PHYSICAL, CHEMICAL AND KINETIC FACTORS ASSOCIATED WITH FIBER DIGESTIBILITY IN RUMINANTS AND MODELS DESCRIBING THESE RELATIONSHIPS

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PHYSICAL, CHEMICAL AND KINETIC FACTORS ASSOCIATED WITH FIBER DIGESTIBILITY IN RUMINANTS AND MODELS DESCRIBING THESE RELATIONSHIPS

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Various trials were conducted to improve the neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin (ADL) methodologies. Enhanced methods increased recovery of the fractions on average by 3%, 11% and 24% for NDF, ADF and ADL, respectively, among forage groups. The improved methods were also applied to both in-vitro and in-situ NDF digestibility methodologies. Since recoveries were higher for NDF residues at longer fermentation times (240 h), the estimation of the indigestible NDF was significantly impacted and recoveries increased from 0-75% among all forages and samples. To better define the indigestible fraction of NDF (iNDF), other approaches for in-vitro and in-situ trials were conducted to verify the in vitro studies. Results from all approaches demonstrated that the iNDF is a unique value for each sample and is not directly related to the chemical composition of the plant. This allowed for a better definition of the relationship between lignin and iNDF. A single value of 2.4 for the ratio iNDF/ADL, on NDF basis, was determined to be invalid for all forages and the ratio was determined to be more variable (between 1.73 and 7.59) and dependent not only on the amount of ADL, but also on the physiological stage at harvest of the plant and most likely the agronomic conditions the plant was grown in. Further, a study to evaluate the chemical linkages

associated with altered digestibility found that the quantification of ester- and ether-linked ferulic and *para*-coumaric acids, with the lignin explained most of the variation (up to 98%) of in-vitro NDF digestibility, rate and extent of NDF digestion, for all groups of forages, except for alfalfa. An experiment was also conducted in lactating dairy cows and confirmed the observations of ester and ether linkages and digestibility from the in vitro studies. The chemistry of the ADF fraction and not the NDF component in this case showed the most interesting and consistently negative correlations with the digestibility parameters. Finally, a mathematical model of NDF digestibility was developed by the estimation of fast and slow digesting pools of NDF, utilizing the improved data on iNDF. The model was developed using a visual modeling tool (Vensim) and a non-linear optimization. This was possible using a composite decay model and no more than two points from a NDF fermentation curve and a forage group-specific range for iNDF. This will allow a better description of the forages used by nutritionists and clarify the understanding of the variation observed in ruminant animal performance as it relates to the primary ingredient in their diet.

BIOGRAPHICAL SKETCH

Emiliano Raffrenato is from Sicily, Italy. He grew up in a family of five. He has always loved everything related to living his life in the larger world and traveling to experience people and places. Loving animals and farming was a natural consequence. He pursued a degree in Tropical and Subtropical Agriculture in Sicily in 1998, focusing on pastures and their effect on quality of dairy products and farm sustainability. He started to work as part of the Data Analysis Center of Corfilac (a Sicilian dairy research center). In 2000 he was offered the opportunity to study at Cornell University where in 2003 he received a M.S. degree in Animal Breeding, studying genotype by environment interaction in dairy cows, under diverse environmental opportunity. After graduation, he returned to Sicily to work at Corfilac where this time he had the opportunity to experience Sicilian dairy farms of various input systems and to learn more about nutrition, through which he was exposed to nutritional challenges on dairy farms. In 2005 he returned to Cornell University to pursue a Ph.D. in Animal Science where he was able to focus on his real interests, dairy cow nutrition, forage quality and modeling. During the last few years he has become interested in diversifying his research, within the scope of animal sciences. He plans to remain in the academic world.

A chi mi ha amato e mi ama veramente.

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LIST OF ABBREVIATIONS

AD	Acid detergent
ADF	Acid detergent fiber
ADL	Acid detergent lignin
aNDF	Amylase neutral detergent fiber
AOAC	Association of Official Analytical Chemist
BMR	Brown midrib corn silage
BW	Body weight
CHD	Conventional high digestibility
CLD	Conventional low digestibility
CA	Coumaric acid
CNCPS	Cornell net carbohydrate and protein system
CP	Crude protein
CS	Corn silage
D	Days
DM	Dry matter
DMI	Dry matter intake
EST	Ester linked
ETH	Ether linked
FA	Ferulic acid
H	Hours
iNDF	Indegistible neutral detergent fiber
$iNDF_{2.4}$	Indegistible neutral detergent fiber obtained using (ADL×2.4)/NDF

$iNDF_{96}$	Indegistible neutral detergent fiber obtained using 96 h of fermentation
$iNDF_{240}$	Indegistible neutral detergent fiber obtained using 240 h of fermentation
$iNDF_D$	Indegistible neutral detergent fiber obtained using the Daisy apparatus
HPLC	High performance liquid chromatography
K_d	Rate of NDF digestion (1/h)
KL	Klason lignin
L	Lag
MSPE	Mean square prediction error
ND	Neutral detergent
NDF	Neutral detergent fiber
NDFD	Neutral detergent fiber digestibility
NDR	Neutral detergent residue
NSC	Non-structural carbohydrate
OM	Organic matter
pCA	*Para*-coumaric acid
pdNDF	Potentially digestible neutral detergent fiber
RMS	Residual mean square
RMSE	Root mean square error
TMR	Total mixed ration
TTD	Total tract digestibility

CHAPTER ONE: LITERATURE REVIEW

Neutral detergent fiber: concentration and digestibility in forages

Forages are used extensively in the animal industry around the world. If a greater percentage of the total potential energy stored in forages was available to the ruminant, it would have a tremendous economic impact and increase profitability through reduced off farm feed purchases. Further, increased forage usage would have positive effects on the environment by allowing for more farm raised feeds to be used, and to allow for greater manure nutrients to be spread on the land used to grow the forage. In many places around the world, there is a growing problem of groundwater quality and maintenance of productive farm land that will require greater emphasis on the effective utilization of forages. Economic productivity to the individual farm also must be improved by using the biomass accumulated in these crops, particularly in farming systems (such as dairy operations) where high production demands high energy inputs.

Animal performance is the product of dry matter intake, nutrient digestibility, and metabolism. Of the variation in digestible dry matter or digestible energy intake among animals and feeds, 60 to 90% is related to differences in intake, whereas 10 to 40% is related to differences in digestibility (Mertens, 1994). Furthermore, digestibility and voluntary intake are correlated and vary according to fermentation time. The maximum correlation with digestibility of temperate forages occurs at about 36 h ($r = 0.83$), perhaps reflecting mean in vivo retention (Van Soest, 1994).

Digestibility of forages is more variable than metabolizability, and feces typically are the greatest loss of ingested nutrients and energy. Extensive research has been devoted to measuring digestibility and relating it to feed characteristics because digestibility can be accurately measured with relative ease compared to dry matter intake, depending on the housing conditions of the animals. Although intake is more important than digestibility in assessing forage quality, progress in understanding the basic factors that affect intake has been difficult because of our inability to measure intake accurately and to separate the effects of animal and diet on intake. In addition to the direct effects on energy intake, digestibility also influences nutrient supply indirectly due to close association between digestibility and intake in ruminants fed forage-based diet.

Although dietary concentration of NDF is related positively to bulk density of feeds and affects feed intake potential (Karkalas, 1985) forage NDF greatly varies in its digestibility in the rumen (Nocek and Russell, 1988) and in vitro (Mertens, 1973). Digestibility of NDF influences animal performance independent of dietary NDF concentration. In animal trials where forages of different in vitro digestibility were fed, at similar dietary NDF concentrations, significant increases in DMI and milk production were reported (Grant et al., 1995; Dado and Allen, 1996; Oba and Allen, 2000). A faster disappearance of the NDF fraction from the rumen because of increased rate of digestion or passage might reduce physical fill in the rumen and allow greater voluntary feed intake (Van Soest, 1994).

Digestion and passage in ruminants can be empirically and mechanistically described by models of varying complexity. Illius and Allen (1994) made a detailed comparison of the structure and assumptions of the

models, which differed principally in the fractioning of feed and in the description of the digestion and passage kinetics. However, accurate and precise predictions of the intrinsic digestion kinetic parameters are critical to the accurate prediction of NDF digestibility and intake. In order to be useful in rumen models, the kinetic parameters should only be limited by the attributes of substrates, i.e. intrinsic characteristics of cell walls. Physical and chemical attributes of the digestion environment should not be limiting factors in the determination of the potential rate and extent of NDF digestion.

Several reviews of digestion kinetics of cell wall carbohydrates (Mertens, 1993a; Mertens, 1993b; Ellis et al., 1994; Ellis et al., 1999) have addressed the problems associated with the estimation of kinetic parameters. The importance of the rate and extent of NDF digestion on OM and NDF digestibility can be demonstrated by simulation with the CNCPS model (Fox et al., 2004; Tylutki et al., 2008). Simulation results clearly demonstrate profound effects of these parameters on digestibility and therefore on the supply of energy and microbial protein.

The earliest attempts to describe the kinetics of digestion have been reviewed by Mertens (1993a, 1993b). The term "rate of digestion" appeared in the 1950s, but the assessments were mainly based on the visual interpretation of digestion curves. Waldo (1972) suggested that if the indigestible residue was subtracted, digestion of the potentially digestible cell walls might follow first order kinetics. From that observation, two primary methods were developed for fitting data to first order kinetic models: linear regression on logarithmic transformations of undigested residues (Ln-linear) and nonlinear estimation of parameters. The regression between the

logarithm of the potentially digestible NDF (pdNDF) against time results in supporting the hypothesis that pdNDF follows the first order digestion kinetics. Therefore, the estimation of the indigestible fraction (iNDF) is critical to the accurate description of digestion kinetics.

Bias can also be associated with early measurement of digestion. Usually, digestion of the pdNDF fraction does not appear to start instantaneously at time zero. Instead there is a lag period during which digestion occurs slowly or not at all (Mertens, 1993a, 1993b). As a result the use of these early fermentation points with the logarithmic transformation and linear regression may result in a biased estimate that is lower than the true rate. Problems associated with the logarithmic transformation-linear regression approach can be overcome by estimating kinetic parameters using non-linear least squares regression procedures (Mertens and Loften, 1980; Van Milgen et al., 1993; Ellis et al., 2005; Huhtanen et al., 2008a). Non-linear models assume an equal error at each fermentation time, whereas the Ln-linear models assume that error is proportional to the size of residue at each time point. Because random errors are typically the largest for early and medium (8-48 h) incubation times, neither of these approaches seems reasonable. Therefore the only apparent discrepancy with the Ln-linear method is during lag when fluxes and variation are low, but residue weights are high. Thus, it does not seem that the multiplicative error distribution associated with logarithmic transformation is a significant problem during parameter estimation.

Currently rates of digestion (k_d) are most commonly measured by exposing samples of a feed to ruminal fluid in-vitro or in-situ (nylon or dacron bags) for specified time intervals or by measuring the volume of gas

produced during the fermentation. Even if the gas production system provides frequent measurements that allow accurate parameter estimation, Firkins (1998) referred to several problems including a correction for changes in fermentation stoichiometry (VFA ratio) over time, evolution of gas from the buffer, errors caused by small sample sizes, an inability of the system to distinguish between different substrates, the contribution of ammonia to the gas pool and problems related to the blank correction. Furthermore, the accuracy of determination of rates of gas production could be any better than the method of verification (typically in vitro or in situ substrate depletion kinetics) (Firkins et al., 1998). Ultimately Van Soest (1994) suggests that measuring gas or VFAs may be less than accurate because these products are produced at the expense of microbial yields and efficiency. For this reason an end point measurement that does not confound substrate with products is preferred.

Rates of digestion for NDF sources are included in most ration analyzers and optimizing models (Fox et al., 2004; Danfær et al., 2006a; Tylutki et al., 2008). However, incorporation of digestion rates as a standard procedure to define the nutritive quality of specific feeds and diets has not been achieved, in part, because of lengthy laboratory analyses and statistical interpretation of fiber digestion rates. As previously mentioned both linear and nonlinear regression require multiple fermentation time points and therefore multiple NDF determinations on the residues of the samples and of the blanks resulting from the in vitro procedure (Goering and Van Soest, 1970). Measurements of digestion rates by commercial laboratories present problems because most laboratories will not provide multiple time point fermentation curves or the user cannot afford the cost of generating such

values. Van Amburgh et al. (2003) presented a mathematical approach for determining rates of NDF digestion. The approach needs to be mathematically compared to a reference procedure, such as a non-linear first order decay to ensure that variance inflation is not a factor in the procedure.

Acid detergent lignin and its measurement

Lignin has been defined in the literature since the 19th century (Klason, 1897). Lignin can be defined either chemically or from a functional role that lignin has within a plant. According to Sarkanen and Ludwig (1971), lignin is a heterogeneous polymeric material composed of phenylpropanoid units derived primarily from three cynnamil alcohols (monolignols): *p*-coumaryl, coniferyl, and sinapyl alcohols. From a functional perspective, lignins impart strength to cell walls, facilitate water transport, and impede the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects, and herbivores. Lignification, which is known to impact rigidity, alters the shear force necessary to fracture plant tissue and decreases digestibility, and is the biochemical process of forming the collective of phenylpropanoid macromolecules termed lignin (Sarkanen and Ludwig, 1971).

Several well-defined procedures to quantify lignin in plant tissues have been developed and approved by AOAC (Hatfield and Fukushima, 2005). The most commonly used method in ruminant nutrition and agronomy is the procedure developed by Goering and Van Soest (1970), in which an acid detergent extraction step is employed to produce acid detergent fiber (ADF).

The ADF is cellulose and lignin with small amounts of pectins and xylans potentially present depending on the sample. A subsequent and sequential step is the isolation of lignin from the ADF through the use of 72% sulfuric acid in hydrolyzing the remaining carbohydrates. Of all procedures, acid detergent lignin (ADL) results in the lowest values of lignin as reviewed by Hatfield and Fukushima (2005), and it can be from 2 to 5 times lower than Klason lignin values (Jung et al., 1999).

For the nutritionist, it has been important to know the lignin value of a feed and forage because it is generally accepted that lignin and lignin cross-linking to carbohydrates are the primary factors responsible for limiting the anaerobic digestion of forages (Besle et al., 1994; Van Soest, 1994). Furthermore, no anaerobic or mammalian enzyme systems are known to exist that degrade polymerized phenolic compounds (Van Soest, 1994), therefore true lignin is theoretically indigestible and should serve well as an internal indicator of digestibility. However, there are inconsistencies in the ability to quantitatively recover lignin (Fahey Jr and Jung, 1983; Cochran et al., 1988) with observed positive and negative recoveries (Fahey Jr and Jung, 1983; Van Soest, 1994). Reports of positive ADL recoveries are frequently attributed to the formation of "artifacts" of ADL during gastrointestinal transit, due to soluble, but apparently indigestible, lignin-carbohydrate complexes (LCC) entering the rumen that precipitate in the acidic environment of the abomasum and are subsequently recovered in the feces (Gaillard and Richards, 1975; Neilson and Richards, 1978). Alternatively, incomplete lignin recoveries have been attributed to either true or apparent digestion of lignin by formation of soluble LCC that pass from the rumen and the gastro-intestinal tract as polymers not recovered in the fibrous

residue of feces (Fahey Jr et al., 1979), or to partial destruction of the fecal lignin fraction by reagents used in the analytical method (Fahey Jr and Jung, 1983). Given the data from the last few decades indicating both degradation and solubilization of lignin in the digestive tract, use of ADL as a marker violates the criteria of an ideal marker (Faichney, 1975) and the use of it as a marker has been viewed with caution.

The original AOAC lignin procedure for crucibles (Van Soest and Wine, 1967) relied on the use of asbestos fiber as a filtering agent, but asbestos was rendered a health hazard in 1989 by the United States Environmental Protection Agency (EPA, 1989, Asbestos Ban and Phase Out Rule) and removed. The sintered glass filter within the crucible is prone to particle loss and plugging with fine particles. Since asbestos was removed, no filtering aids have been used and in the case of the ADL assay, no other filter aids have been used since few are as inert as asbestos and can interfere in the lignin recovery (Van Soest, 1973). Udén (2006) evaluated the use of filtration or centrifugation to study the recovery of NDF and ADF. In that study he tested the use of filter paper with porosity of 6 μm or centrifugation at 9000 × *g* for 5 minutes against crucibles with porosity 2 (40-100 μm) (Udén, 2006). In this evaluation, the recovery of ADF was on average 20 and 24% higher for centrifugation and paper filtration, respectively, for both feeds and feces than through the use of the traditional Gooch crucible (Udén, 2006).

In the paper by Udén (2006) ADL recovery was not evaluated, but it seems apparent that any change in recovery of ADF would potentially impact the recovery of ADL. Our hypothesis was that ADL is lost during both the preparation step (i.e. ADF), as shown by Udén (2006), and the 72%

sulfuric acid hydrolysis step. Furthermore this loss would vary among forage families, and type of sample, because the different physicochemical structure of cell walls would result in specific particle size reduction during the two step procedure.

Currently, glass microfiber filters are available that are manufactured from 100% borosilicate glass that is binder free and chemically inert and have pore sizes that extend into the sub-micron range resulting in retention of very fine particles. Furthermore they can be used at temperatures up to 500°C to facilitate ashing. These characteristics of the glass microfiber filters appear to make them an ideal replacement for the asbestos in the ADL assay and hold the potential of improving upon the use of asbestos. The precision of gravimetric analyses is dependent upon the ability to produce repeatable weights, accurate to at least 0.05 to 0.1% of the respective components being determined. In gravimetric fiber analysis, the residues from the various extractions can be very hygroscopic. The glass of the crucibles can adsorb up to 25 mg of water when cooled to room temperature. Even at 100°C, all absorbed water in materials is not removed. Since most residues or extracts to be gravimetrically assayed are usually 500 mg or less, absorbed water can result in substantial error. Hot weighing at 105 °C is preferred over the use of desiccators because the low ADL contents of some forage species (e.g. bmr corn silages), the variable hygroscopic characteristics of different forages and of the filters used might all contribute to a biased weight or variability that is unacceptable in the assay. The removal of asbestos as a filtering aid may have increased the loss of ADF and ADL, especially in low lignin and highly processed samples and this lack of filtering aid also altered the variation within the assay.

The importance of the indigestible portion of the neutral detergent fiber and the prediction of this fraction

Cell wall carbohydrates can be quantified by determination of neutral detergent fiber (NDF), which includes cellulose, hemicellulose and lignin as the major components (Mertens, 2002). A part of the forage cell wall, is unavailable to microbial digestion in ruminants even if the total tract residence time of fiber could be extended to an infinite time i.e. indigestible NDF (iNDF) (Huhtanen et al., 2006b). The digestibility of the remaining fiber, the potentially digestible NDF (pdNDF = NDF – iNDF), determines the digestibility of NDF. Forage digestibility is thus constrained by the extent and rate of digestion of pdNDF (Van Soest, 1994).

The iNDF fraction has been defined as the most important factor affecting the total diet organic matter digestibility (Nousiainen et al., 2004). Based on the Lucas principle, the iNDF is an ideal fraction since by definition it is digested at a predictable rate of zero.

According to Ellis et al. (Ellis et al., 1999) determination of iNDF should be included in all basic feedstuff analysis because it has a predictable digestibility; it can be used for the estimation of the pdNDF as NDF-iNDF and it has an important role in contributing to rumen digesta load. Furthermore, a close empirical relationship between silage iNDF and OM digestibility (Nousiainen et al., 2003) indicates that iNDF is a useful entity for the prediction of the nutritive value of forages. The importance of the iNDF estimation on OM and NDF digestibility, rumen NDF pool and microbial N flow is also demonstrated by the Nordic model of dairy cow

metabolism "Karoline". Simulation results clearly demonstrate profound effects of these parameters on OM digestibility and consequently on the supply of energy and microbial protein (Danfær et al., 2006a; Danfær et al., 2006b).

Analytically iNDF is estimated by fitting kinetic models that describe the disappearance of NDF over digestion time (Waldo et al., 1972; Robinson et al., 1986; Weimer et al., 1990). Alternatively, iNDF is analytically defined as the undigested NDF remaining after exposure to agents of digestion for a sufficient time, to approximate complete digestion of pdNDF. The estimation of the indigestible fraction is not a mathematical or modeling contrivance, but is a critical biological principle upon which the concept of digestion kinetics and rates are based (Mertens, 1994). Often digestion rates are, for example, calculated without subtracting the indigestible residue or by subtracting one that is determined at too short a fermentation time. Mertens (1977) using Ln-linear approach has shown the effect of the fermentation time chosen to represent the iNDF on digestion rate and if iNDF is over-estimated the rate will increase, and the opposite is true for an under-estimation of the rate. If our objective is to characterize cell wall in terms of its susceptibility to rumen degradation, then direct estimation is essential as size of the degradable fraction obtained by extrapolation varies with maximum time of incubation (Mertens, 1977). Subtraction of large iNDF (early time points) results in prediction of greater than true digestion rates. Conversely, subtraction of small or no iNDF results in less than true rates of digestion, because high value of residues at later fermentation times cause a counter-clockwise rotation of the semi-logarithmic regression line. Therefore any error in estimating indigestibility can bias the estimates of fractional rate

and lag time as they are sequentially estimated using Ln-linear regression (Mertens and Loften, 1980; Moore and Cherney, 1986). According to Mertens (1977), estimation of the iNDF using 96-h residue is effective in estimating rates of digestion (Mertens, 1977). However, observations of long in-situ (up to 40 days; (Robinson et al., 1986)) and in-vitro (up to 240 h) (Van Soest et al., 2005) fermentations has shown how digestion was, in most cases, not completed at 96 h. Furthermore, according to Robinson et al. (1986), fitting models to degradation curves with residuals up to 40 days but using times of incubation progressively reduced indicated that two common misestimates are underestimation of the degradable fraction size and overestimation of their rate constants. If this occurs, then description of NDF residuals in terms of fractions is of limited use as fraction sizes are not real or repeatable. This suggests that extended incubations are necessary in order to estimate iNDF. Assuming that any estimation based on long time fermentations and made at any time other than infinity is an overestimate of the true asymptotic indigestible residue, several attempts to predict iNDF from lignin concentration have been made (Mertens, 1973; Chandler et al., 1980; Conrad et al., 1984; Weiss et al., 1992; Traxler et al., 1998).

Huhtanen et al. (2006a), on the other hand, have suggested that the ultimate extent of NDF digestion may not be reached with in-vitro batch system and the in-situ system estimates may be biased (Huhtanen et al., 2006b) due to crucial drawbacks of the traditional nylon bag procedure as discussed by Nousiainen et al. (2004). Mertens (1993a, 1993b) has presented too several critical aspects of the in-situ method. However these aspects are probably more critical for the determination of the intrinsic rate of digestion than for the determination of the extent of digestion. The close relationship

between in-vivo digestibility and the potential extent of digestion (Nousiainen et al., 2003) suggests that using prolonged incubations and bags with a small pore size may allow the extent of NDF digestion (and iNDF) to be accurately measured. Nousiainen et al. (2004) determined iNDF by in situ incubations for 12 d using nylon bags of small pore size (6-17 μm). The range of 6-17 μm was determined as the best compromise to minimize particle inflow and outflow, but still allowing adequate microbial activities inside the bags to avoid prolonged NDF digestions (Huhtanen et al., 1998; Huhtanen et al., 2006a). To our knowledge only Traxler et al. (1998) and Van Soest et al. (2005) have attempted to determine iNDF with in-vitro fermentations using respectively flasks and filtration (144 hrs) or plastic bottles and centrifugation (240 hrs), respectively, in both cases with samples in direct contact with the rumen fluid and buffer (Goering and Van Soest, 1970). Furthermore Traxler et al. (1998) were the only ones using also the in-vitro apparatus Daisy^{II} (Ankom Technology Corp., Fairport, NY) to estimate iNDF. For this latter case, even if the Ankom bags are characterized by a porosity of 25 μm, the design of the filter bag used (F57) provides a 3-D filter matrix similar to the filtering effect of a glass crucible with porosity of 40 μm (http://www.ankom.com/00_products/- filterbagtech.shtml - Ankom Technology Corp., Fairport, NY).

Lignin is generally accepted as the primary entity responsible for limiting the digestion of forages (Besle et al., 1994; Van Soest, 1994). Chandler (1980) estimated the indigestible fraction as lignin times 2.4, after fermentation between 90 and 120 days in methane digesters. The Cornell Net Carbohydrate and Protein System uses the 2.4 value as ratio between ADL and NDF to estimate iNDF in forages (Fox et al., 2004; Tylutki et al., 2008).

Van Soest et al. (2005) have validated this concept using several forage species and obtaining a high R^2 (0.94) between observed and predicted iNDF. Recently, data from Huhtanen et al. (2006a) did not show a general applicable relationship between permanganate lignin and iNDF measured by 12 days in-situ fermentation, although the overall slope was 2.4. Even though Van Soest assessed a linear relationship between 72% sulfuric acid and permanganate lignin (Van Soest, 1994), that relationship does not hold in the values from Huhtanen et al. (2006a). This is also confirmed by Nousianen et al. (2004) who could not develop an acceptable prediction equation ($R^2 < 0.40$) for iNDF based on permanganate lignin content on different grass silage types. Both previous findings refer to colder climate grasses that might result in different relationship between lignin and iNDF due to environmental interactions, leading to values different than 2.4. However, Robinson et al. (1986) also confirmed that estimation of iNDF, using Klason lignin and long fermentations (up to 40 days), would underestimate the size of the undegradable fraction compared to that observed by Chandler et al. (1980). However, it is important to recognize that Chandler et al. (1980) were dealing with 72% sulfuric acid lignin, i.e. acid detergent lignin (ADL), and not Klason lignin as Robinson et al. (1986) stated. Robinson et al., (1986) also used bags with porosity of 41 μm that could have resulted in material inflow and outflow, biasing the estimates.

Although there are known relationships between lignin and NDF digestibility (Van Soest, 1994), according to Huhtanen et al. (2006) the attempts to predict iNDF have not been successful because of the relatively high proportional errors in lignin and iNDF analyses, as well as differences between forage types in lignin to iNDF ratio, which may also be prone to

climatic factors. Tests for nutritional uniformity indicated an average recovery of 86% for ADL and sintered glass filters with a 40 μm aperture might not achieve complete recovery of fine particles (Robertson, unpublished results; (Udén, 2006)). Furthermore, the original AOAC lignin procedure for crucibles (Van Soest and Wine, 1967) relied on the use of asbestos as a filtering agent, but the asbestos was rendered a health hazard in 1989 by the United States EPA (1989, Asbestos Ban and Phase Out Rule) and removed and another filtering agent was never instituted. The variation in the lignin assay is likely partially a function of the filtering step and recent observations have confirmed this (Raffrenato et al., 2010, submitted). Some of the previously mentioned works have tested different porosity of bags for in-situ fermentations to estimate iNDF, but none has tested a smaller porosity filter for long in-vitro fermentations or for ADL estimations. There is therefore the need to estimate analytically iNDF and to predict the undegradable fiber fraction using its relationship with ADL and NDF, through improved recoveries of both ADL and iNDF.

Cell wall chemistry and its effects on rate and extent of NDF digestion

Forages are the foundation of all ruminant diets and forage digestibility is of significant importance for dry matter intake, energy yield and economic viability. The interactions, both chemical and physical, among lignin and the carbohydrate moieties of plant cell walls impact the rate and extent of NDF digestion. Data generated in our laboratory indicates that corn silages can have nearly identical chemical composition (NDF and lignin) but vary 50%

in NDF digestibility (NDFD). Thus the content of lignin is not directly responsible for digestibility of corn silage but more likely the degree of cross-linking of phenolics with carbohydrate impacts digestibility more than content. Effects of specific linkages among lignin and cell wall carbohydrates was demonstrated by Grabber et al. (2009) where, using a biomimetic model, that ferulate cross-linking was more important than lignin content in evaluating hemicellulose rate and extent of digestion.

The lignins are the only components in cell walls resistant to bacterial and fungal degradation in the rumen and their association with other cell wall matrix components greatly influences properties of digestion, including the enzymatic degradation of structural polysaccharides. Reductions in degradability are partly related to the increased lignin content of cell walls; however variations in three-dimensional structure and composition of lignin and its hydrophobicity, encrustation, and cross-linking to other matrix components have also been implicated (Chesson, 1993; Jung and Deetz, 1993). Even when plant selection is targeted at specific lignin properties or lignin-matrix interactions, compensatory or associative changes in other cell wall characteristics often occur, making it difficult to identify underlying mechanisms controlling cell wall degradability (Grabber, 2005). Plants might, for example, respond to lower lignification by increasing the amount of cross-linking , yielding no net change in digestibility, thus lignin content as a marker of digestibility might be misleading to a nutritionist (Chabannes et al., 2001).

