal medium is unforeseeable and uncontrollable at the molecular level. It is possible to try to minimize this skew by using an appropriate, homogeneous grain size (> 2 mm) for the samples, but the microparticle content and movement dynamic in the *intra-sacco* medium will continue to be unpredictable. The same will happen if microbial contamination is rigorously addressed as it is almost impossible to obtain high repeatability in the correction values for contaminant microbial biomass in bags incubated inside the rumen. Additionally, the pore size may affect the degree of digestion of the proteins inside the bags. The bags that are nor-

mally used are made of dacron with a 50 ±15µ pore size, which supposes that there would be no problems for bacteria to enter the bags. However, ciliate protozoa measuring more than 50 µ (e.g., *Diplodinium, Epidinium, Isotricha*) are known to exist and are therefore excluded from the degradation activity *in sacco*. This method eliminates their possible ecological interactions and hinders the direct digestive action of these protozoa.

In vitro methods involving ruminal fluid (Broderick, 1987; Hristov and Broderick, 1994; Luchini *et al*., 1996; Spanghero *et al*., 2015) are an attractive option from a biological point of view, but they present a series of limitations that increase their inaccuracy in predicting protein degradation. The main difficulty arises in separating feed protein from microbial protein (Broderick, 1987; Robinson *et al.,* 1996), and different corrective variants have been attempt to address this difficulty. One is to use ruminal fluid that is free of live microorganisms (separated by centrifuging), but little proteolytic activity has been observed when this has been tried on different substrates (Luchini *et al*., 1996). The *in vitro* method with inhibited ruminal fluid (Inhibitor *in vitro* method – IIV) developed by Broderick (1987) is based on the inhibition of microbial protein synthesis with *chloramphenicol* and deamination with *hydrazine sulfate*. The protein degradation of the substrate is quantified by measuring the ammonium and total amino acids (TAA) released, discounting the corresponding blanks, but there are various criticisms of this method, some of which are reported by the authors (Broderick *et al*., 2004). Considerable variability in protein degradability was observed between incubations with ruminal fluid from the same donor animal under the same feed regimen, making it necessary to increase the number of replications to reduce the effects of these sources of error. The authors further report that measuring degraded protein by detection of only NH_3 and TAA could underestimate the rate and extent of protein degradation. Wallace *et al*. (1999) and Choi *et al*. (2002) suggested that some peptides may act as important intermediaries during protein hydrolysis in the rumen and should therefore be considered as part of the degraded protein. Another source of variation is that proteolytic activity is generally substrate-induced, so the stimulation of microbial protein synthesis (peptidases) would be increased in the presence of a protein substrate. Therefore, the degradation potential of these microorganisms would then be negatively affected by the inhibition of their synthesis by *chloramphenicol*. One of the most important criticisms of this method is that the inhibition of microbial growth hinders modification of the biodiversity of the microorganisms in response to the nature of the feed to be digested, and furthermore, treatment with *hydrazine* probably provokes the death and lysis of many bacteria. This method is also limited by the short incubation time during which the protein degradability of the feed samples can be evaluated. After more than 8 to 10 hours, the environment in the incubation medium is severely rarefied, and the inhibitory properties of *chloramphenicol* and *hydrazine sulfate* fail (Broderick *et al*., 2004).

Another technique used for studies of proteolytic degradation in the rumen is based on commercial enzymes, basically the fungus *Streptomyces griseus* (Pichard and Van Soest, 1977; Mahadevan *et al*., 1987; Assoumani *et al*., 1992; Velásquez and Pichard, 2010b). Previous authors achieved high levels of protein degradation for various feed substrates using fungal proteases. However, this method has attracted some criticisms that are basically related to the insufficient biological representativeness of the proteolytic events occurring in the rumen because the fungus that gives rise to the proteases has a very different ecological niche compared to that of the microorganisms occurring naturally in the rumen. It also lacks a diverse carbohydrase and protease pool, limiting the enzymatic interactions that are fundamental to protein digestion in the ruminal ecosystem (Roe *et al*., 1991; Luchini *et al*., 1996; Stern *et al*., 1997; Velásquez and Pichard, 2010b).

It is a permanent challenge to find new methods for predicting protein degradation in the rumen that not only provide laboratories with robust predictions but are easy to use, analytically satisfactory for a wide range of feed substrates and present high repeatability (Givens *et al*., 2000; Velásquez and Pichard, 2010a). To satisfy these conditions, an alternative method has been proposed that is based on enzymes extracted from the ruminal fluid (Mahadevan *et al*., 1987; Kohn and Allen, 1993 and 1995; Velásquez and Pichard, 2010a). Nevertheless, the absence of the biological processes involved in protein degradation in the rumen (induction and the microorganism-substrate interaction) would be a limitation of this methodology because the enzyme extracts are free of microorganisms. It has been observed that certain specific adherence and colonization mechanisms are fundamental to the induction of enzyme secretion and activity in microorganisms. Moreover, techniques for enzyme extraction and purification from the ruminal fluid are not always optimal; in some cases, the enzymes are not very representative or occur at low concentrations. There are also problems with maintaining proteolytic activity during storage and with the persistence of hydrolysis during assays. Velásquez and Pichard (2010b) evaluated the proteolytic activity of mixtures of enzyme extracts generated from ruminal microorganisms cultured *in vitro*, and compared them with the method using proteases from *Streptomyces griseus* and the *in situ* technique. The ruminal fluid from which the enzyme extracts were generated was pre-incubated *in vitro* with different substrates to generate a higher enzyme concentration and promote a broad spectrum of hydrolytic activity (endo- and exo-proteases, cellulases, pentosanases, pectinases and amylases). The enzyme extracts were evaluated by incubating 100 mg each of crude protein (CP) from soybean meal, canola meal, sunflower meal, gluten feed, alfalfa meal, berseem clover, perennial ryegrass and oat forage (*Avena sativa)* in 30 ml of Tris-HCl 50 mM (pH 6.5) buffer at 39 ºC for 48 h. The ruminal enzymes presented an average degradation of 75.5% of the CP across the eight feeds, and this value was similar to that measured with proteases of *Streptomyces griseus* (74.6% CP) but significantly lower ($P<0.05$) than with the *in situ* method (84.8% CP). Degradation of soybean meal with ruminal enzymes was 85.6% with a degradation rate (*kd*) of 6.6 %/h, but with the *in situ* method, the values were 93.2% and 7.2 %/h, respectively. In both cases, the differences were significant ($P<0.05$). For alfalfa meal, the observed degradation was 77.6% with enzyme extracts and 84% with the *in situ* method, and the *kd* values for these measurements were 8 and 9.5 %/h, respectively. These methods differed significantly ($P<0.05$) for the previously mentioned kinetic parameters, and the results were generally similar for the other feeds evaluated with great variation in the values of the extent and rate of proteolytic degradation for the same feed when determined with different methods. Table 1 shows measurements of degradation and kinetic parameters in studies of ruminal protein degradability using different methods for the same feed (according to different authors). While errors due the experimental design or the preparation and origin of the feed samples will always arise, it might be expected that the results would be comparable. However, the values differ widely, for example, in soybean meal, perennial ryegrass, canola meal and alfalfa meal. These observed variations between different methodologies present an uncertainty when attempting to predict the real degradability of the proteins from a feed in the rumen. The ideal situation would be for very similar results to be obtained from the different methodological strategies with the same feed so that the inaccuracies of the predictions would be negligible. In reviewing the coefficients of variation (CV) of the data on CP degradation for the same food among the methodologies presented in Table 1, it was generally observed that the *in situ* and enzymatic extracts from the ruminal fluid (ERF) techniques had the lowest values, indicating less heterogeneity between independent measurements using the same food. For example, the CV values for the methods *in situ*-soybean meal, *in situ*-alfalfa meal and *in situ*-canola meal

were 15.79; 18.01 and 9.32%, respectively. In the cases of ERF-soybean meal, ERF-alfalfa meal and ERF-canola meal, these values were 48.52; 33.56 and 10.19%, respectively. In contrast to the *in vitro* methodologies (ruminal fluid)-soybean meal and *in vitro*-alfalfa meal, the CV values were 63.04 and 49.87%, respectively; while for the protease (*Streptomyces griseus*) technique, the CV for soybean meal it was 94.52%. These values suggest that *in situ* and ERF techniques would present greater accuracy and reproducibility in their implementation given the greater homogeneity between the revised observations.