In grasses, hydroxycinnamic acids, namely p-coumaric and ferulic acids are ester and/or ether linked to cell wall polymers. As result of these coupling reactions, arabinoxylans become extensively cross-linked by ferulate

dimerization and by incorporation of ferulate monomers and dimers into lignin. The concentration of alkali-labile ferulates initially increases during primary wall formation and then peaks and declines during secondary wall formation and lignification (Scobbie et al., 1993; Morrison et al., 1998). However, recent data have shown that at least 50-70% of alkali-labile ferulate deposition occurs during secondary wall formation and lignification (MacAdam and Grabber, 2002). According to Grabber et al. (2000), these analyses might then underestimate ferulate and diferulate deposition in secondary walls because coupling of ferulate and diferulates to lignin prevents the recovery of most of these acids by the solvolytic methods used to degrade lignin. In grasses, ferulates are present as esters of arabinose units on xylans, and many of the ferulate molecules become involved in cross-links between arabinoxylans and by formation of diferulate bridges and/or as nucleation sites for the lignin deposition (Hatfield et al., 1999). Both diferulate cross-linking of lignin to arabinoxylans and cross-linking of lignin to arabinoxylan have been shown to reduce maize cell wall degradability (Grabber et al., 1998). As xylans became less substituted with arabinose units during development of the maize internode, the degree of substitution of the arabinose with ferulates increased dramatically from 0 to 45% (Jung and Casler, 2006).

There is evidence that the effects of these compounds on digestibility are dependent on the content and bonding mode in the cell wall structure; but results are not consistent. For example, if the majority of *p*-coumarate (*p*CA) is esterified to lignin and if *p*CA ethers are only linked to lignin (Lam et al., 1992a; Lam et al., 1992a; Lam et al., 1992b; Jung and Deetz, 1993; Lam et al., 2001) , it is probable that these components do not directly affect digestion.

Etherified ferulic acid, a measure of cross-linking between lignin and arabinoxylans, has a negative effect on cell wall digestibility (Casler and Jung, 1999; Lam et al., 2003). However for esterified ferulic acid the results are not consistent (Jung and Casler, 1990; Jung and Casler, 1991) . Casler and Jung (2006) reported negative effects of esterified ferulic acid on in vitro 24 h NDFD of smooth bromegrass and reed canarygrass, but the relationship changed to a positive relationship when digestibility was measured at 96 h. More recently Rodrigues et al. (2007) reported negative correlations between lignin and ester and ether *p*CA in meadow hay. These latter results were surprising since it is documented that most *p*CA is esterified to lignin and that the concentration of etherified *p*-coumarate increases with the maturation process of grasses (Morrison et al., 1998) , the same trend as lignin deposition, and that *p*-coumarate is incorporated to the lignin polymer through ether linkages during secondary cell wall development (Lam et al., 1992a). However, in the study of Morrison et al. (1998) lignin values were reported as permanganate lignin and the acid treatment might have solubilized some of the *p*-coumarate. Rodrigues et al. (2007) also reported a positive effect of etherified ferulate on cell wall digestibility and an ether linkage between ferulic acid and lignin cannot be broken in anaerobic conditions. Further, Jung and Vogel (1992) (Jung and Vogel, 1992) reported occasional negative relationships with NDFD for a series of observations within and among maturity stages and plant parts of switchgrass and big bluesteam. Furthermore Jung et al. (1998) reported negative correlations between etherified ferulic acid and cell wall polysaccharide degradability in young maize internodes, but not in mature plants. Their explanation was that during maturation of the cell wall, cross-linkages with lignin through

incorporation of ether ferulate will occur, but concentration of both etherified and esterified ferulic acids will decrease during plant growth while deposition of lignin and other polysaccharides increase. This dilution effect can mask the impact of ferulate cross-linking, measured by etherified ferulic acid concentrations, on digestibility.

Overall it seems that these acids exert an inhibitory effect on degradation kinetics rather than extent of digestion of cell wall. Casler and Jung (2006) recently showed how *in-vitro* digestibility of cool-season-grass leaf tissue appears to be regulated by different mechanisms at 24 and 96 h, with ferulate cross-linking affecting NDFD at 96 more than 24 h. To our knowledge, there is no work that compares conventional and bmr corn silages, changes with maturity in grasses, and alfalfas. Furthermore there is little work comparing results in-vitro and in-vivo. Further, *p*-coumaric and ferulic acids in ADF have never been estimated and we hypothesize that since ADF recovers cellulose and lignin, phenolic acids should mostly be limited to *p*-coumarate linked to lignin. However the acid detergent might solubilize some of the phenolics since it likely dissolves a great part of the lignin contained in the Klason lignin (Jung et al., 1999).

We are aware of the large genetic variability among cell types and within and especially among forage species and families because of the different speed of cell wall change and reproductive maturity. However there is the need to integrate recent findings relative to phenolic acids and nutritive value of forages. Also, the possible correlations among cell wall components might prevent any type of cause and effect to be determined from these analyses but might lead to a better prediction of fiber digestibility. The evaluation of the effect of ester and ether linked phenolic acids on *in-vitro* NDFD and

whether the presence of measurable ester and ether linkages impacted rates (k_d) as well as extents of NDFD and if these relationships were similar among forages are needed. Further, since many *in-vitro* observations of digestibility do not directly correspond with *in-vivo* data, the investigation of the relationship among different corn silage hybrids selected for digestibility and the correlation with ester and ether linkages and *in-vivo* digestibility will help clarify the differences among methods and chemistry.

Mathematical fractionation of neutral detergent fiber and its description in terms of rates and pools

Neutral detergent fiber (NDF) is the most common measure of fiber used for animal feed analysis, but it does not represent a unique or homogenous class of chemical components. Heterogeneity of the NDF fraction of a plant can be demonstrated by the Lucas test (Lucas, 1964; Van Soest, 1994). The purpose of the Lucas test is to identify ideal nutrition entities that have uniform digestibility over a wide range of feedstuff, by plotting the digestible nutrient concentration in DM against the nutrient concentration in DM. The slope of regression estimates the true digestibility and the intercept is an estimate of the metabolic and endogenous fecal matter. The neutral detergent divides the feed into a soluble fraction that is rapidly and almost completely available and a fiber fraction that is slowly and incompletely degraded by microbial enzymes (Huhtanen et al., 2006b). Furthermore, NDF is also characterized by the presence of a fraction that is unavailable to microbial digestion in ruminants (i.e., indigestible NDF=

iNDF) even if total tract residence time of fiber could be extended to infinite time (Allen and Mertens, 1988; Van Soest, 1994). Thus by definition, iNDF represents a uniform feed fraction with zero true digestibility according to the Lucas test (Lucas, 1964). The potentially digestible NDF (pdNDF) will then result from the difference NDF- iNDF.

Although dietary concentration of NDF is related positively to bulk density of feeds and affects feed intake potential (Karkalas, 1985) forage NDF greatly varies in its digestibility in the rumen (Allen, M.S. and D.R. Mertens,1998) and in vitro (Van Soest, 1994). Digestibility of NDF influences animal performance independent of dietary NDF concentration. Animal trials where forages of different in vitro digestibility but similar NDF concentration have been fed, reported significant increases in DMI and milk production (Grant et al., 1995; Dado and Allen, 1996; Oba and Allen, 2000). A faster disappearance of the NDF fraction from the rumen because of increased rate of digestion or passage might reduce physical fill in the rumen over time and allow greater voluntary feed intake (Mertens, 1994; Van Soest, 1994).

Accurate and precise predictions of the intrinsic digestion kinetic parameters are critical to the prediction of NDF rumen digestibility and intake. The importance of the fractional rate (k_d) and extent of NDF digestion on total tract OM and NDF digestibility can be demonstrated by simulation with the CNCPS model (Fox et al., 1992; Russell et al., 1992; Sniffen et al., 1992; Fox et al., 2004) or with the Nordic model of cow metabolism, "Karoline" (Danfær et al., 2006a; Danfær et al., 2006b). Simulation results clearly demonstrate profound effects of these parameters on digestibility and therefore on the supply of energy and microbial protein.

Digestion rates can be highly variable between feeds and even within the same species (Van Soest, 1994).

One of the main problems in describing digestion kinetics is that residues remaining at any digestion time are a mixture of undigested and indigestible matter (Mertens, 1993). Furthermore, Mertens has indicated (Mertens, 1973; Mertens, 1977; Mertens and Ely, 1979) that overall digestion is better predicted when assuming that the pdNDF fraction is the sum of two digestible fractions each of which are first order but with different rate constants. According to Van Milgen et al. (1991) the assumption of a single fractional digestion rate constant is also untenable because of the chemical and morphological diversity of forages fed to livestock. More recently Ellis et al. (2005) demonstrated an improved fit of two-pool pdNDF models that was conformed to expectations of a composite of lifetimes of two concurrently degrading sub-entities of pdNDF with different degradation rates. Also for in-vitro gas production and NDF digestion, Huhtanen et al. (2008b) has recently shown a marked improvement of the model when pdNDF was assumed to be comprised of rapidly and slowly degradable fractions.

Rates of digestion of NDF are an input in ration analyzers and models (Fox et al., 2004; Tylutki et al., 2008). However, incorporation of digestion rates as a standard procedure to define the nutritive quality of specific feeds and diets has been achieved only recently, in part, because of lengthy laboratory analyses and statistical interpretation of fiber digestion rates. The mathematical approach by Van Amburgh et al. (2003) described a method for determining rates of digestion for a single pool of pdNDF with one time point assuming first order behavior and a fixed iNDF pool. The indigestible

fraction was in that case estimated using the formula (ADL × 2.4)/NDF where the 2.4 was the factor obtained by Chandler et al. (1980).

Hypothesis

Several decades ago (Van Soest, 1963) the neutral detergent system drastically changed the way of looking at fiber definition for ruminants. Since then there have been many studies on this fraction and its effects on intake, digestion, passage, feeding behavior, energy absorption and rumen health. The large variation in results and conclusions from these studies implies that information is missing that would clarify differences related to the methodology, estimation of rate and extent of NDF digestion and NDF chemistry. Our hypothesis is that improved methodologies to describe fiber fractions, and digestibility including new mathematical applications can improve our understanding of NDF and NDF rate and extent of digestibility, especially for use in ration formulation systems and models. Through a better definition of the digestible and indigestible NDF fraction, we will be able to better explain the variation observed in feeding behavior and dry matter intake of NDF and especially to help explain how environmental and agronomic growing conditions of forages alter the nutritional effects of NDF independent of the standard chemical composition.

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CHAPTER TWO: A MATHEMATICAL APPROACH TO USE A SINGLE IN VITRO FERMENTATION TIME POINT TO ESTIMATE RATE OF DIGESTION OF NEUTRAL DETERGENT FIBER

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ABSTRACT

First order parameter estimation of NDF digestion kinetics can be prone to error associated with the in-vitro or in-situ procedures and also with the mathematical approach. It is possible that there are variance inflation errors associated with logarithmic transformation of undigested residues that might reduce the applicability of the approach for use in the field. To evaluate the associated errors between the non-linear (PROC NLIN) and the Ln-linear approach (Van Amburgh et al., 2003), values from in-vitro fermentations were used to obtain simultaneous estimations of rates of digestion, lag times, potentially digestible NDF and the indigestible NDF fractions. Objectives of the study were then to develop a system that allowed for the determination of rates using a minimum of time points and as few as a single time point and to compare the estimates to a non-linear regression. The single time point rate estimations presented the highest correlations when using 24 and 30 h fermentation values. The lowest means square prediction error corresponded to using the 24 h time point with a variable lag and using the last fermentation point (216 h) from in vitro data.

The use of the ratio 2.4 for estimating iNDF/ADL resulted in the highest MSPE, suggesting that the factor is not constant among forage families and possibly within forage type. This suggests further work is needed to determine how much variation exists in the iNDF and how that influences NDF digestion kinetics.

INTRODUCTION

The digestibility of forage is affected by many factors such as genetics, season, and processing. Explaining this variation is usually the job of the nutritionist with tools that are appropriate and cost-effective. Tools designed to help account for some of this variation are nutritional models such as the Cornell Net Carbohydrate and Protein System (CNCPS) (Fox et al., 2004; Tylutki et al., 2008) and the Cornell-Penn-Miner (CPM) Dairy program (Tedeschi et al., 2008). Inherent to the proper use of the CNCPS and CPM, the digestion rate for the carbohydrate and protein pools must be inputted and should be specific to the feeds that are being used.

Since the inception of the CNCPS rumen submodel there has been discordance in the use of Merten's NDF rate data (Mertens, 1973) combined with the use of the 2.4 × lignin ($iNDF_{2.4}$) to calculate the indigestible NDF pool (iNDF) (Chandler et al., 1980). Inherent to the calculation of the rate of digestion is the available pool of fermentable substrate, which Mertens (1973) predicted mathematically from 96 h in vitro data. Chandler's $iNDF_{2.4}$ which is based on 90 to 120 day fermentations in a methane digester came after Merten's calculations of the unavailable fiber pool ($iNDF_{96}$). However, the two approaches were utilized within the framework of the CNCPS

rumen submodel and potentially resulted in both over and under predictions of ruminal digestion for poor and high quality forages, respectively.

Further, an observation from previous work was that the rate of degradation of all the evaluated feedstuffs deviated from first order behavior and this was demonstrated by the observation of "retarding" rates of digestion with changes in the amount of indigestible NDF (iNDF) (Ellis et al., 2005). This has since been more fully understood and is actually due to the presence of two distinctly different pools of fermentable NDF with very different rates of degradation. Further the calculation of iNDF was also discussed and it was suggested that the value of 2.4 as a constant did not appear to be feasible. This has not been completely resolved, however, data generated in our laboratory on 240 h fermentations suggests the value is, on average, more robust and can be used to anchor the estimation of the digestible NDF pool. Subsequently, for the purpose of calculating the digestible NDF pool, the 2.4 × lignin value has been adopted for these derivations.

Digestion and passage in ruminants can be empirically and mechanistically described by models of varying complexity. Illius and Allen (1994) made a detailed comparison of the structure and assumptions of the models, which differed principally in the fractioning of feed and in the description of the digestion and passage kinetics. However, accurate and precise predictions of the intrinsic digestion kinetic parameters are critical to the accurate prediction of NDF digestibility and intake. In order to be useful in rumen models, the kinetic parameters should only be limited by the attributes of substrates, i.e. intrinsic characteristics of cell walls. Physical and chemical attributes of the digestion environment should not be limiting

factors in the determination of the potential rate and extent of NDF digestion.

Several reviews of digestion kinetics of cell wall carbohydrates (Mertens, 1993a; Mertens, 1993b; Ellis et al., 1994; Ellis et al., 1999) have addressed the problems associated with the estimation of kinetic parameters. The earliest attempts to describe the kinetics of digestion have been reviewed by Mertens (1993a, 1993b). The term "rate of digestion" appeared in the 1950s, but the assessments were mainly based on the visual interpretation of digestion curves. Waldo (1970) suggested that if the indigestible residue was subtracted, digestion of potentially digestible cell walls might follow first order kinetics. Two methods are used for fitting data to first order kinetic models: linear regression on logarithmic transformations of undigested residues (Ln-linear) and nonlinear estimation of parameters. The regression between the natural logarithm of the potentially digestible NDF (pdNDF) against time results in supporting the hypothesis that pdNDF follows the first order digestion kinetics. The estimation of the indigestible fraction (iNDF) results therefore critical to the accurate description of digestion kinetics.

Bias can also be associated with early measurement of digestion. Usually, digestion of the pdNDF fraction does not appear to start instantaneously at time zero. Instead there is a lag period during which digestion occurs slowly or not at all (Mertens, 1993a, 1993b). As a result the use of these early fermentation points with the logarithmic transformation and linear regression may result in a biased estimate that is lower than the true rate. Problems associated with the logarithmic transformation-linear regression can be overcome by estimating kinetic parameters using non-linear least

squares regression procedures (Mertens and Loften, 1980; Van Milgen et al., 1991; Ellis et al., 2005). Nonlinear models assume an equal error at each fermentation time, whereas the Ln-linear models assume that error is proportional to the size of residue at each time point. Because random errors are typically the largest for early and medium (8-48 h) incubation times, neither of these approaches seems reasonable. Therefore the only apparent discrepancy with the Ln-linear method is during lag when fluxes and variation are low, but residue weights are high. Thus, it does not seem that the multiplicative error distribution associated with logarithmic transformation is a significant problem during parameter estimation.

Incorporation of digestion rates as a standard procedure to define the nutritive quality of specific feeds and diets has not been achieved, in part, because of lengthy laboratory analyses and statistical interpretation of fiber digestion rates. As previously mentioned both linear and non-linear regression require multiple fermentation time points and therefore multiple NDF determinations on the residues of the samples and of the blanks resulting from the in vitro procedure (Goering and Van Soest, 1970). The result is the inability of commercial laboratories to provide rates of digestion due to the time, labor and cost associated with the traditional approaches.

The objective of this paper is to describe the mathematical approach for determining rates of digestion and the application of this approach to in vitro corn silage NDF digestion data. A further objective was to develop a system that allowed for the determination of rates with a minimum number of time points. A final objective was to compare nonlinear and Ln-linear regression using the same time point fermentations and to describe a mathematical approach for determining rates of digestion.

MATERIALS AND METHODS

Long time period fermentations, in-situ and in-vitro, were necessary in order to accurately estimate the iNDF and then the pdNDF rates of digestion using a non linear model. The focus of this work was to develop a corn silage database, thus conventional and bmr corn silages were used as the primary forages, along with a few samples of alfalfa, wheat straw and various grasses (Table 2.1). Forage samples were analyzed for fiber fractions using the procedure of Van Soest and Robertson (1980), Van Soest, (1973) and Van Soest et al. (1991). Sulfuric acid lignin was also used as the standard lignin procedure (Van Soest and Robertson, 1980). A small preliminary study indicated that fermentations in-situ for 16 days may not be enough to reach the maximum extent of digestion. However extended fermentations were also conducted using the ANKOM in-vitro apparatus (DAISY$^{II;}$ ANKOM Technology Corp., Fairport, NY), because of the ease of re-inoculating the samples. This latter system allowed a better estimation of the intrinsic iNDF of the forage after 16 d. Fermentations were then carried for 16 days using the DAISY system and bags of polyester polyethylene terephthalate with 15 µm porosity and 8.5% open area (ANKOM Technology Corp., Fairport, NY). Jars were re-inoculated with medium every 4 days. Fermentations in-vitro were carried out for 6, 12, 24, 30, 36, 48, 72, 96, 120, 144, 168, 192 and 216 h, in 125 ml Erlenmeyer flasks in a 39 °C water bath under constant CO_2 in Goering and Van Soest buffer (1970), and with renewed medium after 96 h. In-vitro flasks were inoculated with rumen fluid from the same cows used for the DAISY fermentations, fed hay and grain. Blank samples were run for all fermentations and used to correct for

any contamination at each fermentation time. All samples at the end of the fermentations were analyzed for NDF without the use of sodium sulfite and amylase Van Soest et al. (1991).

Values from the in-vitro fermentations were used to obtain simultaneous estimations of rates of digestion (kd_v), lag times (L_v), pdNDF ($pdNDF_v$) and iNDF ($iNDF_v$), through a non-linear first order decay model using PROC NLIN of SAS and the Marquardt algorithm. Initial values for the non-linear iterations were obtained using a linear transformation of the mentioned model (Mertens and Loften, 1980; Moore and Cherney, 1986). The model was:

$$(1) \qquad NDF_t = pdNDF_v \, e^{-kd\,(t-L)} + iNDF_v$$

where

NDF_t = concentration of residual NDF after t hours of fermentation when $t > L$ and NDF_t = pdNDF + iNDF when $t < L$;

pdNDF = concentration of potentially digestible NDF;

kd = fractional rate of pdNDF digestion;

L = discrete lag time;

iNDF = concentration of indigestible NDF.

A new mathematical approach was then developed and used to estimate kd for the samples. The first-order differential equation states that the decrease in available pdNDF (A) per time (t) is governed by a rate constant (k) times the available substrate A:

(2) $-dA / dt = kA$, or also $-dA / A = kdt$

Integration of (2) gives:

(3) $\ln A_0 - \ln A = k (t - L)$

where A_0 is initial pdNDF. A_0 is set to unity and values of A are calculated by subtraction of the indigestible fraction (iNDF) and divided by the initial pdNDF. The value of $\ln A_0$ becomes zero and falls out of the equation:

(4) $-\ln A = k (t - L)$.

Equation (4) converted to logarithms becomes:

(5) $\ln (-\ln A) = \ln k + \ln (t - L)$.

Table 2.1. Composition of forages used for long-term fermentations, mathematical development and evaluation.

Forages	NDF	ADF	Lignin	Ash
	---------- % of Dry Matter----------			
Wheat straw	81.1	55.9	10.61	3.6
Alfalfa 1	51.4	40.7	7.72	8.4
Alfalfa 2	31.3	24.3	5.31	7.8
Timothy 1	81.6	49.7	7.88	4.8
Timothy 2	63.7	37.1	4.62	5.3
	Corn silages			
1 (BMR)	48.7	26.7	1.94	3.1
2	43.4	24.1	2.72	4.0
3 (BMR)	39.4	21.4	1.50	3.1
4	40.3	22.1	2.71	3.3
5 (BMR)	49.6	28.5	3.49	2.8
6	46.2	26.4	2.67	3.8
7	41.4	23.6	2.51	2.8
8	47.8	28.3	2.89	4.1
9	50.1	29.6	3.99	3.7
10	39.8	23.8	2.51	3.5
11	46.9	28.8	3.79	5.5
12	43.5	26.5	3.55	5.3
13	46.6	27.7	3.08	3.0
14	52.3	30.7	3.73	2.6
15	48.0	27.6	3.06	3.0
16	46.2	26.1	2.77	3.6
17	51.5	30.2	3.86	2.9
18	45.6	26.8	2.92	4.0
19	39.9	22.6	2.61	3.0
20	38.7	22.3	2.61	3.1
21	47.3	28.2	2.90	2.8
22	47.3	28.2	2.90	2.8

Since lnA is a negative number –lnA becomes negative and its logarithm can be taken. The requisite equations for lag and rate k are then derived from (5). Lag time is then the amount that needs to be subtracted from fermentation time (t) to produce equality in equation (6). Equation (6) is derived by subtracting equation (5) by itself as the difference between two fermentation times. In this case the logarithm of the 6 h time and the value of available substrate $Ln(lnA_6)$ are subtracted from later times lnt_n and $Ln(-lnA_n)$. In doing so we obtain:

(6) $$\ln (t_n - L) - \ln (6 - L) = \ln (-\ln A_n) - \ln (-\ln A_6).$$

The 6 h value is used in the calculations since it is assumed to be in equilibrium with the kinetic rate and therefore after the discrete lag time. Equation (6) is then solved for L. Solving for L is possible through at least three different algebraic solutions, all giving the same numerical value. One of the solutions that also avoids negative numbers and is easiest to use is:

(7) $$L = [6 (\text{Ln } A_n / \text{Ln } A_6) - t_n] / [(\text{Ln } A_n / \text{Ln } A_6) - 1].$$

The rate k can then be estimated after rearrangement of equation (5):

(8) $$\ln k = \ln (-\ln A) - \ln (t - L).$$

Thus the rate, k_d, can be estimated from a single fermentation value.

Estimations of rates of digestion with this approach were then possible after calculating the pdNDF as NDF – iNDF. Estimates were then found subtracting the 72% sulfuric acid lignin x 2.4 from NDF, according to Chandler (1980) ($iNDF_{2.4}$), or using the residue after 216 h of in-vitro fermentations ($iNDF_{216}$) or using the residue of the DAISY fermentations

($iNDF_D$). This resulted in respectively $kd_{2.4}$, kd_{216} and kd_D. Estimations were therefore done either calculating a lag or using a fixed lag derived from the observed disappearance of NDF during in-vitro digestion. The calculation of the lag was accomplished using 6 and 24 h as the two time points needed, since a preliminary analysis showed the highest correlation with the true lag. As an evaluation of the approach to the minimum of time points, a discrete arbitrary lag of 3 h was used, as a proxy of the average lag observed in our laboratory. This was done to analyze the option of using only one time point to estimate the rate of NDF digestion. The rates of digestion obtained with the new approach were then compared for prediction with the rates obtained from the non-linear estimation. The single point rates of digestion were obtained using the 12, 24, 30 and 36 h time points. The rates are estimated by semi-logarithmic transformation and using the same iNDF as mentioned and were included for comparison. Because the non linear estimation used data up to 216 h, the log-linear transformation estimation was obtained using different ranges of time points. Prediction accuracy was tested and compared using correlations and the mean square prediction error (MSPE) analysis of Theil (1966) and Bibby and Toutenburg (1977). The non-linear estimation allowed us to obtain an approximate 95% confidence interval for the rates that was used as further comparison tool.

RESULTS AND DISCUSSION

Since non-linear regression methods assume equal error at each observation, simultaneously fit all parameters to the data, result in smallest

residual sums of squared deviations and no assumptions are required, they were chosen to be the standard methodology in this case. However, to increase the probability that a non-linear solution is the global minimum, several sets of initial estimates for parameters (for each sample) were derived from the simple linear transformation of the same model, as suggested by Mertens (1994). This results in reasonably confidence that the global solution was obtained for each set of data. Furthermore, our mathematical approach described here is derived from the linearization of the non-linear first order decay and it therefore seems reasonable the comparison to this procedure.

A sample calculation for L is given for the corn silage 1 (Table 2.2). The 6 h value for A is 0.905 and was used as the 6 h base and δ was calculated in Table 2.2 according to equation 7. The four values of L were averaged to give a mean value of 4.21 h and used in the 6 h time point to calculate a k_d. Values of time minus the respective lag and the residual value of A were substituted into equation 8 to calculate Ln k_d. The value of k_d is taken as the antilog.

Table 2.2. Time point fermentation data and calculations from corn silage 1 based on 6 to 36 h fermentations.

Time, h	S[a]	A[b]	δ	Lag, h	kd, %/h
6	0.895	0.884	---	(4.21)	6.91
12	0.594	0.551	4.82	4.43	7.87
24	0.322	0.250	11.17	4.23	7.02
30	0.260	0.182	13.77	4.12	6.59
36	0.215	0.132	16.38	4.05	6.34
			Means:	4.21	6.95

[a]Neutral detergent residues as a proportion of the initial NDF

[b]Unitized values of S calculated according to equation 3. $U_{2.4} = 0.956$

Mathematical equations and procedures are presented that allow direct calculation of lag time and rate of digestion (k_d). For rate determination, a single time point allows calculation of k_d. Lag requires two time points, although an average lag of 3 h can be used with a single point to calculate k_d. The time-point calculation of k_d represents the slope of the logarithmic disappearance curve at a particular time. These procedures have the advantage of requiring minimum data that may be available from commercial laboratories. Since the calculations are direct and use no statistical regression procedures, they are simpler to implement because many observations are not required. If a number of time point digestions are available, means and

standard deviations of the respective lag and rate values can be calculated and their uniformity examined.

The use of an average 3 h lag does not appear to introduce any great error. If a forage had a true lag less than 3 h use of the constant value will slightly enhance the k_d. If the true lag is longer than 3 h use of the constant lag results in a slightly lower value of k_d. Thus the variation of k_d caused by use of a constant lag is consistent with forage quality. During the course of our evaluations we found feeds that calculated to have negative lags. Obviously those values are not kinetically possible. Under those circumstances we first recommend checking your undigested ND values for errors. If the undigested residues are appropriate, then you must set the lag to zero. Based on the data we have generated to date, a negative lag implies that the sample digested faster in the first 6 h than at later times. From the single point 24 h k_d determination of the corn silages, varying the lag from 0 to 5 h results in deviation of ±12% relative to the use of the 3 h fixed lag.

The time-point calculation allows the calculation of k_d at a single time observation, except that lag calculation will require two-time point values. However, if an average lag value is used the single point calculation can be made. An evaluation of the overall time point calculations, and three different approaches are found in Tables 2.3 and 2.4. For the single point 24-h calculation and the two-point (12 and 24 h) calculation, a constant 3 h lag was used. The use of a 6 h digestion value allows for the calculation of lag, which is then used to calculate a 24 h k_d in the third column. All time points (6-36 h) are used to calculate the k_d values in the last column. The correlation between the use of the complete data (6 to 36 h) with the alternative calculations was $r = 0.97$ with a coefficient of variation of 7%.

The 24 h time is advantageous because it lies in the middle of the first pool and is a time that commercial laboratories will find convenient.

Some comparisons of k_d using this mathematical procedure to the values found in Mertens's thesis have been made, but are not shown here. Rate of digestion values of about 12% in Mertens thesis are about 10% using this new mathematical approach. At the lower ranges, k_d values from Mertens at 6% are about 3% in the determinations. Thus the proportional drop in rates estimation is much greater for low quality forages. The drop occurs because of the change in estimated $iNDF_{2.4}$. The value of iNDF affects the rate number mathematically, the lower the iNDF the lower the k_d estimates. Lower values of iNDF yield lower rates because the fermentable pool is increased: Mertens' values of iNDF are the residual near 96 h and contain considerable fermentable substrate from the second pool (Van Soest et al. 2002). The use of the $iNDF_{2.4}$ decreases the estimate of ultimate extent and is largely responsible for the lower determined values. Further, among the corn silages, the relationship between the k_d and $iNDF_{2.4}$ was $r = -0.66$.

Table 2.3. Rates of fermentation k_d (%/h) of selected forages based on various time point calculations, 24 h with fixed lag, 6 and 24 h with variable lag, 12 and 24 with fixed lag and 6 – 36 h with variable lag and the calculated lag from 6-36 h.

Forage	24 h k_d, fixed lag, (3 h)	6 and 24 h k_d variable lag	12 and 24 h k_d fixed lag (3 h)	6 – 36 h k_d, variable lag	Lag, h
Alfalfa 1	5.48	5.51	5.27	5.02	2.74
Alfalfa 2	7.70	7.19	9.43	8.19	1.64
Timothy 1	2.96	2.82	2.52	2.27	0.57
Timothy 2	6.28	6.28	6.89	6.59	2.87
Wheat straw	1.88	1.72	1.97	1.74	1.20

Table 2.4. Rates of fermentation k_d (%/h) of corn silages based on various time point calculations, 24 h with fixed lag, 6 and 24 h with variable lag, 12 and 24 with fixed lag and 6 – 36 h with variable lag and the calculated lag from 6-36 h. Known bmr varieties are noted.

Corn silages	24h k_d, fixed lag (3h)	6 and 24h k_d, variable lag	12 and 24h k_d fixed lag	6 – 36h k_d, variable lag	Lag, h
1 (BMR)	6.61	7.02	6.62	7.10	4.21
2	5.23	5.40	5.47	5.67	3.77
3 (BMR)	6.59	6.85	6.93	7.25	3.87
4	4.81	4.87	5.12	5.02	3.23
5 (BMR)	4.57	4.67	4.26	4.29	3.22
6	6.15	6.45	5.78	6.05	3.82
7	5.09	5.26	4.98	4.99	3.45
8	4.57	4.48	4.48	4.22	2.29
9	4.79	4.85	4.96	4.84	3.13
10	4.56	4.57	4.58	4.37	2.72
11	4.73	4.71	4.06	3.87	2.09
12	4.47	4.50	4.58	4.26	2.83
13	4.35	4.50	3.74	3.66	3.09
14	3.33	3.24	3.48	3.17	2.24
15	3.71	3.22	4.38	3.50	0.06
16	3.20*	2.88	3.91*	3.67	0.00
17	4.02	3.76	4.42	3.83	1.42
18	4.95	4.56	5.43	4.53	1.00
19	3.96	3.62	4.23	3.50	0.72
20	3.96	3.52	4.63	3.69	0.31
21	3.22	2.90	3.90	3.59	1.59

*For these calculations, lag was set to zero.

Comparison of mathematical approaches

Predicted and observed values were highly correlated ($P < 0.01$) for both the non linear and linear procedures, also indicated by the similarity of the residual errors of the models. However even if the overall equations tended to describe the data equally well, our objective is the implementation of the new mathematical approach for commercial use and therefore the analysis of the bias in the actual values of the parameters obtained due to calculation method. Rates of digestion were evaluated for their prediction accuracy and biological relevance, with the preferred prediction having a small regression bias and minimal unexplained variation. The log-linear transformation gave low correlations when using data greater than 48 h fermentation and therefore those rates are not shown here. This is most likely related to the possible presence of two first order pools as shown by Van Soest et al. (2005) and discussed previously by Mertens (1977).

The single time point rate estimations presented the highest correlation for the 24 and 30 h fermentation values (Tables 2.5a and 2.5b). In general the lowest MSPE's were obtained when using 24 h as single time point fermentation with the variable lag and using either the last fermentation point (216 h) for both in-vitro and in-situ. Higher MSPE resulted when comparing the actual k_d, from the non linear model, with the k_d obtained using the log-linear transformation and the residue $iNDF_{2.4}$. The higher MSPE values were primarily due to mean bias, however the slope of the regression was not different than unity. The use of Chandler ratio of 2.4 (Chandler, 1980) to obtain the $iNDF_{2.4}$ generally resulted in higher MSPE,

suggesting that the factor is not constant among forage families and possibly within forage type. The time-point calculation of k_d presented here represents the slope of the logarithmic disappearance curve at a particular time. This procedure has the advantage of requiring minimum data that might be available from field laboratories. Since the calculations are direct and use no statistical regression procedures, they are simpler to implement because many observation are not required. If a number of time point digestions are available, means and standard deviations of the respective lag and rate values can be calculated and their uniformity examined. Even if lag requires two time points, our results show that an average lag of 3 h can be used with a single time point. However a lab-specific value would need to be used to obtain the best rate estimations. Results also show that the net effect of using single time point estimation and the approach presented here cannot be considered biologically different from predicting a k_d using a non linear model and different fermentation points. The primary differences and variation associated with using a single time point to calculate k_d was through the estimation of the iNDF. This suggests further work is needed to determine how much variation exists in the iNDF and how that influences the estimation of rates and potentially the feeding behavior and rumen fill effects of the forage.

Table 2.5a. Comparison of a non linear estimation with fixed, single time point estimations of rates of digestion of neutral detergent fiber. Values in rows of individual forages in bold are the those falling within the 95% confidence interval of the respective rate estimated by the non-linear decay model (1st column). Statistical parameters for all forages are in Table 2.5b.

	Nonlin	**24 h fixed lag**			**30 h fixed lag**			**ln-lin 6-30 h**			**6-24 h**		
Forage*	**kd**	**kd2.4**	**kd216**	**kdD**	**kd2.4**	**kd216**	**kdD**	**kd2.4**	**kd216**	**kdD**	**kd2.4**	**kd216**	**kdD**
1	**6.81**	5.23	5.36	**6.17**	5.04	5.19	**6.16**	5.09	**5.81**	**6.28**	5.32	**5.96**	**6.36**
2	**6.15**	4.82	**5.53**	**5.76**	4.48	**5.25**	**5.51**	4.46	**5.35**	**5.55**	4.80	**5.65**	**5.83**
3	**6.14**	**5.27**	**6.09**	**6.21**	4.76	**5.60**	**5.73**	4.97	**5.90**	**6.04**	**5.47**	**6.39**	**6.52**
4	**5.95**	**4.78**	**5.49**	**6.68**	4.40	**5.15**	**6.50**	4.42	**5.23**	**6.69**	**4.80**	**5.58**	**6.90**
5	**5.54**	4.58	**4.88**	**5.22**	4.27	4.58	**4.94**	**4.45**	**5.11**	**5.19**	**4.73**	**5.36**	**5.44**
6	**6.79**	6.16	**6.43**	**6.83**	5.64	5.94	**6.38**	5.98	**6.41**	**6.81**	**6.49**	**6.89**	**7.27**
7	**5.75**	4.56	**4.86**	**6.00**	4.16	4.46	**5.68**	4.20	**5.47**	**5.84**	4.56	**5.80**	**6.14**
8	**5.29**	**4.71**	**5.41**	**5.66**	**4.41**	**5.16**	**5.45**	**4.62**	**5.46**	**5.77**	**4.84**	**5.61**	**5.90**
9	**4.89**	**4.42**	**5.32**	**5.43**	3.62	**4.38**	**4.48**	3.68	**4.53**	**4.64**	**4.42**	**5.39**	**5.52**
10	**5.45**	**4.36**	**5.46**	**5.89**	3.60	**4.55**	**4.94**	3.95	**5.06**	**5.52**	**4.62**	**5.86**	**6.36**
11	**7.64**	6.60	6.66	6.75	6.32	6.39	**7.05**	6.59	6.72	**7.39**	**6.95**	**7.07**	**7.65**
12	**8.18**	6.59	**7.43**	**7.68**	6.48	**7.59**	**7.95**	6.55	7.24	**7.77**	6.74	7.31	**7.73**
13	**5.05**	**4.57**	**5.07**	**5.35**	**4.36**	**4.91**	**5.23**	**4.36**	**4.94**	**5.28**	**4.52**	**5.05**	**5.36**

*1-10 are conventional corn silages, 11-13 are bmr corn silages;

Table 2.5b. Comparison of a non linear estimation with fixed, single time point estimations of rates of digestion of neutral detergent fiber. Values in rows of individual forages in bold are the those falling within the 95% confidence interval of the respective rate estimated by the non-linear decay model (1st column).

	Nonlin	**24 h**			**30 h**			**ln-lin**					
		fixed lag			**fixed lag**			**6-30 h**			**6-24 h**		
Forage*	**kd**	**kd2.4**	**kd216**	**kdD**	**kd2.4**	**kd216**	**kdD**	**kd2.4**	**kd216**	**kdD**	**kd2.4**	**kd216**	**kdD**
ALFALFA 1	**6.35**	**5.43**	**7.22**	**6.67**	4.77	**6.59**	**6.01**	4.91	**6.00**	**6.29**	**5.49**	**6.59**	**6.87**
ALFALFA 2	**8.18**	**7.69**	**8.72**	**9.46**	**7.26**	**8.55**	**9.61**	**6.48**	**7.03**	**8.87**	**6.88**	**7.36**	**8.78**
TIMOTHY 1	**3.89**	2.96	**3.55**	**3.87**	**3.04**	**3.74**	**4.14**	**3.07**	**4.08**	**4.23**	2.93	**3.78**	**3.90**
TIMOTHY 2	**7.01**	**6.27**	**6.73**	**7.12**	**5.93**	**6.47**	**6.94**	**5.76**	**6.13**	**6.57**	**6.10**	**6.43**	**6.81**
WHEAT STRAW	**2.39**	1.87	**2.20**	**2.42**	**1.88**	**2.24**	2.95	1.76	**2.12**	**2.35**	1.72	**2.05**	**2.26**
Slope		1.02	0.92	0.90	1.09	0.98	0.93	1.14	1.21	0.99	1.04	1.05	0.93
Correlation		0.97	0.94	0.95	0.97	0.94	0.94	0.97	0.98	0.97	0.97	0.96	0.96
mean bias		0.86	0.08	0.00	1.55	0.30	0.01	1.42	0.20	0.00	0.80	0.03	0.04
regression bias		0.00	0.01	0.02	0.01	0.00	0.01	0.03	0.06	0.00	0.00	0.01	0.01
Unexpl. variation		0.12	0.28	0.23	0.15	0.27	0.26	0.13	0.10	0.15	0.12	0.17	0.17
MSPE		**0.98**	**0.38**	**0.26**	**1.71**	**0.58**	**0.28**	**1.58**	**0.36**	**0.15**	**0.92**	**0.21**	**0.23**
RMSE		**4.15**	**1.27**	**0.31**	**5.56**	**2.47**	**0.39**	**5.33**	**1.99**	**0.00**	**3.99**	**0.81**	**0.94**

CONCLUSIONS

Mathematical procedures are presented for the direct calculation of lag and rate of digestion k_d. Rate of digestion can be calculated from a single digestion value and lag from two values at different times. Calculation of k_d from a single time point (24 h) using a constant average lag of 3 h gives a very close estimate of the average k_d, and would allow easy calculation in the field if NDF, lignin and 24 h in vitro digestion of NDF were available.

Comparison of the directly determined k_d in the data of Mertens (1973) gives lower values for k_d than those of Mertens (1973). Values of k_d are proportionally lower for mature forages. Low quality forage has been overvalued by previous systems. This occurs because of the overestimation of iNDF and the existence of a second pool. These lower rates would place a greater penalty upon mature forages when these rates are used in the CNCPS. The high correlations between all of the parameters - lag, rate and ultimate extent validate Merten's original conclusions.

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CHAPTER THREE: IMPROVED METHODOLOGY FOR ANALYSES OF ACID DETERGENT FIBER AND ACID DETERGENT LIGNIN

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ABSTRACT

The objective of this study was to evaluate the methodology of the acid detergent lignin assay in an effort to improve recovery within and repeatability among samples. The original acid detergent lignin method relied on asbestos as a filtering aid, but that was removed in 1989 with the mandate from EPA to eliminate asbestos in the environment. Further, recent work on fiber methodology indicated that pore size in the Gooch sintered glass crucible was too large to trap all of the small particles associated with NDF and ADF. Thus any loss of ADF could potentially result in a loss of acid detergent lignin.

Sixty forages including conventional and bmr corn silages, alfalfas, mature grasses, early vegetative grasses and 9 feces samples, were analyzed for ADF and acid detergent lignin (ADL) using the procedure by Goering and Van Soest (1970) except for the use of the asbestos fiber. A glass microfiber filter with 1.5 μm pore size was chosen as filtering aid because it met the criteria required of the assay – glass, heat resistant, acid resistant, and chemically inert. To compare with the original ADF and ADL assays, the filter was inserted into the crucibles, rinsed with acetone and then the assays

were conducted per Van Soest and Wine (1967). The samples analyzed covered a range from 18.11 to 55.79% ADF and from 0.96 to 9.94% ADL on DM basis. Overall, mean ADF recovery was increased 4.2% and mean ADL recovery was increased 18.9%. Overall, both ADF and ADL values were greater with the use of the filter paper than without ($P < 0.05$) indicating that depending on the sample, use of the Gooch crucible results in particle loss. Use of the filter with lower lignin containing forages, or forages with greater processing, such as feces, increased the recovery of small particles. Repeatability standard deviations using the filters ranged from 0.07 to 0.29% for ADF and from 0.06 to 0.34% for ADL. Values obtained using the filters were greater than without the use of filters ($P < 0.05$). We recommend adoption of the use of a small pore size (1.5 μm) glass microfiber filter to improve filtration and recovery of ADF and ADL and to reduce variation in the ADL assay specifically, especially when sintered glass bottom crucibles are used as the filter. These differences in recovery and repeatability have implications for other fiber and lignin methods.

INTRODUCTION

Lignin has been defined in the literature since the 19th century (Klason, 1897). Lignin can be defined either chemically or from a functional role that lignin has within a plant. According to Sarkanen and Ludwig (1971), lignin is a heterogeneous polymeric material composed of phenylpropanoid units derived primarily from three cynnamil alcohols (monolignols): *p*-coumaryl, coniferyl, and sinapyl alcohols. From a functional perspective, lignins impart strength to cell walls, facilitate water transport, and impede the degradation

of wall polysaccharides, thus acting as a major line of defense against pathogens, insects, and herbivores. Lignification, which is known to impact rigidity, alters the shear force necessary to fracture plant tissue and decrease digestibility, is the biochemical process of forming the collective of phenylpropanoid macromolecules termed lignin (Sarkanen and Ludwig, 1971).

Several well-defined procedures to quantify lignin in plant tissues have been developed and approved by AOAC (Hatfield and Fukushima, 2005). The most commonly used method in ruminant nutrition and agronomy is the procedure developed by Goering and Van Soest (1970), in which an acid detergent extraction step is employed to produce acid detergent fiber (ADF). The ADF is cellulose and lignin with small amounts of pectins and xylans potentially present depending on the sample. A subsequent and sequential step is the isolation of lignin from the ADF through the use of 72% sulfuric acid in hydrolyzing the remaining carbohydrates. Of all procedures, acid detergent lignin (ADL) results in the lowest values of lignin as reviewed by Hatfield and Fukushima (2005), and it can be from 2 to 5 times lower than Klason lignin values (Jung et al., 1999).

For the nutritionist, it has been important to know the lignin value of a feed and forage because it is generally accepted that lignin and lignin cross-linking to carbohydrates are the primary factors responsible for limiting the anaerobic digestion of forages (Besle et al., 1994; Van Soest, 1994). Furthermore, no anaerobic or mammalian enzyme systems are known to exist that degrade polymerized phenolic compounds (Van Soest, 1994), therefore true lignin is theoretically indigestible and should serve well as an internal indicator of digestibility. However, there are inconsistencies in the

ability to quantitatively recover lignin (Fahey Jr and Jung, 1983; Cochran et al., 1988) with observed positive and negative recoveries (Fahey Jr and Jung, 1983; Van Soest, 1994). Reports of positive ADL recoveries are frequently attributed to the formation of "artifacts" of ADL during gastrointestinal transit, due to soluble, but apparently indigestible, lignin-carbohydrate complexes (LCC) entering the rumen that precipitate in the acidic environment of the abomasum and are subsequently recovered in the feces (Gaillard and Richards, 1975; Neilson and Richards, 1978). Alternatively, incomplete lignin recoveries have been attributed to either true or apparent digestion of lignin by formation of soluble LCC that pass from the rumen and the gastro-intestinal tract as polymers not recovered in the fibrous residue of feces (Fahey, Jr et al., 1979), or to partial destruction of the fecal lignin fraction by reagents used in the analytical method (Fahey, Jr and Jung, 1983). Given the data from the last few decades indicating both degradation and solubilization of lignin in the digestive tract, use of ADL as a marker violates the criteria of an ideal marker (Faichney, 1975) and the use of it as a marker has been viewed with caution.

The original AOAC lignin procedure for crucibles (Van Soest and Wine, 1967) relied on the use of asbestos fiber as a filtering agent, but asbestos was rendered a health hazard in 1989 by the United States Environmental Protection Agency (EPA, 1989, Asbestos Ban and Phase Out Rule) and removed. The sintered glass filter within the crucible is prone to particle loss and plugging with fine particles. Since asbestos was removed, no filtering aids have been used and in the case of the ADL assay, no other filter aids have been used since few are as inert as asbestos and can interfere in the lignin recovery (Van Soest, 1973). Udén (2006) evaluated the use of filtration

or centrifugation to study the recovery of NDF and ADF. In that study he tested the use of filter paper with porosity of 6 μm or centrifugation at 9000 × *g* for 5 minutes against crucibles with porosity 2 (40-100 μm) (Udén, 2006). In this evaluation, the recovery of ADF was on average 20 and 24% higher for centrifugation and paper filtration, respectively, for both feeds and feces than through the use of the traditional Gooch crucible (Udén, 2006).

In the paper by Udén (2006) ADL recovery was not evaluated, but it seems apparent that any change in recovery of ADF would potentially impact the recovery of ADL. Our hypothesis was that ADL is lost during both the preparation step (i.e. ADF), as shown by Udén (2006), and the 72% sulfuric acid hydrolysis step. Furthermore this loss would vary among forage families, and type of sample, because the different physicochemical structure of cell walls would result in specific particle size reduction during the two step procedure.

Currently, glass microfiber filters are available that are manufactured from 100% borosilicate glass that is binder free and chemically inert and have pore sizes that extend into the sub-micron range resulting in retention of very fine particles. Furthermore they can be used at temperatures up to 500°C to facilitate ashing. These characteristics of the glass microfiber filters appear to make them an ideal replacement for the asbestos in the ADL assay and hold the potential of improving upon the use of asbestos. The precision of gravimetric analyses is dependent upon the ability to produce repeatable weights, accurate to at least 0.05 to 0.1% of the respective components being determined. In gravimetric fiber analysis, the residues from the various extractions can be very hygroscopic. The glass of the crucibles can adsorb

up to 25 mg of water when cooled to room temperature. Even at 100ºC, all absorbed water in materials is not removed. Since most residues or extracts to be gravimetrically assayed are usually 500 mg or less, absorbed water can result in substantial error. Hot weighing at 105 °C is preferred over the use of desiccators because the low ADL contents of some forage species (e.g. bmr corn silages), the variable hygroscopic characteristics of different forages and of the filters used might all contribute to a biased weight or variability that is unacceptable in the assay.

Our hypothesis was that the removal of asbestos as a filtering aid increased the loss of ADF and ADL, especially in low lignin and highly processed samples and this lack of filtering aid also altered the variation within the assay. The objective of our work was therefore to possibly increase the recovery of both steps of the acid detergent lignin assay, using a filter that would be chemically, acid and heat inert.

MATERIALS AND METHODS

Sixty forages, including conventional and bmr corn silages, alfalfas, mature grasses (straws and hays), early vegetative grasses and nine feces samples were analyzed in duplicate for ADF and ADL using the procedure by Goering and Van Soest (1970), except for the use of the asbestos fiber. Crucibles with smaller porosity (10-15 μm and 4-5.5 μm) were tested for filtering ease, recovery and repeatability, but resulted in filtering problems and increased variation. The filter 934-AH™ by Whatman® (Whatman Limited – GE Healthcare, Maidstone, UK) was chosen as filtering aid

because it met the criteria required of the assay – glass, heat resistant, acid resistant, and chemically inert.

The filters were inserted in 50 ml Pyrex® Gooch crucibles with porosity 40-60 μm and rinsed with acetone. Crucibles with filters were tested for weight loss during the ADF assay, the subsequent 72% H_2SO_4 step and then after ashing at 500 °C in a muffle furnace. Intra-laboratory repeatability (s_r) associated with a single analysis was determined by approaches described by Wernimont (1990). Statistical results were determined for forage groups. The F-distribution was used to test the null hypothesis that the replication population variances were the same for the two procedures by calculating the 95% confidence limits for the ratio of the two variances. An interval including 1 would be meaning that the two variances are similar. Furthermore the Student's *t*-distribution was used to test the differences between the means (paired comparisons) of the two procedures (d_1 and d_2) and the null hypothesis that the populations mean difference value of d_3 is zero. The null hypothesis was accepted if the 95% confidence interval included zero. For the probability distributions, evaluations were done within each forage group and therefore using each group's degrees of freedom.

RESULTS AND DISCUSSION

The samples analyzed covered a range from 18.11 to 55.79% ADF and from 0.96 to 9.94% ADL on DM basis. Evaluation of the filters for weight loss resulted in an average loss of 0.02% for the sequential procedure, evenly distributed among the three steps. Therefore use of blanks to correct

for loss of both crucibles and filters is recommended. A preliminary analysis of blanks, using the original ADL procedure, resulted in an increase in weight after the sulfuric acid treatment, probably due to a tendency of the filter to retain sulfuric acid. Accordingly, two additional washes with hot distilled water eliminated the problem. We therefore suggest washing the crucible with hot distilled water two extra times and this step was suggested in the original procedure (Goering and Van Soest, 1970). The hot weighing procedure of each crucible is done at the lowest weight of the same (with or without sample) that is usually reached between 20 and 30 seconds after removing the crucible from the oven (P.J. Van Soest, personal communication). The presence of the filter resulted in the lowest weight at later times, specifically between 30 and 45 seconds. This is likely due to the hygroscopic characteristics of the glass filter. Therefore more care and time is needed to record the most precise and accurate crucible weight when using glass filters.

Overall, mean ADF recovery was increased from 4.2% and mean ADL recovery was increased 18.9%. (Table 3.1). The change in ADF recoveries observed in this study were not as high as those observed by Udén (2006) but were of a similar magnitude given the range of samples used in this study. The samples highest in ADL, such as wheat straw resulted in the lowest difference in recovery, while the bmr corn silage samples resulted in the greatest differences in recovery, whereas the grass and alfalfas demonstrated similar recoveries overall. Overall, both ADF and ADL values were greater with the use of the filter paper than without ($P < 0.05$) indicating that depending on the sample, use of the Gooch crucible results in particle loss (Tables 3.2 and 3.3). The lower the lignin content of

the forage or feed, and with greater processing, such as was identified in the feces, the greater the recovery of small particles, thus demonstrating the need for the additional filter.

Repeatability standard deviations (s_r) ranged from 0.07 to 0.29% for ADF and from 0.06 to 0.34% for ADL (Tables 3.4 and 3.5). Except for conventional corn silages, s_r values obtained using the filters were similar than without the use of filters ($P < 0.05$) for both ADF and ADL procedure, for all forages. However, the use of filters lowered the s_r ($P < 0.05$) for feces samples from 0.17 to 0.06% and from 0.23 to 0.06% for ADF and ADL, respectively. Small particles that form during both the grinding and the analytical procedure can be lost and compromise the values of both ADF and ADL among forage groups, when using conventional sintered glass crucibles and alter the repeatability of the assays (Table 3.4 and 3.5).

Table 3.1. Percent difference in recovery of acid detergent fiber (ADF) and acid detergent lignin (ADL) when using a glass microfiber filter with 1.5 μm for ADF and ADL procedure, for wheat straws, corn silages (C.S.), alfalfas, grasses, immature grasses and cow feces (ranges in parentheses).