Methods based on *infrared spectroscopy*, notably NIRS (near infrared reflectance spectroscopy) and FTIR (Fourier-transform infrared spectroscopy) have greatly advanced the analysis of the chemical composition of feeds in recent years, particularly the prediction of protein degradability in the rumen. Although these methods are robust and can be applied to a wide variety of feeds, some limitations have been observed. This type of analysis provides chemical-structural information about the feed, allowing the chemical composition of and the kinetic processes that degrade ruminal food to be inferred (Andrés *et al*., 2005; Ohlsson *et al*., 2007). The FTIR technique has recently been developed; it is more sensitive than traditional methods, and data can be obtained more quickly (Belanche *et al*., 2013). These authors, in research on the "*Estimation of feed crude protein concentration and rumen degradability by Fourier-transform infrared spectroscopy*", which used PLS (partial least squares regression) models with SIMPLS algorithms, concluded that the use of this method is promising for the estimation of the concentration of CP and its degradation in the rumen. It was also found that the FTIR method was particularly accurate in predicting the CP concentration in forage and its degradation kinetics in the rumen. However, when the models were used to evaluate concentrates, the results were less robust, and ruminal CP degradation could only acceptably be estimated in protein-rich concentrates. Another interesting conclusion of this study was that the majority of FTIR-based predictions show a similar level of accuracy as the NIRS method, suggesting that FTIR should be considered as a low-cost alternative method for the nutritional evaluation of feeds. Although NIRS has demonstrated some capability for directly predicting the degradability of forage components *in situ* (Nordheim *et al*., 2007; Ohlsson *et al*., 2007), approximately 12,000 dacron bags would need to be processed for calibration purposes (Hoffman *et al*., 1999). Given this complexity, NIRS is proposed as a reliable technique for predicting the composition of incubation residues for which calibration requires fewer observations (Reeves *et al.,* 1991; Berzaghi *et al*., 1997; Andrés *et al*., 2005).

In general, this review allows us to conclude that there is still no single method that complies with all of the requirements of representativeness, precision and accuracy. The ideal would be to establish a method that is sufficiently safe, robust, practical and flexible and that offers universal validity for application in any laboratory and to any feed type and can be adapted to the context of the production system used for the ruminants. Such a methodology would allow for feeding plans to be established that fit the needs of the animals as closely as possible. Table 2 shows a summary of the methods discussed above, together with their advantages and disadvantages and some suggestions for improvement.

Table 1. Measurements of degradation and kinetic parameters in studies on ruminal protein degradability with different methods according to feed and authors.