Group	ADF	ADL
Grasses	0.7 (-0.4 – 5.0)	10.9 (-2.2 – 29.3)
Immature grasses	19.1 (1.9 – 21.1)	38.3 (19.25 – 90.5)
Conventional C.S.	3.3 (-0.5 – 9.9)	23.2 (2.7 – 41.0)
BMR C.S.	7.0 (-1.8 – 15.1)	27.5 (-1.5 – 67.2)
Alfalfas	2.7 (-1.0 – 11.2)	3.2 (-1.6 – 10.1)
Feces	5.9 (2.3 – 10.3)	18.9 (8.4 – 32.0)

Table 3.2. Mean ADF values, as % of DM, of the samples analyzed, without and with filter, and respective differences of replicates (d1 and d2) and procedures (d3).

	without filter		with filter		
Groups	ADF_1	d_1	ADF_2	d_2	d_3
Grasses	44.16	0.27	45.36	0.23	-1.20
	45.78	0.27	45.68	0.33	0.10
	44.71	1.00	44.75	0.85	-0.04
	48.66	0.41	48.69	0.21	-0.03
	49.77	0.56	49.99	0.89	-0.22
	32.68	0.53	34.32	0.32	-1.64
	51.82	0.84	51.72	0.45	0.10
	54.06	0.75	54.00	0.78	0.06
	53.70	0.26	53.48	0.29	0.22
	55.72	0.83	56.12	0.45	-0.40
	55.79	0.20	55.99	0.35	-0.20
	20.01	0.34	21.79	0.32	-1.78
	23.45	0.12	25.69	0.07	-2.24
	19.01	0.32	20.05	0.29	-1.04
	24.23	0.23	25.12	0.14	-0.89
	19.21	0.35	21.79	0.33	-2.58
	22.32	0.13	25.69	0.07	-3.37

Table 3.2. (Continued)

	without filter		with filter		
Groups	ADF_1	d_1	ADF_2	d_2	d_3
Conventional C.S.	23.16	0.18	23.12	0.10	0.04
	21.45	0.21	21.34	0.01	0.11
	25.99	0.63	26.20	1.25	-0.21
	21.80	0.76	22.10	0.46	-0.30
	22.74	0.19	23.01	0.39	-0.27
	21.02	0.51	21.17	0.84	-0.15
	25.88	0.89	28.17	0.61	-2.29
	20.09	0.38	20.58	0.65	-0.49
	24.02	0.67	24.92	0.75	-0.90
	24.20	0.41	25.46	0.68	-1.26
	22.44	0.22	24.08	0.47	-1.64
	19.54	0.22	21.49	0.29	-1.95
	22.59	0.19	23.04	0.17	-0.45
Bmr C.S.	20.11	0.21	20.93	0.10	-0.82
	21.32	0.12	22.28	0.07	-0.96
	18.11	0.23	19.89	0.22	-1.78
	19.65	0.34	19.28	0.23	0.37
	21.54	0.45	22.66	0.29	-1.12

Table 3.2. (Continued)

	without filter		with filter		
Groups	ADF_1	d_1	ADF_2	d_2	d_3
Alfalfas	29.78	0.12	30.48	0.32	-0.71
	24.30	0.66	25.60	0.03	-1.30
	30.41	0.04	32.40	1.34	-1.99
	20.95	0.87	23.30	0.68	-2.35
	28.02	0.62	28.00	0.35	0.02
	24.39	0.21	25.02	0.21	-0.63
	27.05	0.22	28.56	0.39	-1.52
	25.20	0.06	25.65	0.06	-0.45
	25.58	0.45	26.01	0.45	-0.43
	29.98	0.07	30.10	0.07	-0.12
	48.53	0.04	49.01	0.04	-0.48
	34.64	0.42	35.87	0.42	-1.23
Immature grasses	29.75	0.40	30.90	0.22	-1.15
	33.21	0.29	34.25	0.12	-1.04
	27.87	0.22	30.42	0.15	-2.55
	25.63	0.23	27.30	0.18	-1.67
	24.23	0.53	26.32	0.25	-2.09
	25.69	0.24	28.56	0.14	-2.87
	27.89	0.41	30.04	0.40	-2.15
	25.02	0.28	26.89	0.29	-1.87
	27.04	0.14	28.77	0.12	-1.73
Feces	33.71	0.21	35.23	0.12	-1.52
	34.81	0.57	35.61	0.23	-0.80
	36.15	0.12	38.92	0.11	-2.77
	36.13	0.10	36.99	0.09	-0.86
	31.82	0.07	35.12	0.07	-3.30
	31.16	0.10	33.14	0.02	-1.98
	35.29	0.23	38.65	0.15	-3.36
	33.12	0.34	34.81	0.18	-1.69
	37.25	0.45	39.12	0.11	-1.87

Table 3.3. Mean ADL values, as % of DM, of the samples analyzed, without and with filter, and respective differences of replicates (d_1 and d_2) and procedures (d_3).

	without filter		with filter		
Groups	ADL_1	d_1	ADL_2	d_2	d_3
Grasses	3.73	0.03	3.64	0.03	0.09
	4.03	0.24	4.43	0.03	-0.40
	4.06	0.08	4.40	0.10	-0.33
	7.02	0.03	7.78	0.12	-0.76
	4.19	0.35	4.75	0.44	-0.56
	5.46	0.19	6.03	0.03	-0.56
	2.87	0.01	3.21	0.01	-0.34
	2.39	0.02	2.85	0.08	-0.46
	8.30	0.48	10.73	0.21	-2.43
	9.94	0.34	9.82	0.18	0.12
	6.52	0.16	7.18	0.32	-0.66
	3.73	0.03	3.64	0.03	0.09
	4.03	0.24	4.43	0.03	-0.40
	4.06	0.08	4.40	0.10	-0.33
	7.02	0.03	7.78	0.12	-0.76
	4.19	0.35	4.75	0.44	-0.56
	5.46	0.19	6.03	0.03	-0.56

Table 3.3. (Continued)

	without filter		with filter		
Groups	ADL_1	d_1	ADL_2	d_2	d_3
Conventional C.S.	2.19	0.16	3.09	0.18	-0.90
	2.71	0.08	2.78	0.17	-0.07
	3.12	0.49	3.81	0.13	-0.69
	1.85	0.56	2.57	0.09	-0.72
	2.52	0.32	2.99	0.01	-0.47
	2.12	0.32	2.94	0.15	-0.82
	2.15	0.17	2.58	0.24	-0.43
	2.13	0.61	2.47	0.13	-0.34
	1.79	0.16	2.28	0.23	-0.49
	2.36	0.29	2.55	0.09	-0.19
	2.71	0.08	3.32	0.16	-0.61
	2.23	0.05	2.76	0.13	-0.53
	2.33	0.14	2.84	0.05	-0.51
BMR C.S.	1.09	0.08	1.79	0.07	-0.70
	1.11	0.04	1.25	0.04	-0.14
	2.24	0.13	2.57	0.27	-0.33
	1.14	0.02	1.47	0.00	-0.33
	1.24	0.16	1.66	0.04	-0.42
	1.47	0.06	1.60	0.11	-0.12
	1.17	0.01	1.15	0.19	0.02
	1.57	0.00	1.65	0.03	-0.08
	1.14	0.21	1.91	0.02	-0.77
	1.43	0.16	2.26	0.03	-0.83
	1.41	0.02	1.56	0.24	-0.15

Table 3.3. (Continued)

	without filter		with filter		
Groups	ADL_1	d_1	ADL_2	d_2	d_3
Alfalfas	6.42	0.13	6.78	0.09	-0.36
	6.50	0.84	6.93	0.83	-0.43
	7.41	0.39	7.33	0.39	0.09
	7.20	0.27	7.35	0.01	-0.14
	4.59	0.01	4.98	0.40	-0.39
	5.81	0.06	6.21	0.06	-0.40
	5.94	0.06	6.13	0.18	-0.19
	9.11	0.49	9.22	0.21	-0.11
	5.70	0.29	5.61	0.14	0.09
	5.36	0.19	5.28	0.19	0.08
	5.26	0.01	5.28	0.07	-0.02
	5.83	0.07	6.06	0.19	-0.23
Immature grasses	2.34	0.16	3.33	0.10	-0.99
	2.43	0.22	2.99	0.18	-0.56
	1.87	0.18	2.23	0.05	-0.36
	2.14	0.11	2.85	0.26	-0.71
	2.38	0.00	3.08	0.01	-0.70
	2.28	0.09	3.06	0.10	-0.78
	2.25	0.18	2.95	0.18	-0.70
	1.42	0.05	2.13	0.05	-0.71
	1.34	0.05	2.55	0.06	-1.21
Feces	6.38	0.27	8.42	0.05	-2.04
	6.94	0.39	7.77	0.14	-0.82
	5.32	0.32	6.03	0.08	-0.71
	6.43	0.10	8.34	0.05	-1.91
	6.69	0.91	7.77	0.23	-1.08
	5.32	0.45	6.00	0.13	-0.68
	6.38	0.29	8.42	0.21	-2.04
	6.94	0.39	7.54	0.20	-0.60
	5.20	0.18	5.64	0.16	-0.44

Table 3.4. Intra-laboratory study results for the determination of ADF (% of DM) per group of samples analyzed.

Parameter	Grasses	Conventional C.S.	BMR C.S.	Alfalfas	Immature grasses	Feces
Number of samples	11	12	11	16	10	9
Average ADF value w/out filter	49.66*	22.76*	20.81*	28.41*	27.09*	34.48*
Average ADF value with filter	49.90*	23.66*	22.29*	29.13*	28.85*	36.40*
Repeatability standard deviation without filter, s_r1, g/100 g	0.28	0.24	0.11	0.29	0.11	0.17*
Coefficient of variation of s_r1, %	0.56	1.09	0.53	1.02	0.56	0.51
Repeatability limit r ($r = 2.8 \times s_r1$)	0.78	0.69	0.53	0.81	0.31	0.49
Repeatability standard deviation with filter, s_r2, g/100 g	0.25	0.29	0.10	0.35	0.09	0.06*
Coefficient of variation of s_r2, %	050	1.25	0.48	1.20	0.50	0.17
Repeatability limit r ($r = 2.8 \times s_r1$)	0.70	0.82	0.48	0.97	0.27	0.17

*: Statistically different pairwise comparison for mean value or for repeatability standard deviations of the procedures ($P < 0.05$)

Table 3.5. Intra-laboratory study results for the determination of ADL (% of DM) per group of samples analyzed.

Parameter	Grasses	Conventional C.S.	BMR C.S.	Alfalfas	Immature grasses	Feces
Number of samples	11	11	11	16	10	9
Average ADL value w/out filter	5.32*	2.32*	1.36*	6.03*	2.11*	6.18*
Average ADL value with filter	5.89*	2.84*	1.71*	6.22*	2.86*	7.32*
Repeatability standard deviation without filter, s_r1, g/100 g	0.16	0.18*	0.07	0.22	0.07	0.23*
Coefficient of variation of s_r1, %	3.00	8.06	5.31	3.69	3.53	3.72
Repeatability limit r ($r = 2.8 \times s_r1$)	0.44	0.52	0.20	0.62	0.20	0.64
Repeatability standard deviation with filter, s_r2, g/100 g	0.13	0.06*	0.09	0.25	0.08	0.06*
Coefficient of variation of s_r2, %	2.31	2.28	5.56	4.14	2.80	0.95
Repeatability limit r ($r = 2.8 \times s_r1$)	0.38	0.18	0.26	0.72	0.22	0.19

*: Statistically different pairwise comparison for mean value or for repeatability standard deviations of the procedures ($P < 0.05$)

CONCLUSIONS

We recommend using the glass microfiber filters (934-AH™, Whatman® Limited – GE Healthcare, Maidstone, UK) with small porosity to increase recovery during the sequential ADF – ADL procedure. There can be some degradation of the filter during the 72% H_2SO_4 treatment, however, test results indicate the degradation did not impact the precision or recovery and could be managed through the use of blanks. Most of this degradation occurred due to the physical mixing of the ADF and the sulfuric acid with the stir rod. To decrease the chance of filter degradation, an alternative that was tested was to put the sample in a small beaker (50 to 100 ml) and soak it in excess H_2SO_4, stir appropriately and then filter in crucibles with the microfiber glass filters. It is also suggested that vacuum be applied slowly, allowing the sample time to sediment during the rinsing step and then apply vacuum.

In summary, improved recoveries and repeatability are achieved in the ADF and ADL assays with the use of the 1.5 μm pore size microfiber glass filter paper inserted into the Gooch crucible. The application of this filter aid improves the assays and restores the method to be compliant with the first movement of AOAC ADL method by Van Soest (1973).

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CHAPTER FOUR: DETERMINATION OF INDIGESTIBLE NEUTRAL DETERGENT FIBER AND ITS PREDICTION FROM ACID DETERGENT LIGNIN

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ABSTRACT

A portion of the forage cell wall is unavailable to microbial digestion in ruminants and in previous work this fraction was characterized by the relationship between acid detergent lignin and NDF as 2.4 time lignin/ NDF and termed the indigestible NDF (iNDF). Further, this relationship was considered to be a static relationship between ADL and NDF with no genetic variation or observed effects of growing conditions on the plant. The objectives of this work were to further characterize the relationship between lignin and iNDF and to evaluate the concept of a fixed value, assuming that the relationship is more dynamic and dependent on agronomic and environmental factors. One hundred and two forages of several species were analyzed for NDF, ADL and iNDF. The ADL was performed as described by Goering and Van Soest (1970) without the use of asbestos, and with the use of a glass microfiber filter with porosity of 1.5 μm in Gooch crucibles as suggested by Raffrenato and Van Amburgh (2010, submitted). The same filter was used for the NDF analysis as in Mertens (2002). Specifically, amylase (aNDF) and the ashing were applied to the NDF analyses (aNDFom). The in vitro analyses were conducted in 125 mL

Erlenmeyer flasks for 240 h to reach the maximum extent of digestion to determine iNDF. The use of a glass microfiber filters with small porosity increased recoveries between 0 and 75% of the iNDF values obtained without filter. The ratios of iNDF to ADL/NDF ranged between 1.73 and 7.59, demonstrating the dynamic nature of the relationship. Further, an attempt to predict iNDF based on the relationship between ADL/NDF was made and the equations provided R^2 values between 0.22 and 0.96, indicating that more information within forage group and single forages on agronomic and environmental conditions are probably needed to better explain this variation. Overall, the data provided a more robust estimation of the truly available NDF and this data will allow us to further examine the effects of growing season and agronomic conditions on the size of the iNDF pool, estimations of rate of digestion of the available NDF and the effects of varying iNDF on DMI in ruminants.

INTRODUCTION

Cell wall carbohydrates represent the major source of potentially digested nutrients for ruminants. Cell wall carbohydrates can be quantified by determination of neutral detergent fiber (NDF), which includes cellulose, hemicellulose and lignin as the major components (Mertens, 2002). A portion of the forage cell wall is unavailable to microbial digestion in ruminants even if the total tract residence time of fiber could be extended to an infinite time and this fraction is referred to as the indigestible NDF (iNDF) (Huhtanen et al., 2006b). The digestibility of the remaining fiber, the potentially digestible NDF (pdNDF = NDF – iNDF), determines the

availability of NDF. Forage digestibility is thus constrained by the extent and rate of digestion of pdNDF and according to the Lucas principle, the iNDF is an ideal fraction since by definition it is digested at a predictable rate of zero (Van Soest, 1994).

The iNDF has been characterized as the most important factor affecting the total diet organic matter digestibility (Nousiainen et al., 2004). According to Ellis et al. (Ellis et al., 1999) determination of iNDF should be included in all basic feedstuff analysis because it has a predictable digestibility, can be used for the estimation of the pdNDF as NDF-iNDF and has an important role in contributing to the rumen digesta load. Furthermore, a close empirical relationship between silage iNDF and OM digestibility indicates that iNDF is a useful entity for the prediction of the nutritive value of forages (Nousiainen et al., 2003). The importance of estimating iNDF to model and explain OM and NDF digestibility, and the size of the rumen available NDF pool is demonstrated by the Nordic model of dairy cow metabolism (Danfær et al., 2006a; Danfær et al., 2006b). Simulation results clearly demonstrate profound effects of these parameters on OM digestibility and consequently on the supply of energy and microbial protein (Danfær et al., 2006a; Danfær et al., 2006b).

The Cornell Net Carbohydrate and Protein System (Fox et al., 2004; Tylutki et al., 2008) and CPM Dairy (Tedeschi et al., 2008) uses 2.4 as the ratio between ADL and NDF to estimate iNDF in forages, where iNDF is calculated from the data of Chandler et al., (1980). Chandler et al. (1980) estimated the indigestible fraction as lignin times 2.4 after fermentation of feeds and other materials in methane digesters for up to 120 d. This value was determined on a small number of feeds with a limited range in

digestibility and did not represent the range in forages fed to cattle and agronomic conditions associated with the environment many forages are grown in, thus it is likely the value is not a fixed relationship with lignin.

Lignin is generally accepted as the primary entity responsible for limiting the digestion of forages (Besle et al., 1994; Van Soest, 1994). Assuming that any estimation based on long time fermentations and made at any time other than infinity is an overestimate of the true asymptotic indigestible residue, several attempts to predict iNDF from lignin concentration have been made (Mertens, 1973; Chandler et al., 1980; Conrad et al., 1984; Weiss et al., 1992; Traxler et al., 1998). Van Soest et al. (2005) provided support for this concept using several forage species and obtaining a high R^2 (0.94) between observed and predicted iNDF using 2.4 as a constant relationship among all forages. Recently, data from Huhtanen et al. (2006) did not show a general applicable relationship between permanganate lignin and iNDF measured by 12 days in-situ fermentation, although the overall slope was 2.4. Even though Van Soest assessed a linear relationship between 72% sulfuric acid and permanganate lignin (Van Soest, 1994), that relationship does not hold in the values from Huhtanen et al. (2006). This was also confirmed by Nousianen et al. (2004) who could not develop an acceptable prediction equation ($R^2 < 0.40$) for iNDF based on permanganate lignin content on different grass silage types. The observations of both Huhtanen et al. (2006) and Nousiainen et al. (2004) were based on grasses grown in Northern European and thus cooler climates that might result in a different relationship between lignin and iNDF, due to environmental interactions, suggesting this relationship is not a constant with lignin. In the work of Chandler et al. (1980), lignin was measured as 72% sulfuric acid lignin, i.e.

acid detergent lignin (ADL), and that difference could also confound the estimations of Huhtanen et al. (2006) due to the assumption of linearity between permanganate lignin and ADL and cellulose recoveries.

Analytically iNDF can be estimated by fitting kinetic models that describe the disappearance of NDF over digestion time (Waldo et al., 1972; Robinson et al., 1986; Weimer et al., 1990) and this requires multiple time points to fully characterize the disappearance curve. Alternatively, iNDF can be analytically defined as the undigested NDF remaining after exposure to agents of digestion for a sufficient time, to approximate complete digestion of pdNDF. The estimation of the indigestible fraction is not a mathematical or modeling contrivance, but is a critical biological principle upon which the concept of digestion kinetics and rates are based (Mertens, 1994). Often digestion rates are, for example, calculated without subtracting the indigestible residue or by subtracting one that is determined at too short a fermentation time. Mertens (1977) used a Ln-linear approach to show the effect of fermentation time chosen to represent the iNDF on digestion rate. If our objective is to characterize cell wall in terms of its susceptibility to rumen degradation, then direct estimation is essential as size of the degradable fraction obtained by extrapolation varies with the maximum time of incubation (Mertens, 1977). According to Mertens (1977), estimation of the iNDF using 96-h residue is effective in estimating rates of digestion (Mertens, 1977), however, observations of long in-situ (up to 40 d) (Robinson et al., 1986) and in-vitro (up to 240 h) (Van Soest et al., 2005) fermentation have shown how digestion was, in most cases, was not completed by 96 h. Furthermore, according to Robinson et al. (1986), (Robinson et al., 1986)fitting models to degradation curves with residuals up

to 40 days but using times of incubation progressively reduced indicated that two common misestimates are underestimation of the degradable fraction size and overestimation of their rate constants. If this occurs, then description of NDF residuals in terms of fractions is of limited use as fraction sizes are not real or repeatable. This suggests that extended incubations are necessary in order to estimate iNDF. Huhtanen et al. (2006), on the other hand, have suggested that the ultimate extent of NDF digestion may not be reached with in-vitro batch system and the in-situ system estimates may be biased (Huhtanen et al., 2006b) due to crucial drawbacks of the traditional nylon bag procedure as discussed by Nousiainen et al. (2004). Further, Mertens (1993a, 1993b) has presented several critical aspects of the in-situ method. However these aspects are probably more critical for the determination of the intrinsic rate of digestion than for the determination of the extent of digestion. The close relationship between in-vivo digestibility and the potential extent of digestion (Nousiainen et al., 2003) suggests that using prolonged incubations and bags with a small pore size may allow the extent of NDF digestion (and iNDF) to be accurately measured. Nousiainen et al. (2004) determined iNDF by in situ incubations for 12 days using nylon bags of small pore size (6-17 μm). The range of 6-17 μm was determined as the best compromise to minimize particle inflow and outflow, but still allowing adequate microbial activities inside the bags to avoid prolonged NDF digestions (Huhtanen et al., 1998; Huhtanen et al., 2006a). To our knowledge only Traxler et al. (1998) and Van Soest et al. (2005) have attempted to determine iNDF with in-vitro fermentations using respectively flasks and filtration (144 hrs) or plastic bottles and centrifugation (240 hrs), respectively, in both cases with samples in direct contact with the rumen

fluid and buffer (Goering and Van Soest, 1970). Furthermore Traxler et al. (1998) was the only group attempting to utilize the in-vitro apparatus Daisy^{II} (Ankom Technology Corp., Fairport, NY) to estimate iNDF. Ankom bags are characterized by a porosity of 25 μm, the design of the filter bag used (F57) provides a 3-D filter matrix similar to the filtering effect of a glass crucible with porosity of 40 μm (http://www.ankom.com/00_products/filterbagtech.shtml - Ankom Technology Corp., Fairport, NY). Thus, it is possible that within the in vitro system of Ankom, a larger pore size would not be detrimental to identifying the iNDF because of the lack of rumen contractions and rumen conditions that would facilitate particle loss or gain.

According to Huhtanen et al. (2006) attempts to predict iNDF have not been successful because of relatively high proportional errors in lignin and iNDF analyses, as well as differences among forage types in lignin to iNDF ratio, which may also be prone to agronomic factors. Tests for nutritional uniformity indicated an average recovery of 86% for ADL and sintered glass filters with a 40 μm aperture might not achieve complete recovery of fine particles (Robertson, unpublished results; Udén et al., 2006)). Furthermore, the original AOAC lignin procedure for crucibles (Van Soest, 1973) relied on the use of asbestos as a filtering agent, but the asbestos was rendered a health hazard in 1989 by the United States Environmental Protection Agency (EPA, 1989, Asbestos Ban and Phase Out Rule) and removed and another filtering agent was never instituted. We believe that the variation in the lignin assay is partially a function of the filtering step and our recent observations have confirmed this (Raffrenato and Van Amburgh, 2010, submitted).

Our hypothesis is that the relationship between lignin and iNDF is not a fixed value and there are agronomic factors that impact the relationship such that it is more dynamic in nature. Further, the ability to identify this relationship has been obscured by fermentations that were too short and that recoveries of both NDF residues and lignin did not allow for accurate characterization of this relationship. Our objective was also to evaluate both in-situ long term fermentation and the use of the Ankom Daisy incubator system for determining iNDF, when compared to the in vitro reference method.

MATERIALS AND METHODS

One hundred and two forages of several species (grass samples at various vegetative stages, conventional and bmr corn silages and alfalfas) were analyzed for NDF, ADL and iNDF. The forages were chosen simply by availability within the laboratory and from specific requests to various companies for samples of representative forages. The ADL was performed as described by Goering and Van Soest (1970) without the use of asbestos, and with the use of a glass microfiber filter (934-AH™ by Whatman®, Whatman Limited – GE Healthcare, Maidstone, UK) with porosity of 1.5 μm in Gooch crucibles (40 to 60 μm), as suggested by Raffrenato and Van Amburgh (2010, submitted). The same filter was used for the NDF analysis as in Mertens (2002). Specifically, amylase and the ashing at 550°C were applied to the NDF analyses. Long-term fermentations (240 h) were conducted to reach the maximum extent of digestion for NDF (iNDF) and then relate that to the forage ADL content. The in vitro fermentations were

conducted according to Goering and Van Soest (1970) but using 0.75 g of sample in 125 ml Erlenmeyer flasks. Preliminary tests for various forage groups were conducted to estimate when the maximum extent of NDF digestion was obtained and the 240 h time period appeared to be adequate simply because we could not detect any change in disappearance among the variance in recoveries, especially using the filter paper within the crucible. In-vitro fermentations were therefore conducted for 240 h for all forages. Rumen fluid was harvested from two lactating cows from the Cornell University Research Farm, fed a total mixed ration . The fluid was carried in two one-liter thermoses previously filled with warm water to maintain the temperature. Rumen fluid was filtered through 4 layers of cheese cloth and mixed, but not blended. The rumen fluid was then inoculated within 30 minutes of harvest. The flasks were swirled at least once per day. Renovation of the media was performed after 120 h, pouring into the flask exactly the same buffer and inoculum mixture as in the beginning, using the same two cows and the same procedure previously described. At the end of the 240 h, samples were analyzed for aNDFom (Mertens, 2002), using the glass microfiber filter (Whatman 934AH) inserted into the Gooch crucibles. Blanks were created by inoculating the flasks with buffer and rumen fluid and the associated re-inoculation to correct for any particles introduced into the in vitro system with the rumen fluid. All fermentations (iNDF) were conducted in triplicate and all other samples (NDF and ADL) were analyzed in duplicate. The ratio between iNDF and ADL/NDF was back-calculated for each sample and forage and regressed on ADL/NDF. Different regression equations were generated and tested for each forage group and for all forages and compared between the data sets obtained from the two

procedures, without and with the filter. The coefficient of determination (R^2) was used to assess the improvement of the regression equations tested with the two data sets generated.

Two parallel fermentations were performed with a subset of the samples (about 30% of the whole in-vitro data set) to assess reproducibility of the iNDF values obtained by the in-vitro assay. In parallel procedures, an in-situ fermentation and a fermentation using the Daisy^{II} Incubator (in-Daisy) (Ankom Technology Corp., Fairport, NY) were conducted. For both procedures fermentations were carried in bags of polyester polyethylene terephthalate (PPT) with 15 μm porosity and 8.5% open area as indicated by Huhtanen et al. (2006). To have no more than 20 mg × cm^2 (Huhtanen et al., 2006) 0.75 g of each sample was inserted in bags and bags were sealed, for both in-situ and in Daisy incubations. The same fistulated cows were used for the in-situ fermentation. For the Daisy incubator, the inoculums and buffer solutions were the same as used in the in vitro procedure and renovation of the media was also performed after 120 h, pouring into each jar exactly the same buffer and inoculum mixture as in the beginning, using the same two cows. After the long term incubations, bags were washed with cold water for 30 minutes and dried at 60 °C for 48 h. The residues were analyzed for NDF, using amylase, but were not corrected for ash (aNDF). Preliminary tests indicated that 288 h (12 d) and 384 h (16 d) of fermentation were necessary to reach the maximum extent of digestion for the Daisy incubator and for the in-situ incubation, respectively. Pearson correlation coefficients were run between the in-vitro reference method and the two other fermentations (in-situ and in-Daisy) to assess reproducibility of the iNDF values obtained with the in-vitro method. The Student's *t*-

distribution was used to test the differences between the means (paired comparisons) of the in-situ and in-Daisy values, with the values from the in vitro procedure. Values of iNDF obtained with the in-situ procedure and the Daisy incubator were also regressed on the in-vitro results. The significance of the deviation of the intercept from 0 and the slope from 1 was analyzed by *t*-test. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

The percentage differences in recovery by forage group for NDF and iNDF, from the 240 h in-vitro incubations are found in Table 4.1. Values for ADF and ADL can be found in Table 4.2.

Table 4.1. Percent difference in recovery of neutral detergent fiber (NDF) and indigestible NDF (iNDF), from the 240 h incubations, when using a glass microfiber filter with 1.5 μm porosity for conventional corn silages (C.S.), bmr corn silages, grasses, immature grasses and alfalfas (ranges in parentheses).

Group	Samples	NDF	iNDF
Conventional C.S.	30	2.3 (-0.5 – 6.4)	11.9 (0.0 – 40.5)
Bmr C.S.	15	2.8 (-1.8 – 3.1)	11.8 (21.2 – 75.2)
Grasses	13	1.8 (-0.2 – 2.4)	18.2 (2.1 – 48.3)
Mature grasses	11	0.4 (-0.1 – 3.0)	15.2 (3.7 – 46.8)
Immature grasses	15	1.1 (-1.9 – 2.1)	21.3 (13.2 – 56.3)
Alfalfas	18	2.7 (-1.0 – 4.2)	10.1 (0.0 – 35.3)

Table 4.2. Percent difference in recovery of acid detergent fiber (ADF) and acid detergent lignin (ADL) when using a glass microfiber filter with 1.5 μm for ADF and ADL procedure, for conventional and bmr corn silages (C.S.), grasses, mature and immature grasses and alfalfas (ranges in parentheses).

Group	Samples	ADF	ADL
Conventional C.S.	30	3.3 (-0.5 – 9.9)	23.2 (2.7 – 41.0)
Bmr C.S.	15	7.0 (-1.8 – 15.1)	27.5 (-1.5 – 67.2)
Grasses	13	0.7 (-0.4 – 5.0)	10.9 (-2.2 – 29.3)
Mature grasses	11	0.8 (0.0 – 4.3)	8.2 (-1.3 – 23.2)
Immature grasses	15	19.1 (1.9 – 11.1)	38.3 (19.25 – 90.5)
Alfalfas	18	2.7 (-1.0 – 11.2)	3.2 (-1.6 – 10.1)

The use of the small porosity filter resulted in average increased recovery ($P < 0.05$) for iNDF, among all forage groups, with values ranging between 0.0% and 75.2%. Numerically, the highest average recovery was for immature grasses (21%) with the largest values for bmr corn silages (11 to 75.2%). The ADF and ADL recoveries across groups increased as well ($P < 0.05$), ranging on average between 0.7 and 19.1% and 3.2 and 38.3% for ADF and ADL, respectively (Table 4.2). The recoveries of NDF were also numerically positive on average, across all groups, but not different than zero ($P = 0.27$).

A description of the NDF, ADL and iNDF values of the forages by group are found in Table 4.3. When evaluated by forage, recoveries increased as the degree of lignifications decreased or possibly when stage of harvest was earlier in plant development like during the vegetative stage (Table 4.2). This suggests that as the overall lignin content decreases, or

with lower maturity, the degree of cross-linking decreases, and this allows for the formation of smaller particles that have the capacity to flow through the bottom of the sintered glass crucible (40 μm porosity) and be lost to recovery. This was demonstrated by Udén (2006) in NDF sample analyses and further demonstrated by Raffrenato and Van Amburgh (2010, submitted) in the analyses of ADL. In our previous work, the increased recovery of ADL among forages averaged 19% but varied by lignin content, with the lower lignin forages, like bmr corn silages charactetized by the greatest differences in recovery, whereas the grass and alfalfas demonstrated similar recoveries overall.

As previously mentioned, a subset of samples from all forage groups were also fermented in-situ, using the same two cows used to collect the rumen fluid for the in-vitro and Daisy^{II} system. Incubation times needed to reach the maximum extent of NDF digestibility in the in situ fermentation were higher than the in-vitro system because the PPT fabric used, minimizes or prevents the flow of particles through the bag allows only moderate microbial activity within the bags (Huhtanen et al., 1998). However, iNDF values were highly correlated with coefficients of 0.87 and 0.89, between the in-vitro and the in-situ and the in-vitro and the Daisy^{II} incubator system, respectively. Furthermore, pairwise comparisons resulted in no difference between the iNDF values from 240 h in vitro incubation and the values from both the in situ and in-Daisy ($P > 0.05$).

Table 4.3. The neutral detergent fiber (NDF), acid detergent lignin (ADL), indigestible NDF (iNDF) and calculated ratio of iNDF/(ADL/NDF) values of forages analyzed. The iNDF was determined after in vitro fermentation for 240 h and the assays were conducted using a glass microfiber filter with 1.5 μm porosity. Ranges in the calculated ratio of iNDF/(ADL/NDF) are in parentheses.

Group	Samples	NDF	ADL	iNDF	ratio
		%DM	g/kg NDF		iNDF/ADL
Conventional C.S.	30	42.67	72.4	316.8	4.72 (1.73-7.59)
BMR C.S.	15	39.06	43.6	171.7	4.01 (3.14-5.45)
Grasses	15	47.25	62.1	222.8	3.63 (2.51-4.73)
Mature grasses (straws and hays)	11	64.51	84.4	313.8	3.89 (2.60-5.64)
Immature grasses	13	44.06	59.3	232.2	4.16 (2.59-7.40)
Alfalfas	18	36.64	172.6	461.4	2.70 (2.43-2.95)

Regressions of in-vitro iNDF values on iNDF values from both the in-situ and the Daisy incubator show positive intercept values, but not statistically different than zero ($P = 0.38$) (Table 4.4). This demonstrates that the in-vitro system was able to simulate the extent of digestion from the rumen of a cow, with a lower incubation time required to reach an asymptote (240 vs. 384 h). Furthermore the Daisy^{II} incubator can be used to obtain iNDF values using 288 h residual NDF values with the use of the small porosity bags.

Table 4.4. Relationship between the iNDF obtained from the in-vitro and the in-situ and from the in-vitro and the Daisy incubator.

System	Intercept[a]	Slope	R^2
In-situ	24.831	0.8976	0.89
Daisy	21.765	0.8821	0.92

[a]: g/kg NDF

The improvement in recovery of both NDF after fermentation and ADL changes the results of the relationship between ADL, NDF and iNDF and demonstrates that the relationship is not static. These differences in recoveries in both the fermented NDF residues and the ADL provide a new perspective on the concept of iNDF as published by Chandler et al. (1980). In paper, the ratio of iNDF /(ADL/NDF) ranged between 1.5 and 3.0. However, the standard filtering procedures were used (40 μm crucibles) and the loss of particles from both ADL and iNDF might have caused those ratios for the specific feeds analyzed. Recently Weisbjerg et al. (2010) examined potential laboratory methods for iNDF estimation on grasses and legumes and showed ranges of the ratios between ADL and iNDF of 1.27-4.57 and 1.22-3.59 for grasses and legumes, respectively, demonstrating the dynamic behavior of iNDF. Furthermore Weisbjerg et al. (2010) fermented their samples until 288 h in situ, using 12 μm porosity bags, but ADL values were estimated without using a filtering aid.

The ratios of ADL/NDF residue after 240 h demonstrate several observations that can impact the availability and subsequent feeding value of NDF. The first observation is that the new data demonstrates the value of 2.4 published by Chandler et al. (1980) cannot be considered valid among all

forage groups, and it represents only an average, but with a large range. The only forage close to that range were the alfalfa forages and alfalfa showed the lowest average ratio and the smallest range, with the ratio being numerically the closest to the 2.4 at approximately 2.7 and this was also confirmed by Weisbjerg et al. (2010). All other forages averaged about 4, with a range of 1.7 to 7.6. Further, Huhtanen et al. (2006) confirmed that the present data do not support a general applicable relationship between permanganate lignin and iNDF measured by 12 days in situ fermentation, although the overall slope was 2.4. In that case slopes for individual forages species varied between 2.8 and 5.5, and a general regression equation predicted iNDF with an unsatisfactory accuracy (R^2=0.56; RMSE=27.4 g/kg dry matter). Only using forage specific relationships lowered the RMSE for iNDF to 14.9 g/kg dry matter.

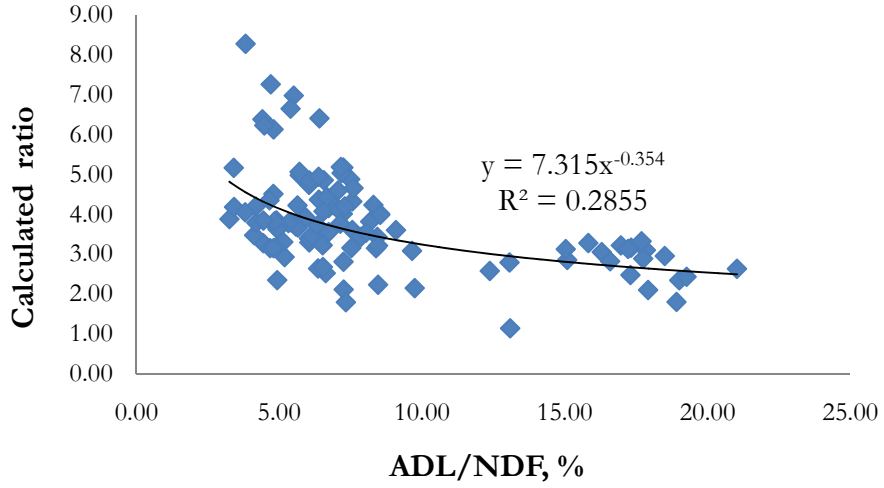
This is a significant departure from the previous data and strongly suggests that there are agronomic and genetic effects of lignification and cross-linking that make the ratio dynamic by growing conditions and season. For example, a low lignin forage like bmr corn silage shows a relatively wide range, from 3.14 to approximately 5.5, which indicates that although bmr forages are lower in lignin, the percent of unavailable NDF is relatively high and if used in the estimation of rate of degradation, would cause an increase in rate of digestion, due to the relatively high degree of unavailability.

Grasses showed the greatest range and this range was due to the inclusion of grasses at many stages of maturity. The very young, early vegetative grasses were in the 1.7 to 2.0 range for ratio of ADL/NDF but increased to values greater than 7. Thus, there are two overarching factors affecting this development of the iNDF pool, the stage of maturity and the agronomic

conditions (e.g. light, heat, water stress, soil type) that interact with genetics to impact the digestibility and extent of digestion and potential rate of digestion of the NDF. This would help explain why forages, for example corn silage, could be nearly identical in chemical composition, but have widely different digestibilities and result in different cow feeding behavior.

Among the equations generated, a power function of the type $y=ax^b$, with y representing the ratio and x being ADL/NDF, resulted to be the best fit based on R^2. Overall, R^2 ranged between 0.10 and 0.69 when not using the filter, and 0.22 and 0.96 when using the filter. All groups where characterized by a numerical higher R^2 when using the filter. Prediction of the ratio still presents much variability. However the data clearly show a dynamic behavior of the ratio among and within forage groups. Maturity and physiological stage of the plant at harvest seem to determine the link between the lignin content of the cell wall and how much that affects the extent of digestion, even with the same lignin content. This appears to be true across forage groups and within groups (Figure 4.3).

A



B

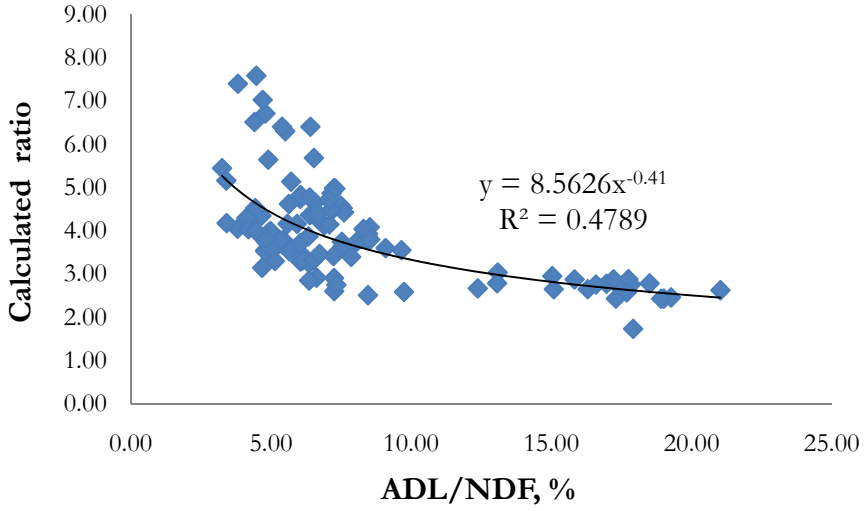


Figure 4.1. Ratios of iNDF/(ADL/NDF) back-calculated after obtaining iNDF, ADL and NDF without (A) or with (B) using a filtration aid, vs. ADL/NDF, for all forages in the data set with power function generated and R^2.

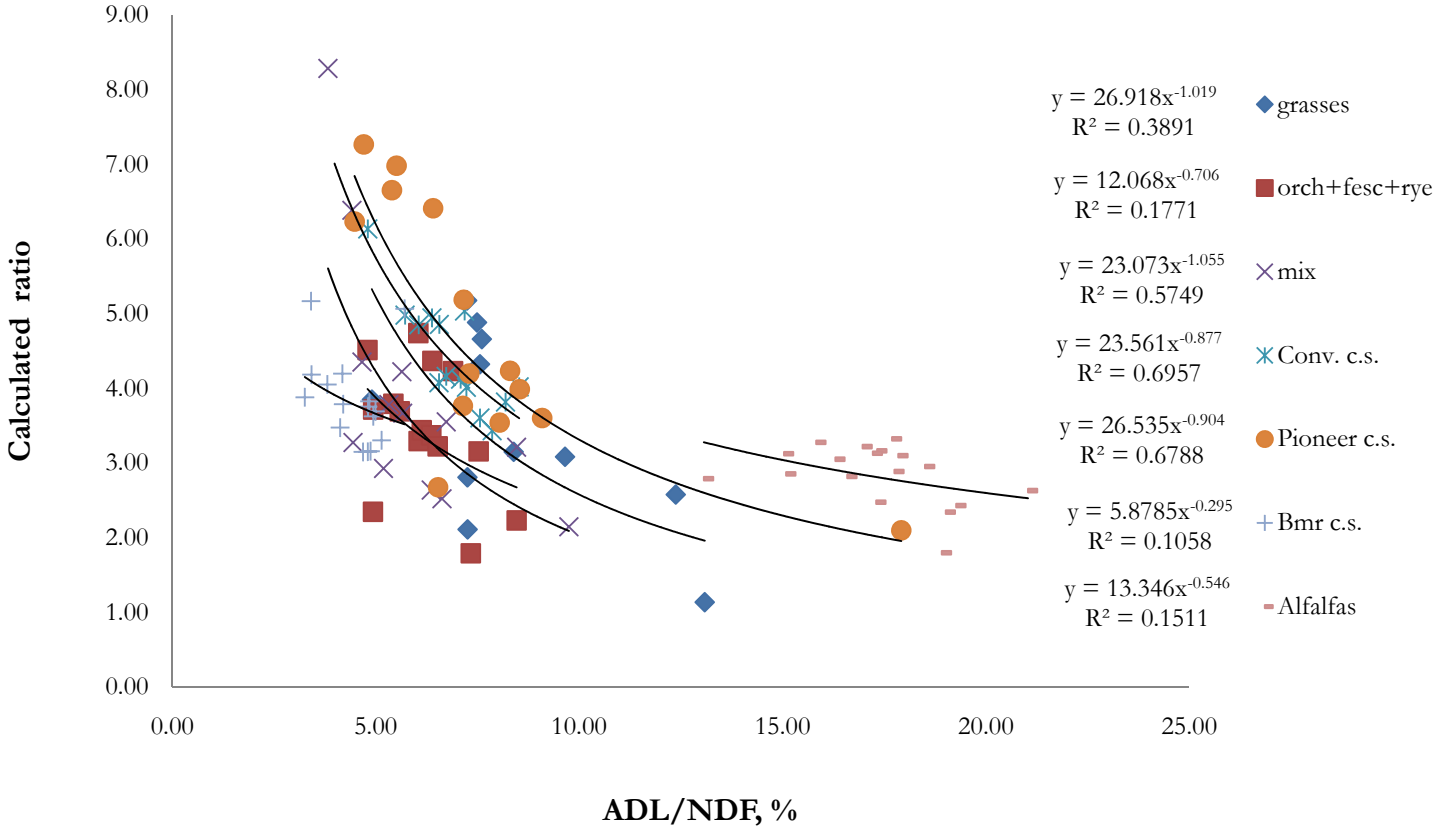


Figure 4.2. Ratios of iNDF/(ADL/NDF) back-calculated after obtaining iNDF, ADL and NDF without using any filtration aid, vs. ADL/NDF with forage group-specific equation generated and respective R^2.

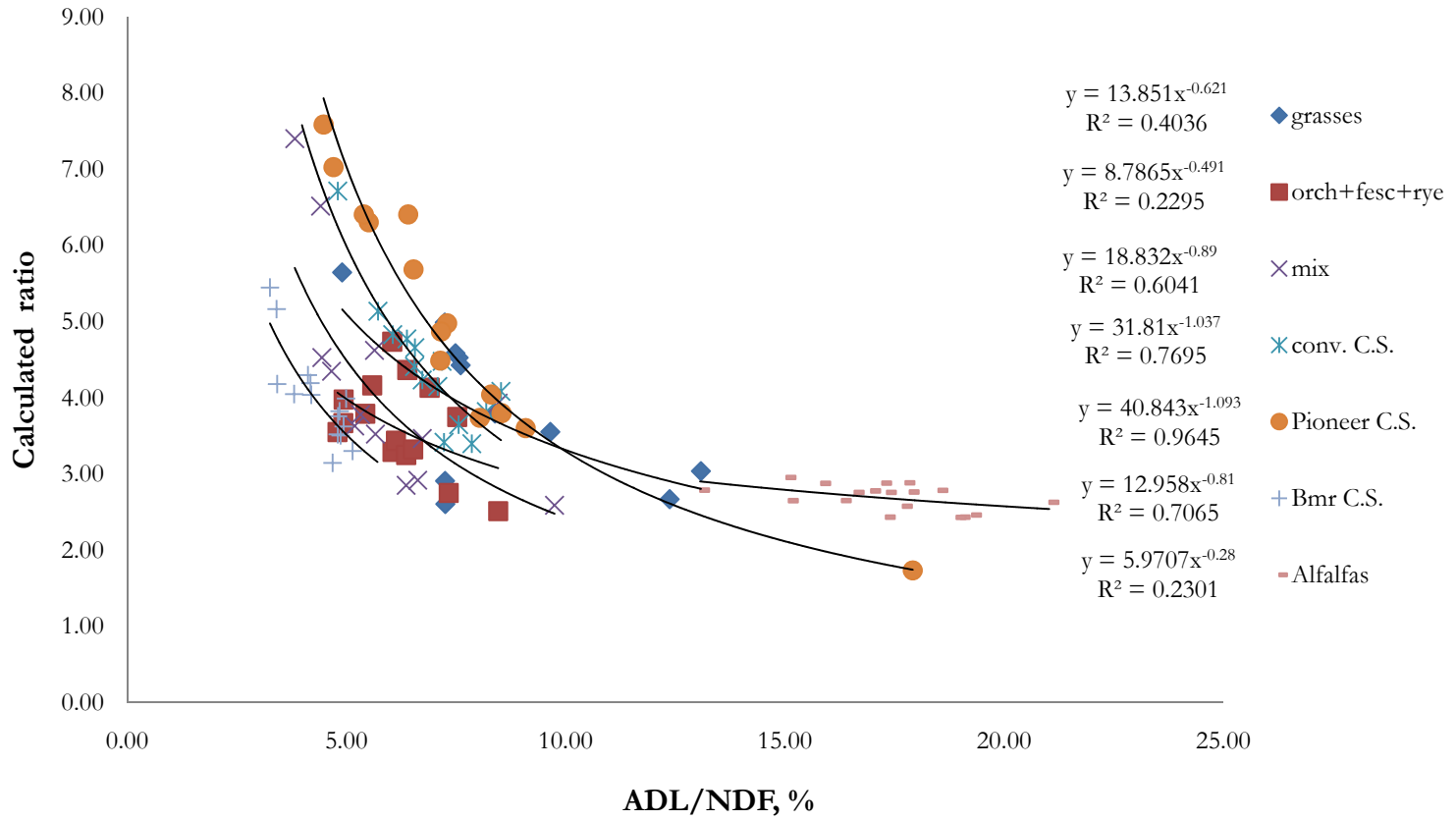


Figure 4.3. Ratios of iNDF/(ADL/NDF) back-calculated after obtaining iNDF, ADL and NDF using a glass microfiber filter (934-AH, Whatman), vs. ADL/NDF with forage group-specific equation generated and respective R^2.

CONCLUSIONS

These data form the basis for an improved approach to describe the variation in indigestible NDF among and within forages allow us to better explain the dynamic behavior of iNDF among and within forage species. A single value of the ratio iNDF to (ADL/NDF) among and within forages does not properly estimate the size of the potentially digestible NDF, resulting in many cases of an under-estimations of the rate of NDF digestion. Further, this data and laboratory approach should encourage the adoption of the glass microfiber filter to increase recoveries of both ADL and iNDF.

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CHAPTER FIVE: EFFECT OF LIGNIN LINKAGES WITH OTHER PLANT CELL WALL COMPONENTS ON IN VITRO AND IN VIVO NEUTRAL DETERGENT FIBER DIGESTIBILITY AND RATE OF DIGESTION OF FORAGES

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ABSTRACT

Content of acid detergent lignin (ADL) does not always account for the observed variability in NDF digestibility. The chemistry of ADL cross-linkages with cell wall polysaccharides rather than amount of ADL alone has been suggested as a better predictor of NDF digestibility (NDFD). The objective of our work was to evaluate the effects of ester- and ether-linked *p*-coumaric (*p*CA) and ferulic acid (FA) on in-vitro and in-vivo NDFD. Thirty forages including conventional and brown midrib corn silages, alfalfa, and immature and mature grasses were incubated in-vitro for measurement of 24 h and 96 h NDFD. Undigested and digested residues were analyzed for NDF, acid detergent fiber (ADF), ADL and Klason lignin (KL), and ester- and ether-linked *p*CA and FA were determined in these fractions. Additionally, three of the corn silages were fed to 6 ruminally fistulated cows for 3 wk in three iso-NDF diets in a completely randomized design. Diet, rumen, urine, feces, and plasma samples were taken every 3 h for three d at

10 d from the start of the study. Intact samples, NDF, and ADF residues were analyzed for ester- and ether-linked *p*CA and FA. Phenolic acid content (total *p*CA and FA) was highest for corn silages (30.62 mg/g NDF), among all forage groups. The content of ester and ether linked *p*CA and FA in both NDF and ADF residues were variably correlated with NDF digestibility parameters, reflecting the competitive effect of these linkages on digestibility. Also, both Klason lignin and ADL were negatively correlated with ether-linked ferulic acid on an NDF basis. Correlations among esterified ferulic acid, esterified *p*CA, etherified *p*CA and all fiber fractions were consistently negative. However, lignin content and chemical linkages explained most of the variation in both rate and extent of NDF digestion, but not uniformly among forage groups, ranging from 56% to 99%, except for alfalfa that resulted in no linkages explaining NDF digestion. The analyses from the in-vivo study confirmed the in-vitro results, demonstrating the highest total tract NDFD (70%) was for the corn silage with the lowest phenolic acid and ester-linked *p*CA content in the ADF fraction. Digestibility of forage fiber is influenced not by the gross chemical composition of the plant, but more specifically the linkages among lignin and the carbohydrate moieties and this varies by plant species and most likely by the agronomic conditions the plant is grown under.

INTRODUCTION

Forages are the foundation of all ruminant diets and forage digestibility is of significant importance for dry matter intake, energy yield and economic viability. Interactions, both chemical and physical, among lignin and the carbohydrate moieties of plant cell walls impact the rate and extent of NDF digestion. Data generated in our laboratory indicates that corn silages can have nearly identical chemical composition (NDF and lignin) but vary 50% in NDF digestibility (NDFD) (Van Amburgh, personal communication). Thus, the content of lignin is not directly responsible for digestibility of corn silage, but more likely it is the degree of cross-linking of phenolics within the cell-wall carbohydrate that impacts digestibility more than lignin content alone. Effects of specific linkages among lignin and cell wall carbohydrates was demonstrated by Grabber et al. (2009) who used a biomimic model to show that ferulate cross-linking was more important than lignin content in evaluating hemicellulose digestion rate and extent of digestion.

The lignins are the only components in cell walls resistant to bacterial and fungal degradation in the rumen and their association with other cell wall matrix components greatly influences properties of digestion, including the enzymatic degradation of structural polysaccharides. Reductions in degradability are partly related to the increased lignin content of cell walls; however, variations in three-dimensional structure and composition of lignin and its hydrophobicity, encrustation, and cross-linking to other matrix components have also been implicated (Chesson, 1993; Jung and Deetz, 1993). Even when plant selection is targeted at specific lignin properties or lignin-matrix interactions, compensatory or associative changes in other cell

wall characteristics often occur, making it difficult to identify underlying mechanisms controlling cell wall degradability (Grabber, 2005). Plants might, for example, respond to lower lignification by increasing the amount of cross-linking , yielding no net change in digestibility, thus lignin content as a marker of digestibility might be misleading to a nutritionist (Chabannes et al., 2001).

In grasses, hydroxycinnamic acids, namely *p*-coumaric (*p*CA) and ferulic (FA) acids are ester and/or ether linked to cell wall polymers. As result of these coupling reactions, arabinoxylans become extensively cross-linked by ferulate dimerization and by incorporation of ferulate monomers and dimers into lignin. In grasses, ferulates are present as esters of arabinose units on xylans, and many of the ferulate molecules become involved in cross-links between arabinoxylans and by formation of diferulate bridges and/or as nucleation sites for the lignin deposition (Hatfield et al., 1999). Both diferulate cross-linking of lignin to arabinoxylans and cross-linking of lignin to arabinoxylan have been shown to reduce maize cell wall degradability (Grabber et al., 1998a). As xylans became less substituted with arabinose units during development of the maize internode, the degree of substitution of the arabinose with ferulates increased dramatically from 0 to 45% (Jung and Casler, 2006).

Etherified ferulic acid, a measure of cross-linking between lignin and arabinoxylans, has a negative effect on cell wall digestibility (Casler and Jung, 1999; Lam et al., 2003). However for esterified ferulic acid the results are not consistent (Jung and Casler, 1990; Jung and Casler, 1991). Casler and Jung (2006) reported negative effects of esterified ferulic acid on in vitro 24 h NDFD of smooth bromegrass and reed canarygrass, but the relationship

changed to a positive relationship when digestibility was measured at 96 h. If the majority of *p*CA is esterified to lignin and if *p*CA ethers are only linked to lignin (Lam et al., 1992ab; Jung and Deetz, 1993; Lam et al., 2001) , it is probable that these components do not directly affect digestion. More recently Rodrigues et al. (2007) reported negative correlations between lignin and ester and ether *p*CA in meadow hay and positive correlations with etherified ferulate and cell wall digestibility.

Negative correlations between etherified FA and cell wall polysaccharide degradability in young maize internodes, but not in mature plants have been reported by Jung et al. (1998). Their explanation was that during maturation of the cell wall, cross-linkages with lignin through incorporation of ether ferulate will occur, but concentration of both etherified and esterified FA will decrease during plant growth while deposition of lignin and other polysaccharides increase. This dilution effect can mask the impact of ferulate cross-linking, measured by etherified FA concentrations, on digestibility.

Overall it seems that these acids exert an inhibitory effect on degradation kinetics rather than extent of digestion of cell wall. To our knowledge, there is no work that compares conventional and brown midrib (bmr) corn silages, changes with maturity in grasses, and alfalfa. Furthermore, there is little work comparing results in-vitro and in-vivo. Jung et al. (1991) compared recoveries of esterified phenolic acids after in vitro fermentation with previous in vivo data (Jung et al., 1983), and they obtained greater recoveries with increasing concentrations of esterified phenolic acids in both cases. In the latter work Jung et al. (1983) wanted to assess the changes that phenolic monomers of forages undergo during digestion, rather their direct effect on in vivo digestibility. Further, *p*CA and FA in ADF have never been estimated

and we hypothesize that since ADF recovers cellulose and lignin, phenolic acids should mostly be limited to *pCA* linked to lignin. However the acid detergent might solubilize some of the phenolics since it likely dissolves a great part of the lignin contained in the Klason lignin (Jung et al., 1999). We are aware of the large genetic variability among cell types and within and especially among forage species and families because of the different speed of cell wall change and reproductive maturity. However, our objective is to try to integrate recent findings relative to phenolic acids and nutritive value, focusing only on phenotypic correlations. Also, the possible correlations among cell wall components might prevent any type of cause and effect to be determined from these analyses but might lead to a better prediction of fiber digestibility.

Our objective was therefore to evaluate the effect of ester- and ether-linked phenolic acids on *in vitro* NDFD and whether the presence of measurable ester and ether linkages impacted rates of NDF digestibility (k_d) as well as extents of NDFD and if these relationships were similar among forages. Further, since many *in vitro* observations of digestibility do not directly correspond with *in vivo* data, we wanted to investigate the relationship among different corn silage hybrids selected for digestibility and the correlation with ester and ether linkages and *in vivo* digestibility.

MATERIALS AND METHODS

In-vitro study

Thirty forages including conventional and bmr corn silages, alfalfa, immature and mature grasses were dried at 60°C for 48 h in a forced-air oven, and ground to pass through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). The ground samples were stored in screw-topped plastic containers at room temperature. All forages were analyzed for NDR (Mertens, 2002), ADF (Van Soest, 1963a), ADL (Van Soest, 1963b) and Klason lignin (KL) (Theander et al., 1995). The use of sodium sulfite was omitted in the NDF procedure to prevent lignin bonds from being cleaved (Robertson and Van Soest, 1981; Moir, 1982) and biasing the phenolic acids values however, amylase was used. Forages were incubated in-vitro for 24-h and 96-h NDFD (Goering and Van Soest, 1970) in 125-ml Erlenmeyer flasks. The 24-h in-vitro NDFD (ivNDFD) values were used to calculate the rates of NDF digestibility, k_d (Van Amburgh et al., 2003). All samples were filtered through a glass microfiber filter with porosity of 1.5 μm (934-AH™, Whatman® Limited – GE Healthcare, Maidstone, UK), inserted in 50-ml Pyrex® Gooch crucibles. In the case of ADF and ADL procedures, the filter replaced the original asbestos that was rendered a health hazard in 1989 by the United States Environmental Protection Agency (EPA, 1989, Asbestos Ban and Phase Out Rule) and removed, but never replaced (Raffrenato and Van Amburgh, submitted). The ADL analyses were conducted on ADF residues, and ADF measured on intact samples. The forages were selected based on previous analyses that

demonstrated wide differences in digestibility among the samples. Preliminary analyses resulted in no detection of phenolic acids in ADL, therefore ADL residues were not analyzed for *p*CA or FA for the study. Intact samples, NDF, ADF, and KL residues were analyzed for ester- and ether-linked *p*CA (Es-*p*CA, Et-*p*CA) and ester- and ether-linked FA (Es-FA, Et-FA) using modified procedures by Iiyama et al. (1990) and Jung and Shalita-Jones (1990). Extraction of *p*CA and FA was by 2*N* and 4*N* NaOH. Extracted samples were then stored at -20 °C. Phenolic acid separation was done in a Waters Spherisorb® ODS-2 (4.6 × 250 mm, 5 μm; Waters, Milford, MA) column with a Supelco Pelliguard LC-18 (2 cm, Supelco, Inc., Bellefonte, PA) guard column. Temperature of the column was set at 50°C. Samples (20 μm) were eluted with a with a mobile phase consisting of a solution of 97.7% water, 2% butanol and 0.3% glacial acetic acid (solution A) and methanol (solution B) as follows: isocratic elution 100% A, 0-20 min; linear gradient from 100% A to 80% A/20% B, 20-27 min; linear gradient from 80%A/20%B to 100% A, 28-31 min. Flow rate was 1.8 ml/min and the injection volume was 20 μm. The monitoring wavelength was 320 nm with a 4 nm bandwidth. PCA and FA were detected at 320 nm, with a 4 nm bandwidth, and quantified using the external calibration method.

In-vivo study

To determine if the observations from the in vitro study could be replicated through in vivo digestibility, a parallel study was conducted using lactating dairy cattle fed three of the corn silage hybrids also used in the in vitro study and were chosen based on expected differences in NDF

digestibility. A conventional low digestibility (CLD), a conventional high digestibility (CHD), and a bmr corn silage (BMR) were used in the in vivo study (Chase et al. 2010). The corn silages resulted in 46.1, 47.1 and 56.5 24 h NDFD for the CLD, CHD and BMR, respectively (Table 5.5). After calving the cattle went on a common post-calving total mixed ration. Approximately 10 DIM the cows were then assigned to one of three treatments, transitioned onto the treatment diets and the study was conducted for 10 wk (Chase, 2010). Six ruminally fistulated, multiparous cows were housed in tie-stalls and at approximately 60 DIM were sampled for the in vivo study. The treatment was assigned to cows in a completely randomized design. Diets were formulated to be iso-NDF and fed as total mixed rations (TMR) to 10% refusal. The rations contained 35.8, 37.8 and 35.4% NDF and were 59% corn silage, 5.5% wheat straw and 35.5% concentrate for the CLD, CHD and BMR diet respectively. Dry matter intakes of the cows were 26.53, 25.33 and 26.91 kg/day for the CLD, CHD and BMR diet, respectively (Table 5.6). Samples of TMR, feces, urine, plasma, and rumen samples were taken for 3 d every 3 h. Acid insoluble ash was used to estimate total tract dry matter digestibility (TTD) and feces excreted (Van Keulen and Young, 1997). Creatinine in urine was used to estimate total daily urine excreted (Chen et al., 1992). Phenolic acids in forages, TMR, rumen and feces samples were analyzed using the procedures as described above. Phenolic acids in urine and plasma were analyzed using the procedure by Zhao et al. (1990).