Method	Substrate	Incubation time (h)	Degradation (% CP)	Kd^{10} (%/h)	Reference
In situ	Soybean meal	24	66.50		Assoumani *et al*. (1992)
In situ	Soybean meal	24	75.80		Calsamiglia *et al*. (1992)
In situ	Soybean meal	48	99.30	9.4	NRC (2001)
In situ	Soybean meal	48	93.17	7.2	Velásquez and Pichard (2010b)
In situ	Soybean meal	48	98.20	12.9	Habib *et al*. (2013)
In situ	Soybean meal	48	90.20		Tuncer and Sacakli (2003)
In situ	Soybean meal	48	98.20	12.9	Habib *et al*. (2013)
In situ	Soybean meal	48	62.00	8.4	Akbarian *et al*. (2014)
In situ	Roasted soybean	48	50.70	6.1	Akbarian *et al*. (2014)
In situ	Roasted soybean	48	50.70	6.1	Akbarian *et al*. (2014)
In situ (ERD)[1]	Soybean meal	48	54.60		Tuncer and Sacakli (2003)
In situ (ERD)[1]	Soybean meal	48	75.00	12.9	Habib *et al.* (2013)
*In situ (*ERD)[1]	Soybean meal	48	75.00	12.9	Habib *et al.* (2013)
In situ (FTIR)[2]	Soybean meal	96	61.00	4.4	Belanche *et al*. (2013)
In vitro (RFI)[3]	Soybean meal	4	48.00		Broderick *et al.* (2004)
In vitro (RFI)[3]	Soybean meal	4	18.40		Broderick (1987)
Enzymatic (ERF)[4]	Soybean meal	16	47.30		Kohn and Allen (1995)
Enzymatic (ERF)[4]	Soybean meal	6	37.90		Kohn and Allen (1995)
Enzymatic (ERF)[4]	Soybean meal	6	25.50		Mahadevan *et al.* (1987)
Enzymatic (ERF)[4]	Soybean meal	48	83.00	5.9	Velásquez and Pichard (2010a)
Enzymatic (ERF)[4]	Soybean meal	48	85.58	6.6	Velásquez and Pichard (2010b)
Proteases Sg[5]	Soybean meal	48	84.52	5.9	Velásquez and Pichard (2010b)
Proteases Sg[5]	Soybean meal	6	16.80		Mahadevan *et al.* (1987)
CFP[6]	Soybean meal		94.46		Velásquez and Pichard (2010b)
CFP[6]	Soybean meal		68.30		Akbarian *et al.* (2014)
CFP[6]	Roasted soybean		92.20		Akbarian *et al.* (2014)
In situ	Alfalfa meal	48	90.60	10.8	NRC (2001)
In situ	Alfalfa meal	48	83.95	9.5	Velásquez and Pichard (2010b)
In situ	Alfalfa meal	48	67.35	9.0	Cobellis *et al*. (2015)
In situ	Alfalfa meal	48	57.37	26.0	Cobellis *et al*. (2015)
In situ	Alfalfa meal	48	70.27	14.0	Cobellis *et al*. (2015)
In situ (ERD)[7]	Alfalfa meal	48	64.24		Cobellis *et al*. (2015)
In situ (ERD)[7]	Alfalfa meal	48	56.80		Cobellis *et al*. (2015)
In situ (ERD)[7]	Alfalfa meal	48	65.35		Cobellis *et al.* (2015)
In vitro (RFI)[3]	Alfalfa meal	4	21.20		Broderick *et al.* (2004)
In vitro (RFI)[3]	Alfalfa meal	6	44.30		Peltekova and broderick (1996)
Enzymatic (ERF)[4]	Alfalfa meal	16	41.70		Kohn and Allen (1995)
Enzymatic (ERF)[4]	Alfalfa meal	6	38.20		Kohn and Allen (1995)
Enzymatic (ERF)[4]	Alfalfa meal	48	55.30	5.9	Velásquez and Pichard (2010a)
Enzymatic (ERF)[4]	Alfalfa meal	48	77.58	8.0	Velásquez and Pichard (2010b)
Proteases Sg[5]	Alfalfa meal	48	76.45	7.2	Velásquez and Pichard (2010b)
Proteases Sg[5]	Alfalfa meal	48	75.90		Mathis *et al.* (2001)
CFP[6]	Alfalfa meal		87.64		Velásquez and Pichard (2010b)