Statistical analyses

To determine how much variation in 24 h and 96 h NDFD and k_d was explained by the effect of lignin linkages and phenolic acids, a multiple regression with stepwise selection was run within each forage group using the independent variables lignin type (ADL or KL) and the difference between them on an NDF basis to determine Pearson correlations among selected variable. Similarly another multiple regression with stepwise selection was run within each forage group using phenolic acids with their specific linkages also on an NDF basis to establish correlations among phenolic acids and NDFD and k_d. The collinearity between explanatory variables was assessed with variance inflation factors (VIF) and tolerance values. Only variables with VIF lower than 2.5 and tolerance larger than 0.40 were allowed in the models to control for collinearity (Neter et al., 1996). Significance for the associative effects was declared at $P < 0.10$ and specific P values are stated. Differences among treatment means for the in-vivo study were detected using Tukey procedure (Neter et al., 1985). Significance was declared at $P < 0.05$ unless otherwise stated. All statistical analyses were conducted using SAS (version 9.1.3; SAS Institute, 2008).

RESULTS AND DISCUSSION

In-vitro study

The chemical composition of the forages analyzed along with the in-vitro NDFD at 24 and 96-h and the cell wall phenolic acid contents (FA and

*p*CA fractions) of Es-*p*CA, Et-*p*CA, Es-FA and Et-FA are presented in Table 5.3. Consistent with literature values, the bmr corn silages were lower in NDF, ADF and lignin while the NDFD was approximately 35% greater than the conventional corn silages (Oba and Allen, 2003). Similarly, the phenolic acid content was higher for corn silages ($P < 0.05$) for Es-*p*CA Et-*p*CA, Es-FA and Et-FA. In particular, while conventional corn silages contained higher levels of both *p*CA linkages, FA content was higher in bmr corn silages on an NDF basis, but lower on DM basis ($P < 0.05$) and based on the data of Grabber et al. (2009) this should indicate lower potential NDF digestibility. The higher concentration of *p*CA for the corn silages has been observed before and is explained by the observation that C4 plants have higher *p*CA content than C3 grasses (Cherney et al., 1989; Ford and Elliott, 1987; Grabber et al, 1991). In particular, bmr mutants are known to have lower lignin values and a lower frequency of syringyl lignin (S) units (Méchin et al., 2000; Marita et al., 2003). Therefore, fewer *p*CA esters are consistent with the preferential acylation of S units by *p*CA. The higher content of both Es- and Et-FA for BMR corn silages compared to conventional hybrids was unexpected, primarily because cross-linking of lignin to arabinoxylan has been shown to reduce maize cell wall degradability (Grabber et al., 1998a, 1998b). This relationship appeared to be counterintuitive for a higher digestibility plant, however, more recently Marita et al. (2003) obtained a greater amount of FA esterified to arabinoxylans in younger plants of bm3 cell walls, compared to wild types. This strengthens the hypothesis that the bm3 gene is not involved in FA biosynthesis and linkage. Most *p*CA is now thought to be ester-linked to S units in lignin, a view consistent with the commonly made observation that

*p*CA is deposited in parallel with lignin (Scobbie et al., 1993; Ralph et al., 1994). Estimates of Et-*p*CA are made by difference and are prone to error since the presence of Et- *p*CA might in part arise because of difficulties in fully hydrolyzing *p*CA esters in lignin (Hartley and Morrison, 1991).

Of interest was the phenolic acid composition of the corn silages which contained the highest values of Et-*p*CA among the groups. If this is the result of underestimated values of total *p*CA or the presence of Et-*p*CA is unknown – or it might reflect the use of the entire corn plant in our experiment, whereas most other experiments evaluated only the stem and leaf sections (Morrison et al., 1998). We are aware that these types of analyses can underestimate ferulate and diferulate deposition in secondary walls because radical coupling of ferulate and diferulate to lignin prevents the recovery of most of these acids by solvolytic methods used to degrade lignin (Grabber et al., 2000; Grabber et al., 2004). The similarity in Es-FA content and the difference in Et-FA ($P < 0.05$) between immature and mature grasses supports the concept that ferulate esters of arabinoxylan are deposited in the primary wall of grasses and ether cross-links to lignin form later (Ralph et al., 1998; Jung and Casler, 2006). A similar difference in Es-*p*CA content between immature and mature grasses, and between conventional and BMR corn silages supports previous observations that most *p*CA esters are on syringyl monolignol units (Ralph et al., 1994) and that only very small quantities of *p*CA can be esterified to arabinoxylans in immature tissues (Morrison et al., 1998; Vailhe et al., 2000).

Correlation coefficients among fiber fractions and esterified or etherified *p*CA and FA content among all forages are found in Table 5.4. Neither KL nor ADL were correlated with Et-FA on a DM basis, and they were both

negatively correlated with Et-FA on an NDF basis ($P < 0.05$). These negative correlations result from observation that both lignin and Et-FA were positively correlated with NDF on a dry matter basis. Thus, both components contribute to NDF concentration, but somewhat competitively. Of significance are the consistent negative correlations, on both a DM and NDF basis, among Es-FA, Es-*p*CA and Et-*p*CA, and all fiber fractions analyzed. This suggests that the various processes leading to secondary cell wall development and cessation of cell growth are synchronized consistently in all genotypes (Casler et al., 2008), but also lignin and phenolic acids might negatively affect NDF digestibility in a competitive manner (Casler and Jung, 1999) with specific cross-linking playing a bigger role than lignin content per se (Grabber et al. 2009).

The analysis of *p*CA and FA in the intact forages, fiber fractions and lignin types allowed for the comparison of recoveries, within forage group, in each of these fractions. The amount of *p*-CA and FA, respectively, recovered in each of the fractions when compared to intact sample amounts are shown Figures 5.1 and 5.2 and expressed as a total of the recovered phenolic acids the intact sample. No phenolic acids were detected in the ADL fraction and this demonstrates the irreversible polymerization of phenolic acids through the lignification phase of plant development. Overall, the ND treatment resulted in a loss of both *p*CA and FA from 0 and 25%, among all samples except for alfalfa, with no differences between ether and ester linkages. However, the ND extraction resulted in the alfalfa samples losing between 80 and 90% of the phenolic acids, especially FA, from the intact forage. Given the relatively high levels of pectin in alfalfa and pectin solubility in ND solution, this loss is primarily from the removal

of phenolics contained in the pectic side-chains. Loss of pectic substances from the plant cell wall by ND extraction could account for some of the differences in yield of phenolic acids, especially FA between the intact sample and the ND residue (Jung and Shalita-Jones, 1990). Previous data has shown that FA, and some *p*CA, is esterified to arabinose and galactose units of spinach pectic substances and feruloylated pectins also have been reported in sugar beet pulp (Fry, 1982; Fry, 1983; Rombouts and Thibault, 1986), thus, the observed differences were most likely due to solubilization of the pectin in the ND. The loss of phenolic acids due to the ADF and Klason lignin procedures was larger ($P < 0.05$) for esterified than etherified FA, presumably because during those procedures FA, esterified to arabinose substitutions, was solubilized. Several studies have shown that a proportion of FA might act not only as a cross-linking agent between lignin and carbohydrates but also among carbohydrates to strengthen the cell wall (Eraso and Hartley, 1990; Ishii, 1991; Iiyama et al., 1994; Bartolomé et al., 1997a; Garcia-Conesa et al., 1999; Saulnier et al., 1999; Iiyama and Lam, 2001).

One of our objectives was to integrate our knowledge about digestibility of different forage groups with their phenolic acid characteristics. The analysis was conducted among all forages to determine if any specific phenolic acid or linkage affected the rate and extent of fiber digestibility. Correlation coefficients among digestibility variables and lignin types and phenolic acids on a NDF and ADF basis are found in Table 5.5. The highest and most significant correlations were obtained when pooling all forages. However , the NDF digestibility values and rate coefficients were not consistent among forages. When all the forage data were pooled, the

ADL and KL were positively correlated with rate of digestion but all of the ester and ether linked FA and *p*CA were negatively correlated with rate. In these analyses, except for alfalfa, ADL always resulted in a negative correlation with k_d, 24-h NDFD and 96-h NDFD, however, this was significant ($P < 0.05$) only for grasses and immature grasses. Within bmr corn silages, the correlation for digestibility when expressed on an NDF basis, with Es-FA, Et-FA, and Es-*p*CA, was positive, significant, and somewhat surprising. This observation is not consistent with the data of Grabber et al. (2009) that demonstrated in a biomimic model that ferulate cross-linking had the greatest negative impact on fiber digestibility. The current data would suggest that within the bmr plant, the FA esters and ethers bonds are formed, producing measurable FA but that a final polymerization step is not occurring, thus not negatively impacting digestibility. Bmr corn silages have higher levels of FA in NDF but this concentration is not a good indicator of digestibility. Further, Eth-FA in NDF is not a good indicator of polysaccharide-lignin cross-linking in bmr corn silage and this is inverted in ADF, unlike other forages except for immature grasses that behave similarly suggesting that the bmr gene maintains immature behavior – low level cross-linking and polymerization in a mature plant.

Generally, the mature grasses and the conventional corn silages demonstrated similar behavior among linkages and measures of digestibility. There were some differences among the mature and immature grasses, especially for KL, Et-FA and Et-*p*CA where the immature grasses demonstrated positive digestibility with KL and etherified phenolic linkages and the opposite for the mature grasses. Overall, grasses evaluated on an

NDF basis resulted in positive correlations between Es-FA and all digestibility measures whereas the correlations became negative on ADF basis.

The relationships among the phenolic acids and 24-h NDFD on an NDF and ADF basis are found in Figures 5.3a and 5.3b and for comparison, the same relationships with 96 h NDFd are found in Figure 5.4a and 5.4b. The relationships for most of the FA and *p*CA linkages were similar on an NDF and ADF basis when evaluated at 24 h. The two most significant differences were in the relationship among Es-*p*CA and digestibility on an NDF or ADF basis (Figure 5.3a panel A vs B).

On an NDF basis there was a strong positive correlation with Es-*p*CA in bmr corn silages, whereas on an ADF basis, the relationship was negative and suggests that the linkages do not impede digestion when associated with the arabinoxylans associated with NDF. Further, this relationship is only observed in bmr corn silages and demonstrates differences in how linkages are altered in the bmr plant. There were also differences among the forage families between 24-h NDFD and Es-FA and Et-FA where bmr corn silage and mature grasses demonstrate positive relationships with digestion whereas other forages there were negative associations with the phenolic acid and digestibility. These observations are not consistent with the biomimic model of cell wall digestibility and FA from Grabber et al. (2009). These differences in the relationship between cell wall digestibility and FA might be related to the use of an intact plant that also includes the grain and cob. Similar observations were made of forage digestibility and phenolic acid linkages when evaluating them at 96-h of digestion (Figure 5.4a and 5.4b). The positive relationship between Es-*p*CA and 96-h digestibility on an

NDF basis was not expected in any of the forages, however the bmr corn silages were positively correlated (Figure 5.4a A) however, again on an ADF basis, this was a negative relationship (Figure 5.4a B). Again for the grasses and the bmr corn silages, there was a positive relationship in 96 h NDFD on an NDF basis and Es-FA (Figure 5.4a C). According to Grabber et al. (2004), correlative studies dealing with ferulates, like this, are often hampered because the quantity of ferulates recovered after alkaline or acid hydrolysis represent only a small and variable proportion of the total ferulates deposited in cell walls. Our analysis of the different fractions was designed to help clarify this recovery problem by providing more information on the location and respective differences in ferulates among the fractions. The different recoveries of both *p*CA and FA in NDF among forage groups demonstrates how the amount of phenolic acids in intact samples might bias these correlations (Jung and Shalita-Jones, 1990). Higher solubility of *p*CA in bmr corn silages is consistent with higher digestibility with less *p*CA incorporation and smaller sized *p*CA-lignin complexes.

One factor not accounted for is that the greater solubility of *p*CA associated with the ND solubles could potentially confound estimates of NDFD and alter the energy derived from the ND soluble and this is similar for immature grasses. These soluble components were identified by Gaillard et al. (1975).

Previous findings in corn plants support the contention that selection of grasses to reduce ferulate cross-linking would enhance the enzymatic hydrolysis and subsequent utilization of structural polysaccharides for nutritional and industrial purposes (Grabber et al., 1998a and 1998b; Jung and Ralph, 1980). However our findings in grasses and corn silages do not

fully support this idea for both rate and extent of NDF digestion. Most of those correlations, significant only for grasses, become negative when Es-FA is analyzed in ADF residues. This different behavior is also demonstrated by the fact that feruloyl esterases can cleave diferulate cross-links in soluble xylans or simple model substrates (Faulds et al., 2003; Garcia-Conesa et al., 1999a), but there is no evidence that these enzymes break a significant proportion of cross links within lignified cell walls, therefore their use is currently limited. The negative correlations for ADF residues also show how the AD treatment might not remove those ester linkages that are not degradable by rumen bacteria. Furthermore the AD treatment seems to have a similar behavior of a xylanase; therefore developing potent microbial xylanases can be more promising than feruloyl esterase treatments for enhancing forage cell wall digestion (Grabber, 2005).

A multiple regression with stepwise selection was run within each forage group using the independent variables lignin type (ADL or Klason lignin) and their difference on an NDF basis and phenolic acids with their specific linkages also on an NDF basis to examine the primary factors affecting rate and 24 and 96 h NDFD among the forages (Table 5.6). There were no significant effects identified for the alfalfa forages ($P < 0.10$), demonstrating and reinforcing that NDF digestibility cannot be easily explained in legumes by any these factors. Although legumes have higher lignin content than grasses, they are very low in esterified hydroxycinnamic acids (Hartley and Jones 1977; Hartley and Haverkamp, 1984) and high in Syringyl and Guaiacyl units. This would suggest that legumes have less condensed types of lignins and therefore relatively less impact on cell wall degradation and protection, and therefore degradation cannot be explained as it is in grasses

(Besle et al., 1994). The procedure identified significant relationships among the rate and extent of digestion and phenolic acids and linkages for all the other forages with R^2 that varied between 0.56 and 0.99 (Table 5.6). Results demonstrate that for each forage or stage of maturity, the factors affecting rate and extent of fiber digestion are not the same and the properties of the linkages play an integral role in the digestion behavior of the plants. For example, in the grasses ADL and Et-FA were both significantly negative for rate and 96 h extent of digestion but were not identified as significant factors for bmr corn silage digestion and in fact at 96 h NDFD Et-FA was positively related to NDF digestibility. This data indicates that the KL has the most negative impact on rate of digestion of bmr corn silage and 24 h extent of digestion whereas conventional corn silage is most negatively impacted by ADL and Es-FA (Table 5.6), consistent with the data of Grabber et al. (2009). Both Es-pCA and Et-FA in ADF had a clear negative effect on NDF digestibility and acid detergent treatment likely solubilizes phenolic acids not involved in cell wall "protection" similar to data from Lowry et al. (1994).

Whether these data support an overall genetic effect or a potential effect of the agronomic conditions from which our samples were generated is unknown. The agronomic conditions, light, heat and water stress could potentially create such differences among plant due to changes in enzyme activity during growth that results in variable cross-linking of phenolics and carbohydrates, but that is unknown since we have little information on the specifics of the growing conditions. More likely, these data demonstrate the diversity of factors specific to each forage group and would provide a more robust approach for evaluating genetic selection of forage species. This data

clearly demonstrates that that factors affecting rates and extents of digestion of NDF vary by forage type and most likely maturity.

In-vivo study

The bmr mutant is associated with improving plant stover digestibility and this was observed as previously described. However, at times studies are equivocal relative to the increased digestibility of bmr varieties or the in-vivo responses. For example data published by Barrière et al. (2004) did not result in increased energy efficiency or balance expected from these forages and this was also observed by Tine et al., (2001). Normal corn silage lines and hybrids also display substantial phenotypic and genetic variability for lignin and degradability traits, at times rivaling that observed with bmr varieties (Jung and Buxton, 1994; Argillier et al., 1996; Méchin et al., 2000). In these and other studies with grasses (Casler, 2001), degradability was negatively associated to lignin concentration, but this is not always the case as demonstrated in this paper and by others (Grabber et al., 1998a).

The nutritional characteristics of the corn silages used and the diet parameters along with the total tract dry matter digestibility (TTD) of the three iso-NDF diets provided to the fistulated cows are shown on Table 5.5 and 5.6, respectively. Only the bmr corn silage resulted in a higher 24 h NDFD ($P<0.05$). As expected TTD followed the observed ivNDFD values of the corn silages, with TTD values of 63%, 66% and 70% for the CLD, CHD and BMR TMR, respectively. Analyses of phenolic acids in all ingredients of the TMR, feces, urines and plasma allowed us to determine the total amount of both FA and *p*CA (with respective linkages) ingested and

excreted, using estimated excretion of urine and feces excreted as described in the previous section. We were not able to detect any phenolic acid in plasma, and the urines were characterized by a small amount of phenolic acids (<0.50 grams of total FA or *p*CA per cow) among diets ($P = 0.56$).

The total amount of ingested and excreted phenolic acids in the NDF and ADF fractions of the diets and feces are found in Figure 5.5. The lower Es-*p*CA concentration in the bmr diet compared to the two diets was inversely related to differences in TTD (Figure 5.5 A and 5.5 B). Esterified hydroxycinnamic acids are partly digested in the ruminant digestive tract, however the lower content of Es-FA in the bmr based diet and feces was again inversely correlated with TTD (Figure 5.5 C and 5.5 D) and resulted in consistent differences among all three diets between intake and excretion of the phenolic acids and ranged between 31 and 41 grams and 21 and 37 for intact and NDF samples respectively. The amount of FA (<0.50 grams total FA per cow) in urine represent only a very small portion of the phenolic acid ingested and therefore it is digested in the digestive tract as indicated by Besle et al. (1995). Ferulic acid is more extensively degraded than *p*CA both in vivo (Giger, 1985) and in vitro, where it is metabolized at the same rate as hemicellulose (Jung, 1989).

This difference between TMR and feces is decreased in the ADF samples (Figure 5.5 D) for the CLD and the BMR diets. The NDF samples of the diets show again how Et-FA is not a good proxy for digestibility (Figure 5.5 E), however this changes when considering ADF samples (Figure 5.5 F), as shown in the in vitro results. The acid detergent solution is potentially again acting to "clean-up" those "surface linkages" easily cleaved by bacteria, and leaving the linkages that cause a decrease in NDF digestibility. Previous data

describing the phenolic acid content of ADF residues are not available and therefore comparisons with previous literature are not possible. Recently Taboada et al. (2010) studied the digestibility of silages in relation to their hydroxynnamic acid content and lignin. In their case, in vivo DM digestibility ranged from 46 and 76% and all the phenolic fractions had in general a negative effect on digestibility. We observed a low correlation (P = 0.67), between both Es-Fa and Eth-Fa in NDF and total tract digestibility, and this observation was confirmed by Taboada et al. (2010). They found no correlation between the ferulic acid fractions (on an NDF basis) and in vivo digestibility, for both dry and organic matter. Only ferulic ethers, which may act as cross-linkages between hemicellulose and lignin, seemed to partially explain the differences in in vivo digestibility, in agreement with previous studies (Argillier et al., 1996; Jung et al., 1994). In our in vivo study the analysis of phenolic acids in the ADF residues provided some explanation for the in vivo differences in TTD for all phenolic acid fractions, especially for FA. This difference in digestibility follows by the observation that FA is released more quickly from the cell wall during digestion than *p*CA. In fact, FA was found to be among the most rapidly and extensively removed constituents from plant cell walls (Faulds et al., 1998). The acid detergent treatment may have in our case a similar effect in removing those FAs that are removed more quickly during digestion.

Table 5.1. Chemical composition of the forages analyzed in the in-vitro portion of the study, described per group on a dry matter and neutral detergent fiber basis.

Forage group	n	NDF	ADF	KL	ADL	ivNDFD 24 h	ivNDFD 96 h	Es*p*CA	Et*p*CA	EsFA	EtFA
		-------% DM------		% NDF		-------% NDF--------		---------- --mg/g NDF ----------			
Conv. Corn silage	8	39.61	25.42	18.73	7.17	44.9	67.6	16.52	2.35	7.95	3.80
BMR corn silage	11	35.57	22.32	16.00	1.88	61.1	80.3	12.25	1.41	9.55	5.00
Alfalfa	7	36.37	29.91	28.00	7.11	47.4	55.7	0.42	0.12	0.41	0.25
Grasses	6	73.56	47.82	19.09	8.54	38.7	66.7	3.25	1.05	3.05	3.72
Imm. grasses	5	43.91	29.12	20.96	6.80	66.3	78.9	1.67	0.32	3.43	1.55

Table 5.2. Pearson correlation coefficients among the major variables investigated and among all forage groups. Correlations are expressed on a DM basis above the diagonal and NDF basis below the diagonal.

	Variable							
Variable	NDF	ADF	ADL	KL	Es-FA	Et-FA	Es-*p*CA	Et-*p*CA
NDF		0.93*	0.60*	0.79*	-0.20	0.63*	-0.11	0.28
ADF	-0.18		0.78*	0.89*	-0.40*	0.50*	-0.27	0.29
ADL	-0.02	0.78*		0.86*	-0.65*	0.17	-0.43*	0.17
KL	-0.09	0.79*	0.88*		-0.58*	0.26	-0.40*	0.16
Es-FA	-0.35*	-0.54*	-0.78*	-0.76*		0.31	0.84*	0.15
Et-FA	0.10	-0.61*	-0.72*	-0.76*	0.78*		0.36*	0.42*
Es-*p*CA	-0.30	-0.45*	-0.61*	-0.62*	0.88*	0.71*		0.32*
Et-*p*CA	-0.08	-0.48*	-0.46*	-0.52*	0.60*	0.56*	0.73*	

* Significantly different from zero: $P < 0.05$.

Table 5.3. Pearson correlation coefficients among digestibility variables and lignin types and phenolic acids on a neutral (NDF) and acid detergent (ADF) basis.

Forage	ADL	KL	Es-FA	Et-FA	Es-FA	Et-FA	Es-*p*CA	Et-*p*CA	Es-*p*CA	Et-*p*CA
	----------NDF%----------				-----ADF%----		-------NDF%----		--------ADF%------	
Bmr corn										
k_d	-0.01	0.03	0.73*	0.54*	-0.17	0.24	0.82*	-0.40	-0.81*	-0.73*
24hNDFD	-0.07	-0.10	0.78*	0.54*	-0.26	0.28	0.79*	-0.33	-0.88*	-0.67*
96hNDFD	-0.14	-0.21	0.60*	0.76*	0.00	-0.09	0.52*	-0.48	-0.78*	-0.54*
Conv. corn										
k_d	-0.13	-0.21	-0.65*	0.11	-0.31	0.27	-0.70*	-0.04	-0.62*	0.03
24hNDFD	-0.27	-0.36	-0.28	-0.37	-0.27	-0.12	-0.42	-0.32	-0.80*	-0.42
96hNDFD	-0.09	-0.21	-0.50	-0.07	0.18	0.20	-0.66*	0.04	-0.66*	0.34
Alfalfa										
k_d	0.44	0.16	-0.40	-0.42	0.42	-0.31	-0.38	-0.67*	-0.35	-0.17
24hNDFD	0.56	-0.16	-0.50	-0.58	0.45	-0.51	-0.41	-0.41	-0.35	-0.60
96hNDFD	-0.68*	-0.37	0.13	-0.14	0.15	-0.21	0.14	0.03	0.18	0.10

* Values with asterisk differ significantly from zero ($P < 0.05$).

Table 5.3. (Continued)

Forage	ADL	KL	Es-FA	Et-FA	Es-FA	Et-FA	Es-*p*CA	Et-*p*CA	Es-*p*CA	Et-*p*CA
	----------NDF%----------				-----ADF%----		-------NDF%----		--------ADF%------	
Grasses										
k_d	-0.72*	-0.54	0.71*	-0.68*	-0.49*	-0.36	-0.72*	0.51	-0.60	-0.49
24hNDFD	-0.90*	-0.66	0.94*	-0.30	-0.74*	0.00	-0.93*	0.14	-0.30	-0.72
96hNDFD	-0.90*	-0.79	0.72*	-0.41	-0.75*	-0.14	-0.88*	0.17	-0.64	-0.58
Immature grasses										
k_d	-0.63*	0.22	-0.61	0.86*	-0.41	-0.24	0.24	0.06	0.16	0.21
24hNDFD	-0.93*	0.40	-0.49	0.66	-0.02	0.12	-0.22	-0.35	-0.34	0.40
96hNDFD	-0.89*	0.56	-0.53	0.79*	-0.11	0.10	-0.22	-0.11	-0.25	0.20
All families										
k_d	0.43*	0.49*	-0.37*	-0.57*	-0.53*	-0.67*	-0.50*	-0.54*	-0.54*	-0.53*
24hNDFD	-0.42*	-0.27	0.36*	0.06	-0.21	-0.39*	0.02	-0.22	-0.34*	-0.16
96hNDFD	-0.84*	-0.70*	0.68*	0.57*	0.17	0.06	0.37*	0.19	-0.02*	0.16

* Values with asterisk differ significantly from zero ($P < 0.05$).

Table 5.4. The independent variables selected for each forage group and dependent variable by a multiple regression with stepwise selection within each forage group using the independent variables lignin type (ADL or Klason lignin) and their difference (NDF basis) and phenolic acids with their specific linkages (NDF basis) examining the primary factors affecting rate and 24 and 96 h NDFD. Significance was not achieved when evaluating alfalfa forages so data is not shown.

Dependent	Forage group							
Variable	Grasses		Imm. grasses		Conv. corn		Bmr corn	
k_d	ADL:	-0.72	Et-FA:	0.87	ADL:	-0.74	KL:	-0.45
	Et-FA:	-0.68			Es-FA:	-1.07	Es-*p*CA:	1.04
R^2		0.97		0.75		0.79		0.84
24h ivNDFD	ADL:		ADL:	-1.12	Et-*p*CA:	-0.75	KL:	-0.62
	Et-FA:		Es-*p*CA:	0.36			Es-*p*CA:	1.09
R^2		0.98		0.97		0.56		0.92
96h ivNDFD	ADL:	-0.90	KL:	0.16			(KL-ADL):	-0.57
	Et-FA:	-0.40	ADL:	-0.59			Et-FA:	0.70
			Et-FA:	0.48			Es-*p*CA:	0.34
R^2		0.97		0.99				0.84

Significance level = 0.10; k_d = rate of digestion, %/h; ivNDFD = in vitro neutral detergent fiber digestibility; ADL = acid detergent lignin; KL = klason lignin; Et-FA = etherified ferulic acid; Es-FA = esterified ferulic acid; Et-*p*CA = etherified *p*-coumaric acid; Es-*p*CA = esterified *p*-coumaric acid.

Table 5.5. Chemical composition of the corn silages used in the feeding and in vivo study.

	Corn silage		
Item	CLD[1]	CHD[2]	BMR[3]
DM%	36.3	32.9	32.9
	---------- (% of DM) ----------		
CP	7.8	8.3	8.4
ADF	23.2	24.8	22.2
NDF	38.3	41.3	38.6
Starch	39.0	33.6	36.5
ADL	2.35	2.28	2.04
Ash	3.74	4.12	4.05
pH	3.86	3.86	3.82
24 h NDFD	46.1	47.1	56.5

[1]Conventional corn silage selected for comparably lower NDF digestibility.
[2]Conventional corn silage hybrid selected for higher NDF digestibility.
[3]Brown midrib corn silage selected for higher NDF digestibility.

Table 5.6. Ingredients and chemical composition of experimental diets fed in the in vivo study.

	Corn silage		
Item	CLD[1]	CHD[2]	BMR[3]
Ingredient, % of DM			
CLD[1]	59.25	-	-
CHD[2]	-	59.25	-
BMR[3]	-	-	59.25
Wheat straw	5.55	5.55	5.55
Soybean hulls	5.55	5.55	5.55
Molasses	1.38	1.38	1.38
Soybean meal	10.18	10.18	10.18
Soy Plus	6.48	6.48	6.48
Blood meal	0.92	0.92	0.92
Animal-vegetable fat	0.92	0.92	0.92
Mepron	0.07	0.07	0.07
Sodium Bicarbonate	1.11	1.11	1.11
Urea	0.18	0.18	0.18
Mineral-vitamin mix	2.85	2.85	2.85
Composition, % of DM			
DM, %	42.3	44.2	46.6
CP	15.0	14.8	15.8
RUP	7.21	7.33	7.67
ADF	21.34	22.95	20.59
NDF	35.8	37.8	35.4
Starch	25.8	24.6	25.4
ADL	2.21	2.46	1.97
Ash	8.02	7.80	8.53
DM intake, kg	24.19	23.48	26.09

[1]Conventional corn silage selected for comparably lower NDF digestibility.
[2]Conventional corn silage hybrid selected for higher NDF digestibility.
[3]Brown midrib corn silage selected for higher NDF digestibility

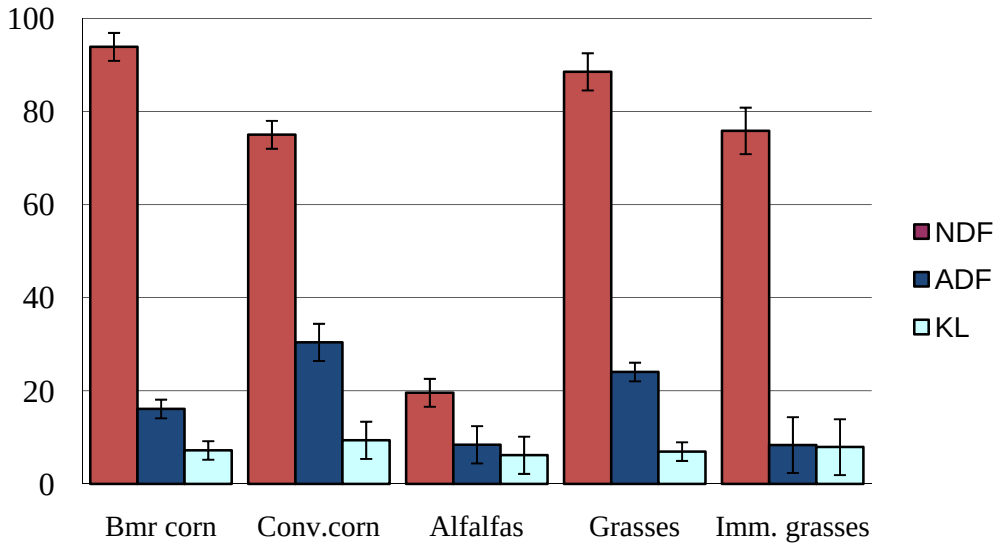

Figure 5.1. *P*-coumaric acid recovered in NDF, ADF and Klason lignin as percent of the total measured in the intact sample and respective standard error.

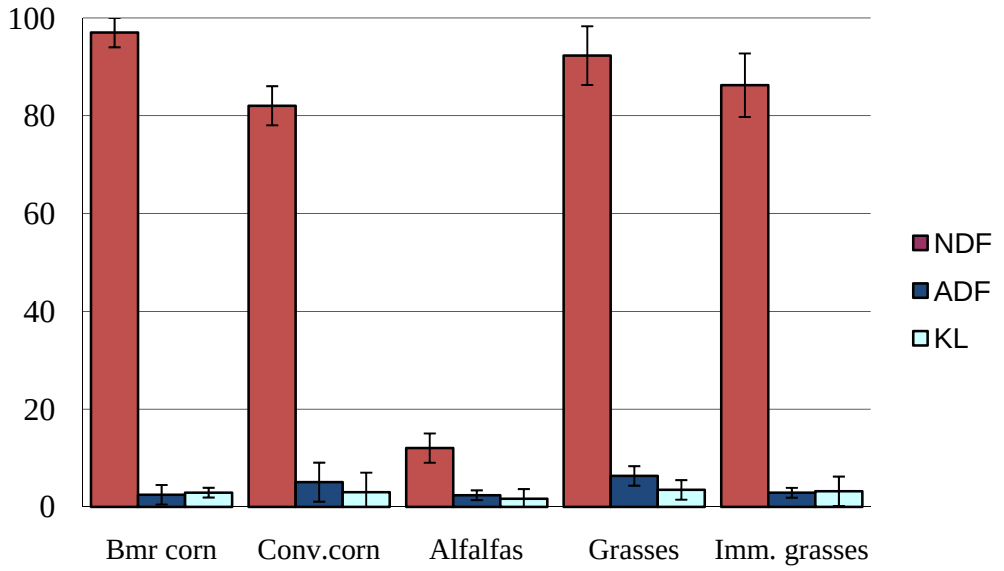

Figure 5.2. Ferulic acid recovered in NDF, ADF and Klason lignin as a percent of the total measured in the intact sample.

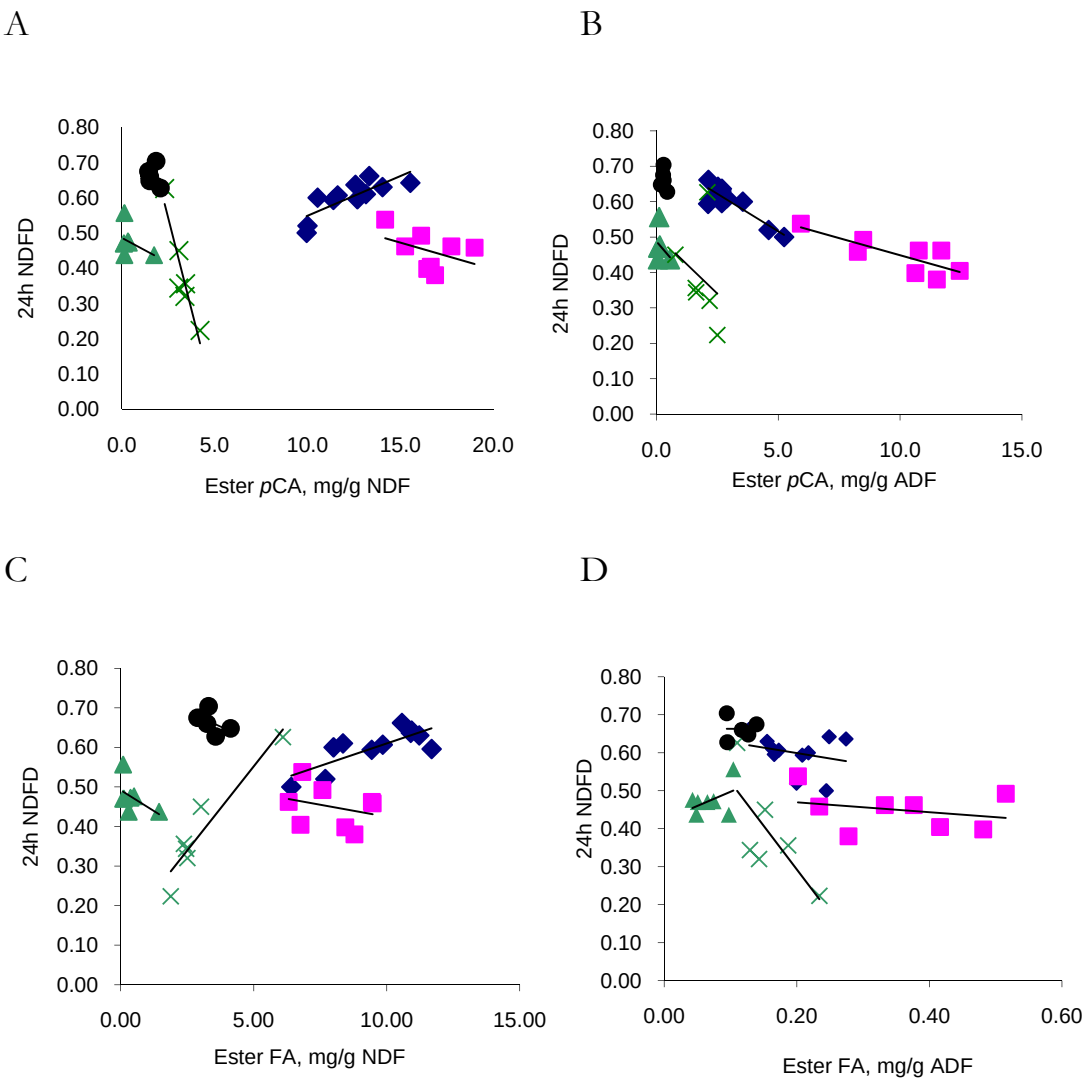

Figure 5.3a. Regressions between ester- linked pCA and FA in NDF or ADF residues and 24 h NDF digestibility for conventional (■) and bmr (♦) corn silages, alfalfas (▲), grasses (×) and young grasses (●).

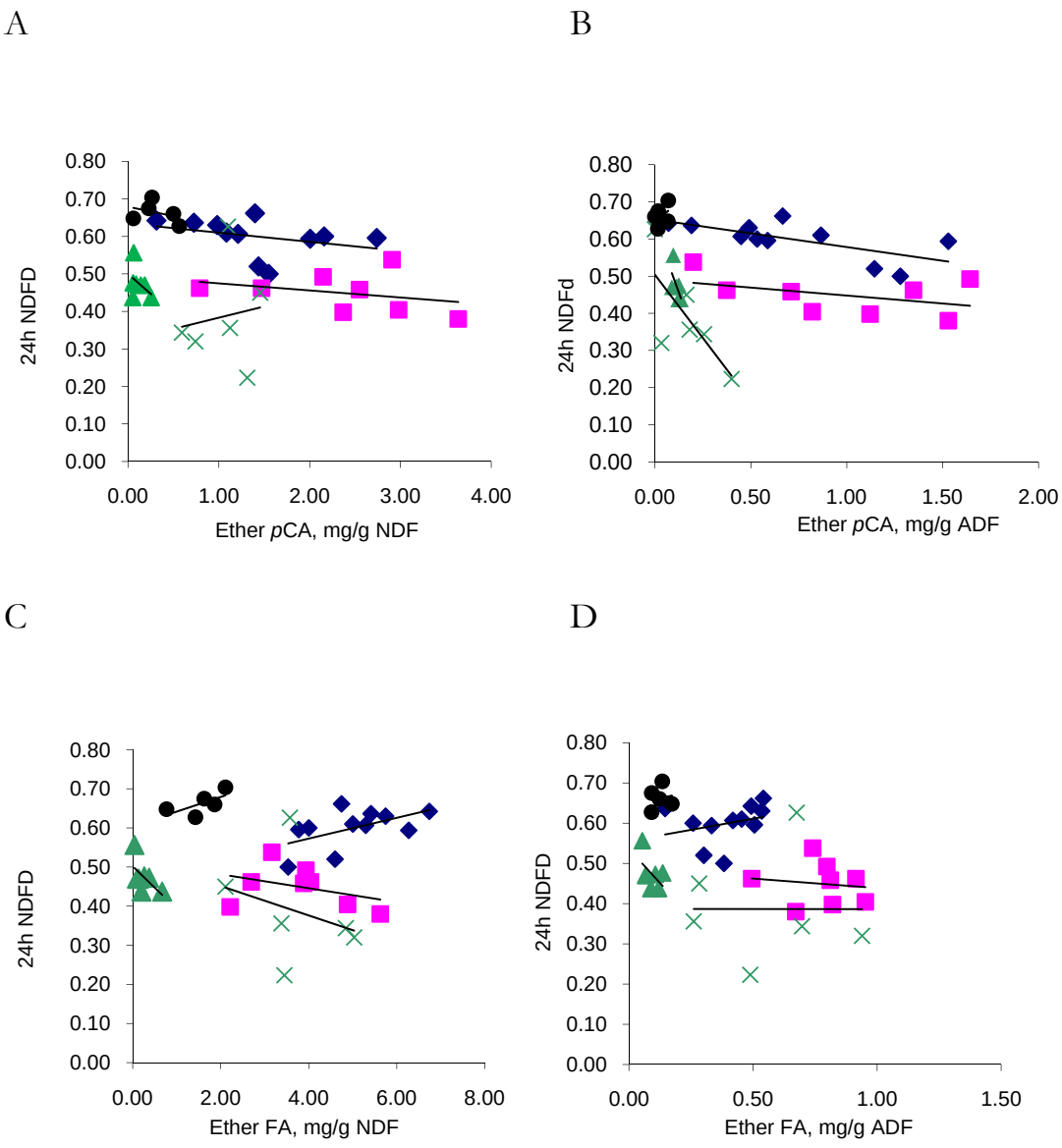

Figure 5.4b. Regressions between ether- linked pCA and FA in NDF or ADF residues and 24 h NDF digestibility for conventional (■) and bmr (♦) corn silages, alfalfas (▲), grasses (×) and young grasses (●).

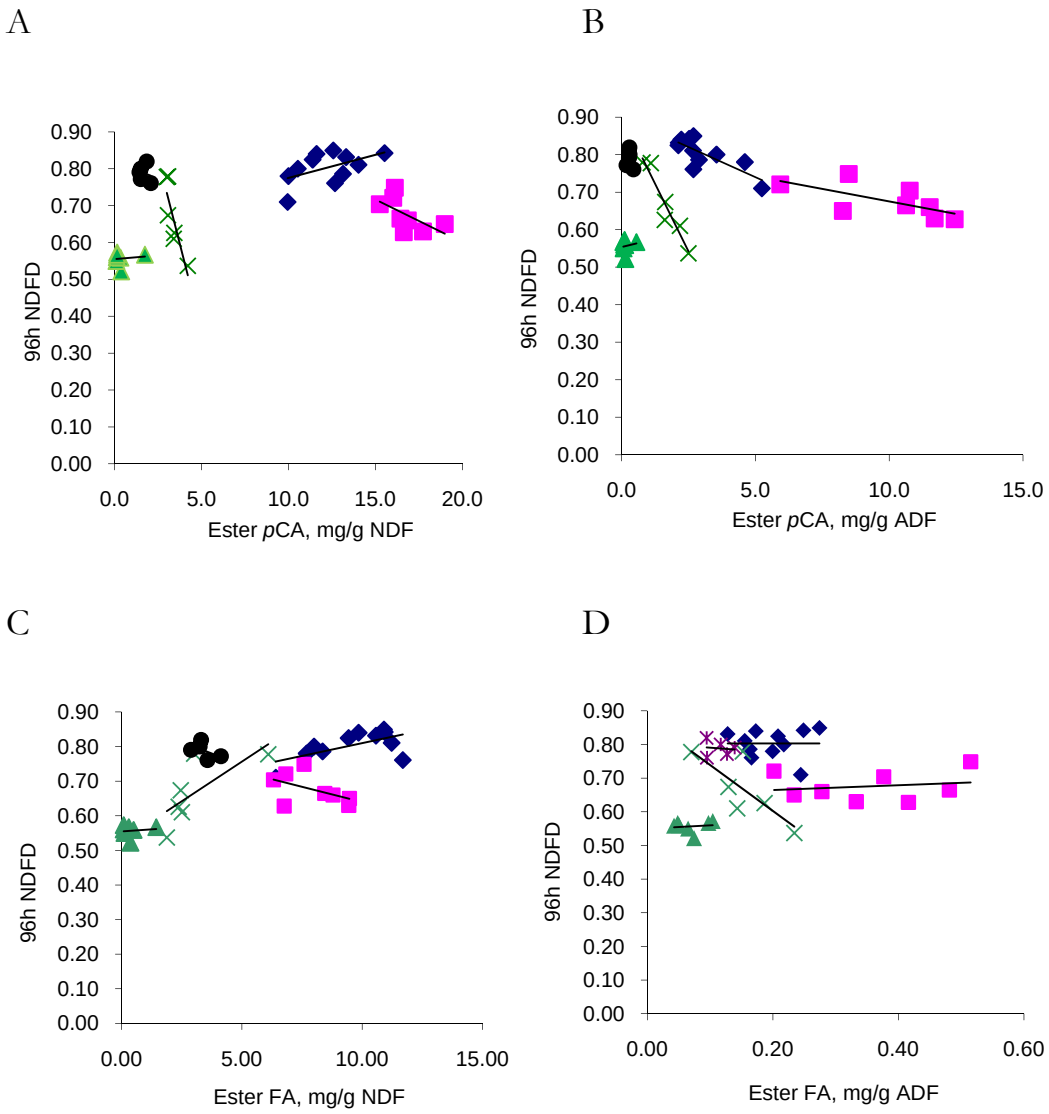

Figure 5.5a. Regressions between ester- linked pCA and FA in NDF or ADF residues and 96-h NDF digestibility for conventional (■) and bmr (♦) corn silages, alfalfas (▲), grasses (×) and young grasses (●).

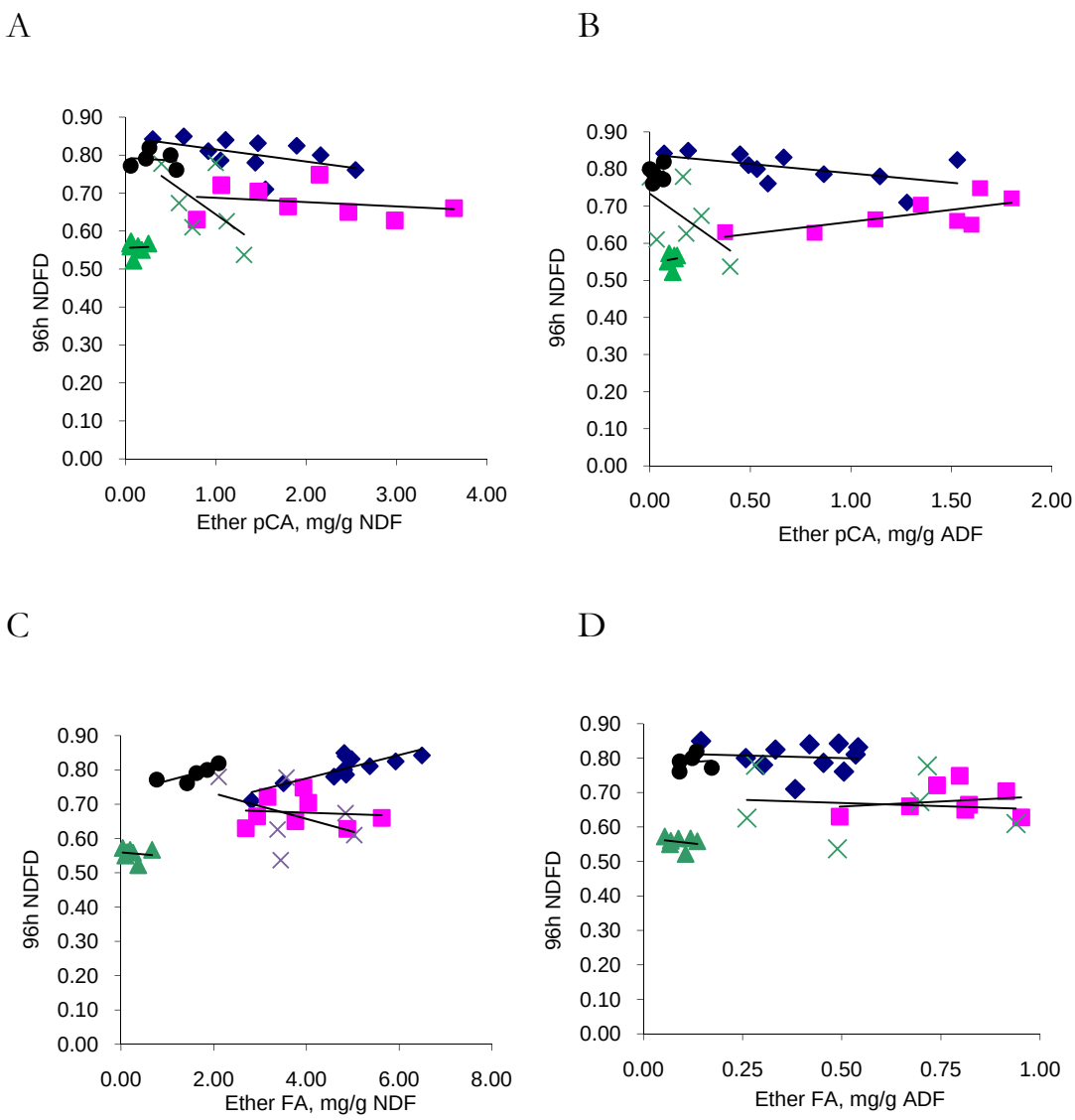

Figure 5.6b. Regressions between ether- linked pCA and FA in NDF or ADF residues and 96-h NDF digestibility for conventional (■) and bmr (♦) corn silages, alfalfas (▲), grasses (×) and young grasses (●).

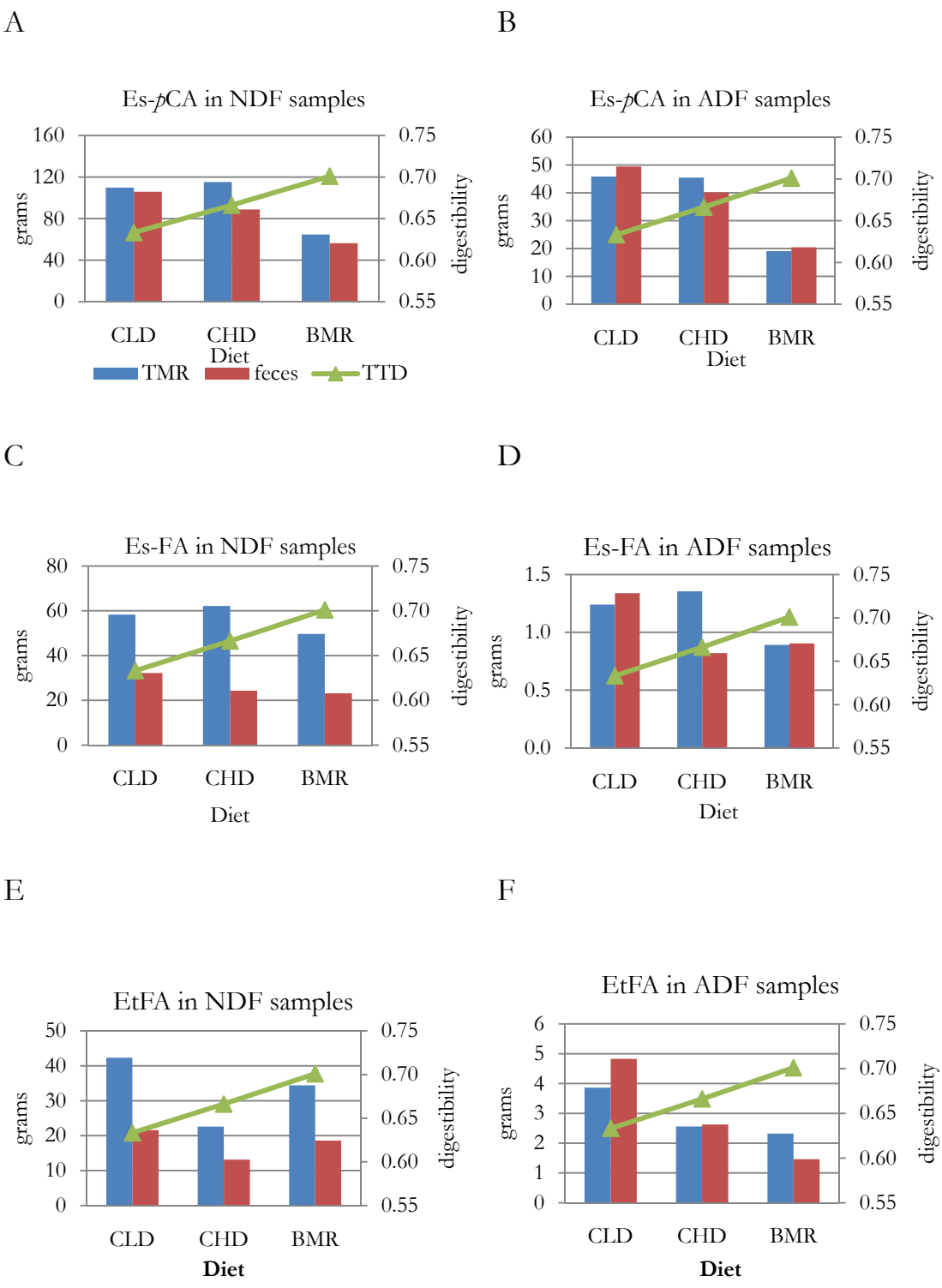

Figure 5.7. Total amounts of phenolic acids ingested and excreted after ND and AD treatment of the samples per single diet (CLD: conventional corn silage lower digestibility diet; CHD: conventional corn silage higher digestibility diet; BMR: bmr corn silage diet).

CONCLUSIONS

Digestibility is one of the most important characteristics of a forage, in terms of its nutritional value. In this study, the relationship among phenolic acids and in vitro digestibility and extent of digestion was investigated in intact plants and various fractions. Lignin type and linkages of phenolic acids explained most of the variation in NDFD, but not uniformly among species and stage of maturity indicating that use of the same factors to explain digestibility in all forages and among agronomic conditions will most likely lead to erroneous characterizations. Ferulic acid and *p*-coumaric acid esters and ether linkages among lignin-carbohydrate complexes appear to be distributed in the different fractions of the plant but not uniformly across forage families, and therefore there is the need of consistency in reporting their respective amounts in plants. In particular the concentration of ADF has been used in the past as an indirect predictor of forage digestibility (Undersander et al., 1993). In this study the acid detergent solubilized the phenolic acids not directly involved in negatively affecting digestibility. The use of phenolic acids content in ADF, and their respective chemical linkages, offers an opportunity to avoid dependence of different cell wall fraction measures, since results were more consistently associated to digestibility across forage groups, when compared to dry matter or NDF basis.

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CHAPTER SIX: DEVELOPMENT OF A MATHEMATICAL MODEL TO PREDICT SIZES AND RATES OF DIGESTION OF A FAST AND SLOW DEGRADING POOL AND AN INDIGESTIBLE NDF FRACTION

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ABSTRACT

Many models that predict rate and extent of digestion of neutral detergent fiber (NDF) in the rumen assume first-order processes, in which the rates of digestion and passage are proportional to the mass of carbohydrate in the rumen. Van Amburgh et al. (2003) described a mathematical approach for determining rates of digestion (k_d) for NDF with one time point assuming first order behavior and a fixed indigestible NDF pool. Recently, we demonstrated a more dynamic relationship between NDF digestibility and ADL in the estimation of the indigestible NDF (iNDF) among forage types (Raffrenato et al., 2009). Our objective was to improve the prediction of digestible NDF and to quantify, using few fermentation points, two pools of digestible NDF, pdNDF1 and pdNDF2, and their respective rates. Based on fermentations from 0 to 240 h among 34 forages (grasses, conventional and bmr corn silages, alfalfas) three pools were described by NDF_t = pdNDF1 * $e^{-k1(t-L)}$ + pdNDF2 * $e^{-k2(t-L)}$ + iNDF, where NDF_t is the residue at time t; L is the lag; k1 is the rate of digestion of pdNDF1; k2 is the rate of digestion of pdNDF2; and iNDF the indigestible NDF on NDF basis. A

non-linear estimation allowed the computation of the pool size and respective rates. Vensim® (Ventana Simulation Environment; Ventana Systems Inc., Belmont, MA, 2005) is an interactive software environment that allows the development, exploration, analysis, simulation and optimization of dynamic models. Using three points on the curve, with 240 h as the proxy for iNDF, we optimized the same model in Vensim to obtain rates and pools. In addition, the same optimization was also performed with two points and a forage group-specific range for iNDF. Parameters (with and without iNDF) obtained per forage by Vensim were compared in fitting kinetics data from the non-linear ones, using R^2 and RMS at convergence for ranking purposes, for the whole equation, and RMSE and MSPE, for specific parameters. The highest R^2 (0.98) and lowest RMS (0.0010) were obtained when using 48, 120 and 240 h of NDF residual or when using 30 and 120 h and a range for the forage group-specific iNDF (R^2 =0.92; RMS= 0.0021). Correlations were in both cases consistently high for all kinetic parameters ranging from 0.76 to 0.99. Results demonstrate that a better description of the heterogeneity of NDF disappearance is possible without multiple fermentation time points. The equation was fit to all the forage data generated, however, because of the variable nature of the pool sizes and rates, forage specific equations should be developed for better estimations of the forage specific pool sizes and iNDF estimation. This study further verifies the heterogeneous nature of NDF disappearance and provides an approach for estimating the individual pool sizes and rates for application in diet formulation.