Continuation Table 1

In vitro (RFI)[3]	BSA	4	54.00		Broderick (1987)
Enzymatic (ERF)[4]	BSA	6	73.60		Velásquez and Pichard (2010a)
Enzymatic (ERF)[4]	BSA	12	78.50		Velásquez and Pichard (2010a)
In situ	Canola meal	48	99.00	4.4	NRC (2001)
In situ	Canola meal	48	89.08	7.0	Velásquez and Pichard (2010b)
In situ	Canola meal	48	78.70	17.0	Xin and Yu (2014)
In situ	Canola meal	48	90.30		Tuncer and Sacakli (2003)
In situ (ERD)[1]	Canola meal	48	77.20		Tuncer and Sacakli (2003)
Enzymatic (ERF)[4]	Canola meal	48	78.56	6.4	Velásquez and Pichard (2010b)
Enzymatic (ERF)[4]	Canola meal	48	68.00	5.5	Velásquez and Pichard (2010a)
Proteases Sg[5]	Canola meal	48	76.09	5.8	Velásquez and Pichard (2010b)
CFP[6]	Canola meal		93.52		Velásquez and Pichard (2010b)
In situ	Perennial Ryegrass	48	75.38	11.0	Velásquez and Pichard (2010b)
Enzymatic (ERF)[4]	Perennial Ryegrass	48	46.80	6.4	Velásquez and Pichard (2010a)
Enzymatic (ERF)[4]	Perennial Ryegrass	48	68.72	10.1	Velásquez and Pichard (2010b)
Proteases Sg[5]	Perennial Ryegrass	48	67.46	7.7	Velásquez and Pichard (2010b)
CFP[6]	Perennial Ryegrass	-	81.30		Velásquez and Pichard (2010b)
In situ	ASTFR[8]	72	94.00		Geron *et al*. (2007)
CFP[6]	ASTFR[8]		99.50		Geron *et al.* (2007)
In situ	FSTFR[9]	72	96.00		Geron *et al.* (2007)
CFP[6]	FSTFR[9]		99.70		Geron *et al*. (2007)
In situ	Sunflower meal	48	91.70	24.7	Habib *et al.* (2013)
In situ (ERD)[1]	Sunflower meal	48	82.60	24.7	Habib *et al.* (2013)
In situ	Corn gluten meal	48	98.00	6.0	Habib *et al.* (2013)
In situ (ERD)[1]	Corn gluten meal	48	71.20	6.0	Habib *et al.* (2013)
In situ	Wheat bran	48	88.20	28.1	Habib *et al.* (2013)
In situ (ERD)[1]	Wheat bran	48	82.30	28.1	Habib *et al.* (2013)
In situ	Fish meal	48	81.00	5.4	Habib *et al.* (2013)
In situ (ERD)[1]	Fish meal	48	59.60	5.4	Habib *et al.* (2013)

[1]ERD, Efective rumen degradability: ruminal passage rate=0.05/h.
[2]FTIR, Fourier-Transform Infrared Spectrometers: calibration.
[3]RFI, Ruminal Fluid Inhibitor *in vitro* method – IIV.
[4]ERF, Enzymatic extracts from ruminal fluid.
[5]Proteases Sg, proteases from *Streptomyces griseus.*
[6]CFP, Chemical fractionation of proteins.
[7]ERD, Efective rumen degradability: ruminal passage rate= 0.02 /h.
[8]ASTFR, Acid silage of tilapia filleting residue.
[9]FSTFR, Fermented silage of tilapia filleting residue.
[10]*Kd*, degradation rate.

Table 2. Methods for predicting ruminal protein degradability and their principal advantages and limitations.

Method	Advantages	Limitations	References
Chemical protein fractioning	-High repeatability. -Standardized method with a high degree of control over empirical conditions. -No restrictions on the number and type of feeds to be evaluated.	-No biological representativeness. -Variability in the results of analyses of the same feed. -Probable skews in the capacity for peptide precipitation by TCA. -Difficulty in calculating hydrolysis rates (*kd*).	Sniffen *et al*. (1992); Licitra *et al*. (1996).
In vivo: -Analysis of feces. -Ruminal and duodenal fistula	-High biological representativeness of the ruminal ecosystem. -Allows for more real evaluation by studying forage under grazing conditions.	- Need for ruminal and duodenal fistulation. -Microbial contamination in the feces and endogenous N in the undigested residues. -Laborious protocols. -Control of consumption of the feed sample to be evaluated. -Variability in N measurement by microbial markers.	Lu *et al*. (1988); Satter (1986); Seymour *et al.* (1992).
In situ	- Directly representative of the ruminal environment. - Simultaneous evaluation of different feed samples under *in sacco* conditions. -No restrictions on the type of feeds to be evaluated.	- Particle loss from the bags. - Microbial contamination of the residue *in sacco.* - Rarefication of the environment *in sacco.* - Lack of a balanced diet to maintain a stable environment in the rumen. -Variability in N measurements with microbial markers.	Mehrez and Ørskov (1977); Ørskov (1992); Pawelek *et al*. (2008).
In vitro with ruminal fluid (with and without inhibition)	-Easy to operate and obtain results. -Uses live ruminal microorganisms under simulated ruminal conditions. -Allows for a large number of samples to be evaluated simultaneously; no restrictions on the type of feed to be evaluated.	-Difficulty of separating microbial protein from that present in the feed. -Variability in the extent and rate of protein degradation of a feed between incubations with ruminal fluid from the same donor animal. -Measuring degraded protein by detection of only NH_3 and total amino acids (TAA) released could underestimate protein degradation. -Inhibiting microorganism growth prevents alterations in the microbial biodiversity in response to the type of feed digested. -The useful evaluation time is very short (8-10 hours) because the incubation medium becomes rarefied, and the inhibitory properties of *chloramphenicol* and *hydrazine sulfate* fail.	Broderick (1987); Luchini *et al.* (1996); Broderick *et al*. (2004).
In vitro (commercial enzymes)	- Easy empirical application. - No animals needed for empirical use. - No limitations on the number of samples and type of feed.	- Limited hydrolytic enzyme spectrum. - Origin of enzymes is not representative of the ruminal ecosystem. - Lack of biological actions of the organisms due to the absence of the colonization, adherence and digestion process.	Pichard and Van Soest (1977); Mahadevan *et al*. (1987); Assoumani *et al*. (1992).

Continuation Table 2

In vitro (ruminal enzymes)	- Practical method; easy to use with good repeatability. - No animals needed for empirical use. - Good biological representativeness; the enzymes come from ruminal microorganisms. - No limitations on the number of samples and the type of feed.	- No biological processes associated with the substrate-microorganism interaction. - Insufficient variability and concentration of hydrolytic enzymes (proteolytic-carbohydrolytic). - Deficient extraction and purification of enzymes from the ruminal fluid. - Loss of enzyme activity and hydrolytic persistence during storage.	Mahadevan *et al*. (1987); Kohn and Allen (1993 and 1995); Velásquez and Pichard (2010a and 2010b).
NIRS; FTIR	- Practical method with high repeatability of results. - No animals or other biological material (enzymes, microorganisms) needed for application. - No restriction on the number of samples evaluated. - Can be applied to any type of feed.	- Large number of samples needed for calibration. - Low protein content in the sample may limit the effectiveness of the technique.	Andrés *et al*. (2005); Nordheim *et al*. (2007); Ohlsson *et al*. (2007); Belanche *et al*. (2013).

Resumen

A. Velásquez, J. Rivero, y P.G. Marnet. 2016. Atributos y limitaciones empíricas de metodologías para predecir la degradabilidad ruminal de las proteínas. Cien. Inv. Agr. 43(2):171-189. El objetivo de esta revisión es analizar las metodologías comúnmente usadas para estimar la degradabilidad de las proteínas en el rumen, resaltando sus atributos y limitaciones en orden a ofrecer algunas sugerencias para mejorar su utilización. Esta información es esencial para seleccionar los tipos de alimentos cuando se formulan las dietas alimenticias. Una confiable predicción de la digestibilidad de las proteínas en el rumen constituye una información básica para optimizar el uso de los recursos nitrogenados, pudiendo por un lado traducirse en una mayor producción de leche, proteína láctea, carne o lana, y por otro, en una menor excreción de compuestos nitrogenados al ambiente; esta mayor eficiencia también tiene un impacto sobre la salud y el bienestar animal. Tradicionalmente, la digestibilidad ruminal de las proteínas de los alimentos ha sido predicha a través de métodos *in vivo*, *in situ* e *in vitro*. Sin embargo, otras técnicas han sido desarrolladas, basadas sobre espectroscopía infraroja, destacando los métodos NIRS y FTIR. Todas estas técnicas presentan limitaciones, factores perturbadores o fuentes de error, las cuales podrían generar predicciones sesgadas e inseguras. Los métodos *in situ* e *in vitro*, utilizando extractos enzimáticos de origen ruminal, y FTIR, probablemente poseen las mayores ventajas, no obstante, estas técnicas requieren ser perfeccionadas a través de futuras investigaciones.

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