INTRODUCTION

Neutral detergent fiber is the most common measure of fiber used for animal feed analysis, but it does not represent a unique or homogenous class of chemical components. Heterogeneity of the NDF content of a plant can be demonstrated by the Lucas test (Lucas, 1964; Van Soest, 1994). The purpose of the Lucas test is to identify ideal nutritional components that have uniform digestibility over a wide range of feedstuff, by plotting the digestible nutrient concentration in DM against the nutrient concentration in DM. The slope of regression estimates the true digestibility and the intercept is an estimate of the metabolic and endogenous fecal matter. The neutral detergent divides the feed into a soluble fraction that is rapidly and almost completely available with a digestibility of 0.98 and a fiber fraction that is slowly and incompletely degraded by microbial enzymes (Huhtanen et al., 2006). Furthermore, NDF is also characterized by the presence of a fraction that is unavailable to microbial digestion in ruminants (i.e., indigestible NDF= iNDF) even if total tract residence time of fiber could be extended to infinite time (Allen and Mertens, 1988; Van Soest, 1994). Thus by definition, iNDF represents a uniform feed fraction with zero true digestibility according to the Lucas test (Lucas, 1964). The potentially digestible NDF (pdNDF) will then result from the difference NDF- iNDF.

Although dietary concentration of NDF is related positively to bulk density of feeds and affects feed intake potential (Karkalas, 1985) forage NDF greatly varies in its digestibility in the rumen (Allen and Mertens, 1988) and in vitro (Mertens, 1973). Digestibility of NDF can influence animal performance independent of dietary NDF concentration. Animal trials

where forages of higher in vitro digestibility but similar NDF concentration have been fed, reported significant increases in DMI and milk production (Grant et al., 1995; Dado and Allen, 1996; Oba and Allen, 2000). A faster disappearance of the NDF fraction from the rumen because of increased rate of digestion or passage will reduce physical fill in the rumen over time and allow greater voluntary feed intake (Mertens, 1994; Van Soest, 1994).

Accurate and precise predictions of the intrinsic digestion kinetic parameters are critical to the estimation of NDF rumen digestibility and intake. The importance of a single fractional rate (k_d) and extent of NDF digestion on total tract OM and NDF digestibility can be demonstrated by simulation with the CNCPS model (Fox et al., 1992; Russell et al., 1992; Sniffen et al., 1992; Fox et al., 2004) or with the Nordic model of cow metabolism, "Karoline" (Danfær et al., 2006a; Danfær et al., 2006b). Simulation results clearly demonstrate profound effects of these parameters on ruminal and total tract NDF digestibility and therefore on the supply of energy and microbial protein.

One of the problems in describing digestion kinetics is that residues remaining at any digestion time are a mixture of undigested and indigestible matter (Mertens, 1993). Furthermore, Mertens has indicated (Mertens, 1973; Mertens, 1977; Mertens and Ely, 1979) that overall digestion is better predicted assuming that the pdNDF fraction is the sum of two digestible fractions that can be described by two first order functions but with different rate constants. According to Van Milgen et al. (1991) the assumption of a single fractional digestion rate constant for NDF is also untenable because of the chemical and morphological diversity of forages fed to livestock. More recently Ellis et al. (2005) demonstrated an improved

fit of a two-pool pdNDF model that assumed two concurrently degrading sub-entities of pdNDF with different degradation rates. Also for in-vitro gas production and NDF digestion, Huhtanen et al. (2008b) has shown a marked improvement of the model when pdNDF was assumed to be comprised of rapidly and slowly degradable fractions.

Rate of digestion of NDF is an input in feed formulation systems and nutrition models (Fox et al., 2004; Tylutki et al., 2008). However, incorporation of digestion rates as a standard procedure to calculate the nutritive quality of specific feeds and diets has been achieved only recently, in part, because of lengthy laboratory analyses and statistical interpretation of fiber digestion rates. The mathematical approach by Van Amburgh et al. (2003) described a method for determining rates of digestion for a single pool of pdNDF with one time point assuming first order behavior and a fixed iNDF pool. The iNDF fraction was estimated using the formula (ADL × 2.4)/NDF where the 2.4 was the factor obtained by Chandler et al. (1980). The primary limitation of this approach was related to the use of the fixed value of 2.4.

In a previous paper we demonstrated, through improved recoveries and definitions of both ADL and iNDF that the relationship between iNDF and ADL is more variable and can be predicted using forage group-specific ranges (Raffrenato et al., 2009; Raffrenato et al., 2010, submitted) based on the value of ADL/NDF of the forage evaluated.

Our hypothesis is that the improved methodology to determine ADL and the refined estimation of iNDF can result in a better description of pdNDF pools, and this can be accomplished with a minimal number of fermentation points. Therefore, our objective was to predict the proportions

of the fast and slow degrading pools of NDF, by computing their respective fractional digestion rates and sizes using 240 h of fermentation as the endpoint for iNDF and to identify the best and least number of time points necessary for this to be conducted by commercial laboratories. A secondary objective was to assess the accuracy of predicting the same parameters when the iNDF was not available and a forage-group-specific range for the ratio (iNDF/NDF)/(ADL/NDF) was used instead. Finally, we wanted to demonstrate how a single weighted k_d for the whole pdNDF fraction, from the fast and slow fractions, can be obtained and used when the definition of fast and slow pool is not yet implemented in nutrition model systems. The approach was to develop composite decay models to describe the heterogeneous behavior of NDF digestion.

MATERIALS AND METHODS

Thirty five forages, including grasses, conventional and bmr corn silages and alfalfas, were analyzed in duplicate for NDF, ADL. All samples were ground through a 1 mm screen in a Wiley Mill (Thomas Scientific, Swedesboro, NJ). For the fermentations, 0.75 g of each sample was weighed into in 125 mL Erlenmeyer flasks, and inoculated with buffer and rumen fluid and residues analyzed for NDF, from 0 to 240 h according to Goering and Van Soest (1970). Rumen fluid was harvested from two cows being fed a TMR and housed at the Cornell University research farm and combined to form the inoculum. Residual ash free NDF of the fermented samples was obtained at 0, 6, 12, 24, 30, 36, 48, 72, 96, 120, 144, 216 and 240 h based on the procedure of Mertens (2002). Use of sodium sulfite for the

NDF analyses was omitted. The samples that fermented longer than 120 h were re-inoculated at 120 h with the same amount of the initial rumen liquor/medium mix. Blank flasks were used to correct for any particle accumulation from the inoculation and were analyzed similarly to the samples. A glass microfiber filter (934-AH™ by Whatman®, Whatman Limited – GE Healthcare, Maidstone, UK) was used in all analyses as suggested by Raffrenato and Van Amburgh (2010), to avoid particle loss and increase recovery. Composite decay models are formed by the sum of several exponential functions and have been used to describe various physical phenomena and the non-linear least-squares-fit is the most common method in use to solve them (Villuendas and Pelayo, 1987). Assuming there are more than one fraction of pdNDF that can be described mathematically (Ellis et al., 2005; Van Soest et al., 2005; Huhtanen et al., 2008a), a composite decay model was used to estimate a fast and a slow degrading pool and the respective rates of digestion and an indigestible fraction. Therefore the residual NDF at time t was described by:

Eq. 1: $NDF_{(t)} = pdNDF_{1(0)} * e^{-k1(t-L)} + pdNDF_{2(0)} * e^{-k2(t-L)} + iNDF$

where $pdNDF_{1(0)}$, and k_1 are the size at time 0 and the fractional rate of the fast pool; $pdNDF_{2(0)}$, and k_2 are the size at time 0 and the fractional rate of the slow pool, respectively, L is the lag and iNDF is the indigestible NDF. Simultaneous estimations of the parameters $pdNDF_1$, $pdNDF_2$, k_1, k_2, iNDF and L were initially obtained using PROC NLIN of SAS (SAS Institute, Inc., Cary, NC) and the Marquardt algorithm. The Marquardt algorithm was selected to improve the efficiency of providing least-squares estimation for

the non-linear curve fitting approach. Non-linear regression was chosen as the standard procedure because the method assumes equal error at each observation and by simultaneously fitting all parameters to the data, the result provides the smallest residual sums of squared deviations. The necessity of establishing initial parameters values for the non-linear estimations was solved using a linear approach to seed the non-linear estimation as done by Grant and Mertens (1992). We used the log-linear approach of Van Soest et al. (2005) to generate the initial values for each sample to parameterize the composite decay model, including a fast, a slow and an indigestible pool for the model (1) using 240 h residual NDF to estimate the pdNDF. The mathematical approach used here (Van Soest et al., 2005) to obtain initial values is derived from the linearization of the non-linear first order composite decay and it therefore seems reasonable to make comparisons to this procedure and provides also a simple cross-check of procedures, especially to compare the integrated rate of digestion for a first order model application.

The statistical model, like the non-linear model described, uses all available data observations at different time points and is therefore regarded as more accurate, and perhaps more precise because it relies on using all of the data points and thus requires the use of the entire decay curve. Use of statistical models would require significant effort by a commercial laboratory. Development of an inexpensive and less labor-intensive approach for daily use would be beneficial, therefore, prediction of three NDF fractions with the least possible number of fermentation points for use in commercial laboratories was an objective.

Vensim® (Ventana Simulation Environment; Ventana Systems Inc., Belmont, MA, 2005) is an interactive software environment that allows the development, exploration, analysis, simulation and optimization of dynamic models. A model was built and run in Vensim® 5.5 (Ventana Simulation Environment; Ventana Systems Inc., Belmont, MA, 2005) in calculation intervals of 0.0625 h, and initial and final time points of 0 and 240 h, respectively. The model (Figure 6.1, model 2) does not require all the data points required of the statistical model and allows the use of fewer data points to estimate the desired parameters. Furthermore, it is structurally consistent with conservation of mass and thus avoids negative values for the stocks or pools which might occur for the unconstrained statistical model. The Vensim modeling language works with the concept of stocks (pools) and flows (disappearance or digestion) and this terminology will be interspersed in this paper.

The structure in model 2 of the slow pool stock depicts a goal-seeking behavior, with the goal being in this case represented by the iNDF. The slow degrading pool is calculated as $pdNDF_2 = 1 - (pdNDF_1 + iNDF)$. Furthermore, the model was parameterized by the condition: if $0 \leq t \leq L$ then $NDF_t = pdNDF_{1(0)} + pdNDF_{2(0)} + iNDF$, otherwise equation (1) was applied (Mertens and Loften, 1980; Moore and Cherney, 1986). This was done to avoid having a separate non-linear model for $t \leq L$, assuming the stabilization of the fermentation was at $t = L$. Concentrations were expressed in terms of fraction of the initial NDF. The system of differential equations for the model depicted in model 2, with FPS and SPS representing the fast pool stock and the slow pool stock, respectively, is:

Eq. 2: at $t < L$: $dFPS_1/dt = 0$ and $dSPS_2/dt = 0$

Eq. 3: and $diNDF/dt = 0$.

Eq. 4. At $t \geq L$: $dFPS/dt = -k_1FPS$,

Eq. 5: $dSPS/dt = -k_2(SPS-iNDF)$

and $diNDF/dt = 0$.

The model performs calculations based on the equivalent integral equations, which are given by:

Eq. 6: at $t < L$: $FPS_{(t)} = FPS_{(0)}$

Eq. 7: and $SPS_{(t)} = SPS_{(0)}$

Eq. 8: $iNDF_{(t)} = iNDF_0$

Eq. 9: with $SPS_{(0)} = 1 - FPS_{(0)} - iNDF_{(0)}$

Eq. 10: and thus $NDF_{(t)} = pdNDF_{1(0)} + pdNDF_{2(0)} + iNDF_0$.

Eq. 11: At $t \geq L$: $FPS_{(t)} = pdNDF_1 \exp(-k_1[t-L])$,

$SPS_{(t)} = pdNDF_2 \exp(-k_2[t-L]) + iNDF$

$iNDF_{(t)} = iNDF_{(0)}$

Eq. 12: and thus $NDF_{(t)} = pdNDF_{1(t)} + pdNDF_{2(t)} + iNDF_{(t)} =$

$pdNDF_{1(0)} \exp(-k_1[t-L]) + pdNDF_{2(0)} \exp(-k_2[t-L]) + iNDF_{(0)}$.

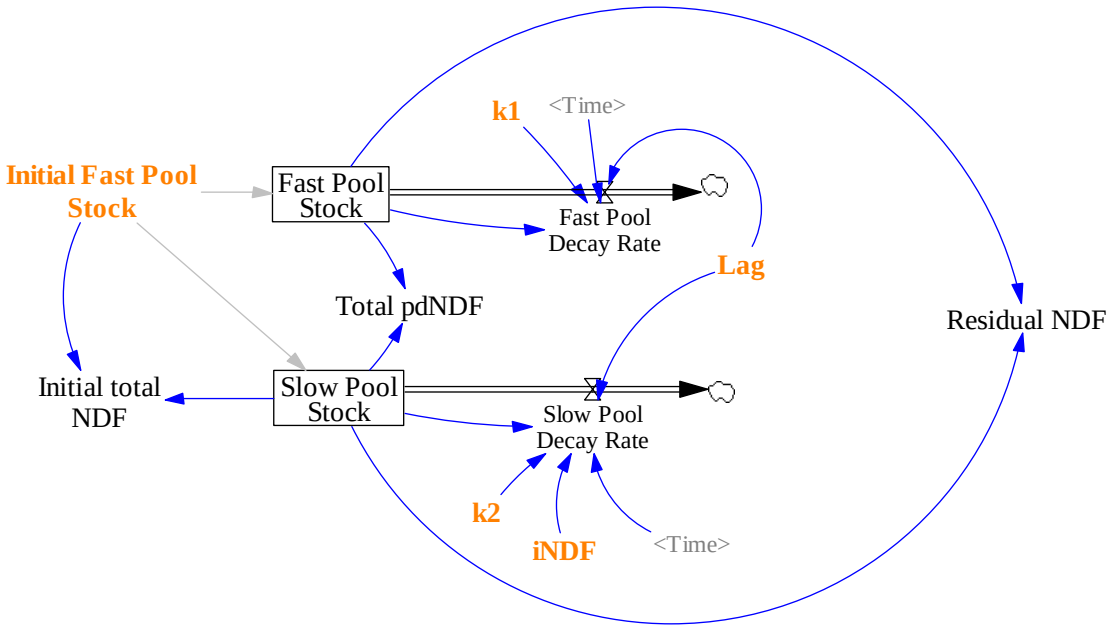

Figure 6.1. Diagrammatic representation of the composite decay model described in the text. The slow pool stock is in this case equal to $pdNDF_2$ + iNDF and the slow degrading pool follows a goal seeking behavior with the goal being iNDF. In orange are the parameters that need to be optimized. The slow degrading pool is calculated as $pdNDF_2 = 1 - (pdNDF_1 + iNDF)$.

Optimization methods can be used to estimate parameters of this model. In our procedure, optimizations were run using different fermentation endpoints and the choice of the endpoints was made by evaluating the time points that represented the three fractions with the least amount of error. Previous work by Van Soest et al. (2005) showed that the fast pool was exhausted by 48 h, so the choice was made for a time point up to 48 h to represent the first part of the curve (fast pool, slow pool, indigestible fraction), a point between 48 and 216 h to represent the second part of the curve (slow pool and the indigestible fraction), and the 240 h to represent the iNDF fraction. All combinations of the points available (6, 12, 24, 30, 36 and 48 for the fast pool; 72, 96, 120, 144 and 216 for the slow pool), and 240 for the iNDF resulted in 30 possible combinations to be examined.

A modified Powell hill-climbing algorithm (Powell, 1964) was used to perform search for the parameters optimization as defined in Vensim® 5 Reference Manual (Ventana Systems Inc., Belmont, MA, 2006). To optimize we defined a "payoff", which is a single number reported for each simulation. The NDF residue from zero to 240 h was chosen as the payoff because this is a function of the parameters that need to be estimated in the model (i.e.: fast pool stock and decay rate, slow pool stock and decay rate, lag and iNDF). The optimization chooses parameter values that minimize the sum of squared differences between the actual data points and the model predictions for the NDF residue value. Maximizing the payoff means reaching a value as close to zero for the squared difference and this procedure is similar to regression analysis, but with additional constraints on the model structure and therefore the parameter values. Parameters of the model that were optimized for were: L, $pdNDF_1$, $pdNDF_2$, k_1, k_2, and iNDF.

The range of values for parameters was constrained as: $0 < iNDF < iNDF_{(240)}$ with $iNDF_{(240)}$ being the indigestible fraction estimated as the residual NDF at 240 h from the laboratory observation for each forage fermented and $1.5 < L < 4.5$ based on the results from the non-linear estimation in SAS (SAS Institute, Inc., Cary, NC) and on our previous work (Van Amburgh et al., 2003). Optimizations were run for individual forages and values in Vensim® of fractional tolerance and tolerance multiplier were left as default values, i.e. 0.0003 and 21, respectively, which means the model will attempt to solve the non-linear extrapolation within 0.03% of the actual values with a range of ±0.063%.

To determine the optimal time points from the fermentation curve, among all combinations, constants obtained from the optimization with Vensim® were compared to the values obtained with the non-linear estimation from equation (1) from PROC NLIN in SAS (SAS Institute, Inc., Cary, NC). To evaluate the performance of the optimizations, the goodness of fit was compared using the variance accounted for (R^2) and the residual mean squares (RMS) at convergence for ranking purposes for each optimal combination of time points, as in Ellis et al. (2005) and Huhtanen et al. (2008a), using the average values obtained by pooling all forages analyzed and then by forage group (conventional and bmr corn silages, grasses, straws and hays, and alfalfas). For each combination, the evaluation was then made separately for each parameter ($pdNDF_1$, $pdNDF_2$, k_1, k_2, L and iNDF) predicted by the optimization and computations were made as suggested by Piñeiro et al. (2008), with the results of the non-linear estimation being the "observed" values, since we assume those to be the most accurate and precise values. The root mean square errors (RMSE) between the observed

and predicted values for each parameter, using the least number of fermentation endpoints, were calculated as follows:
RMSE = $\sqrt{\sum}$ (observed – predicted) ^{2}/n, where n is the number of forages. The mean square prediction error (MSPE) was divided into components resulting from mean bias, slope bias, and random or unexplained variation around the regression line according to analysis of Theil (1966) and Bibby and Toutenburg (1977). Significance of the deviation of the intercept from 0 and the slope from 1 was analyzed by *t*-test.

Finally, to make this approach more applicable to commercial laboratories, an analysis and estimation of extent of NDF digestion and iNDF was conducted using forage-family specific endpoints for iNDF as a starting point, knowing there is a range within the iNDF value within and among forage families and there is a possibility that a laboratory might not have or would generate 240 h iNDF values. Therefore, assuming that the residue at 240 h represents the true iNDF fraction, we defined specific ranges of the ratios iNDF/ADL (on NDF basis) for each forage group and determined the range and variance associated with the iNDF value within our dataset. The same evaluation using Vensim as described above was then performed using these specific ranges to determine the time points needed to predict the same parameters. The iNDF was then constrained to fall within the range defined for each forage group, based on the ADL/NDF ratio. The ratios (iNDF/NDF)/(ADL/NDF) were constrained in the following manner: 3.0-5.5 for conventional corn silages, 2.0-6.5 for bmr corn silages, 2.0-6.0 for grasses, and 2.0-3.0 for alfalfas (Raffrenato and Van Amburgh, submitted). The other constraints were as previously described. Goodness of fit was evaluated as above mentioned, when using the 240 h as

observed iNDF. The slow pool stock is again equal to pdNDF2 + iNDF and the slow degrading pool follows a goal seeking behavior with the goal being iNDF. The indigestible fraction is in this case determined by constraining the optimization using the forage group-specific ratio iNDF/ADL and ADL (both on NDF basis) specific for the individual forage optimized. Optimizations were run in the same manner described above for the model using 240 h as endpoint of the fermentation.

RESULTS AND DISCUSSION

Descriptive values of the forages by group are found in Table 6.1. The non-linear model resulted in high average R^2 (>0.98) for all forages and the average values of pool sizes and fractional rates obtained from the non-linear estimation are shown in Table 6.2. The values from this non-linear statistical model are considered the averages of "observed values" and are used to compare the Vensim outputs. The standard errors of all parameter values were small, ranging from 1.6 to 2.1% of the mean value, except for L (8.1%). An overall rate of digestion for the whole feed NDF was obtained from the weighted average of the two pdNDF pools. The pools sizes and rates obtained from the non-linear estimation allowed the extrapolation of the residual pools (example in Figure 6.3) for the entire curve and it was observed that residues of $pdNDF_1$ are exhausted in most cases by 48 h and always by 72 h of fermentation, meaning that the fast pool (pdNDF1) was exhausted between 48 and 72 h for all forages analyzed, confirming the previous data (Van Soest et al., 2005). Forages such as bmr corn silages and alfalfas forages exhausted their fast pool earlier (36 to 48 h) in the

fermentation curve, when compared to conventional corn silages and grasses.

Table 6.1. Mean values of neutral detergent fiber (NDF) (% of DM), acid detergent lignin (ADL, g/kg NDF), indigestible NDF (iNDF, g/kg NDF) determined after 240 h of fermentation, and the calculated ratios of iNDF/ADL and respective ranges of the forages used in the analyses per group of conventional and bmr corn silages (C.S.), grasses and alfalfas(ranges in parentheses).

Group	Samples	NDF	ADL	iNDF	iNDF/ADL
		% of DM	----g/kg NDF----		
Conventional C.S.	7	44.04	68.6	232.4	3.38 (3.23-5.46)
BMR C.S.	6	39.06	34.2	123.2	3.60 (2.14-5.78)
Grasses	6	64.03	80.9	286.3	3.53 (2.59-6.53)
Straws and hays	4	77.25	90.0	343.2	3.45 (2.60-4.39)
Alfalfas	7	36.64	169.1	428.2	2.53 (2.43-2.95)

Table 6.2. The pool sizes and rates obtained from the simultaneous non-linear estimation for the respective NDF digestion components. The standard errors for each variable are in parenthesis. The k_d represents a weighted average of $pdNDF_1$ and $pdNDF_2$ (and the k_d of iNDF is equal to zero by definition).

Group	n	$pdNDF_1$	$pdNDF_2$	iNDF	k_1	k_2	k_d
		----------% of NDF----------			----------1/h----------		
Conv. C.S.	7	60.7 (3.09)	18.7 (3.18)	20.6 (1.25)	0.073 (0.005)	0.016 (0.006)	0.060 (0.004)
BMR C.S.	6	73.8 (2.64)	13.1 (2.81)	13.1 (1.56)	0.087 (0.007)	0.024 (0.012)	0.078 (0.005)
Grasses	6	54.4 (4.59)	24.4 (5.06)	21.1 (2.15)	0.094 (0.036)	0.016 (0.005)	0.067 (0.018)
Straws & hays	4	58.7 (2.42)	10.3 (2.29)	32.3 (4.08)	0.040 (0.006)	0.007 (0.003)	0.035 (0.004)
Alfalfas	7	48.7 (4.91)	8.7 (3.44)	42.5 (6.30)	0.134 (0.018)	0.024 (0.012)	0.113 (0.013)

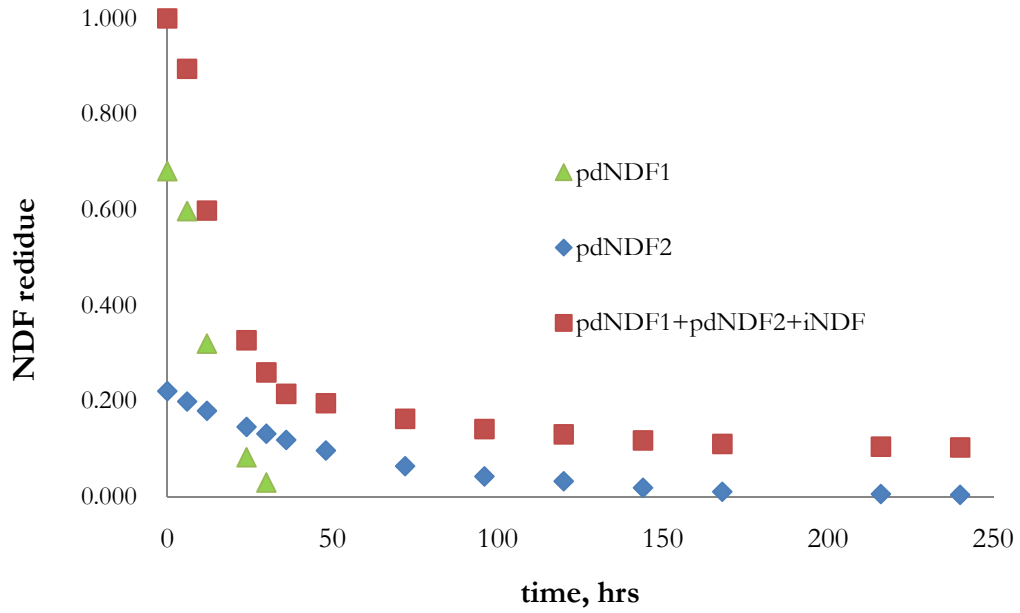

Figure 6.2. The residual NDF from the in-vitro fermentation, from 0 to 240 h, and the extrapolated amounts of the fast and the slow pool for a conventional corn silage as an example.

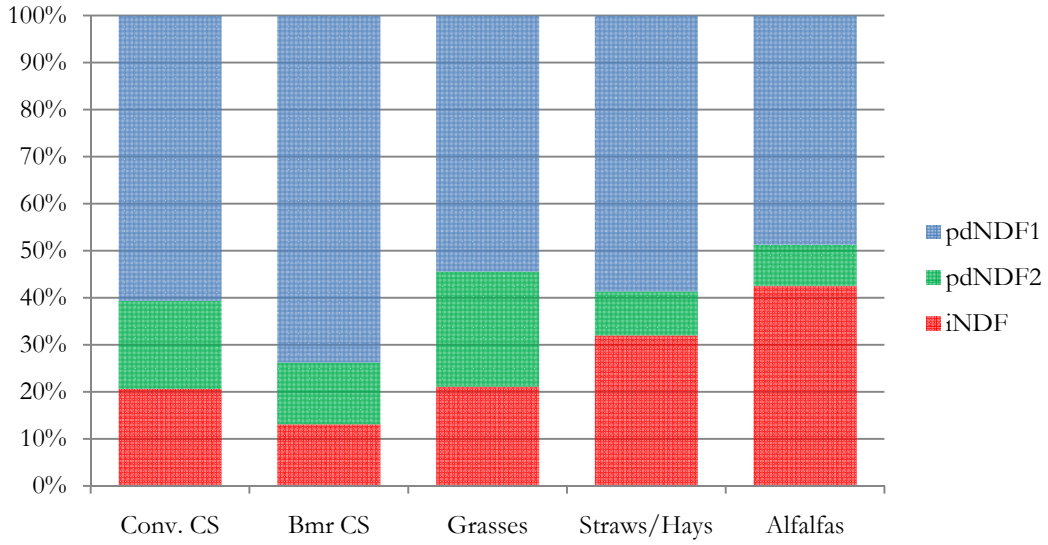

Figure 6.3. The average distribution, per forage group, of the fast ($pdNDF_1$) and slow ($pdNDF_2$) pools and indigestible fractions (iNDF) of the forages analyzed.

Based on average R^2 and RMS across forages, the combinations 48-96-240, 36-120-240, and 48-120-240 h provided the best fit from the model. When compared to the observed values from the non-linear procedure in SAS, parameters were predicted with a different goodness of fit and precision, resulting in different RMSE and MSPE. The differences among the combination of time points and how the RMSE and MSPE ranked the combinations differently across predicted parameters ($pdNDF_1$, $pdNDF_2$, k_1, k_2 and iNDF) are shown in Table 6.3.

Table 6.3. Goodness of fit of the combinations 48-96, 36-120 and 48-120 h as intermediate points, and 240 h as an end-point, when pooling all forages using the model defined in Vensim.

	Time point combinations					
	48-96		36-120		48-120	
Parameter	RMSE	MSPE*	RMSE	MSPE*	RMSE	MSPE*
$pdNDF_1$	0.0381	0.1673	0.0420	0.2205	0.0304	0.0927
$pdNDF_2$	0.0345	0.1188	0.0401	0.1762	0.0308	0.0948
k_1	0.0058	0.0034	0.0071	0.0051	0.0063	0.0029
k_2	0.0021	0.0004	0.0034	0.0011	0.0021	0.0039
iNDF	0.0148	0.0219	0.0138	0.0192	0.0136	0.0184

*: $\times 10^3$

The parameter values from the optimization in Vensim, using the time point combinations previously identified, and the estimated values from the non-linear procedure were highly correlated ($P < 0.01$), and ranged in correlation between 0.88 and 0.99, 0.53 and 0.99, and between 0.89 and 0.99 for the combinations 36-120-240 h and 48-120-240 h, and 48-96-240 h, respectively. The highest correlations resulted from the direct use of the iNDF fraction in the model (240 h), with values of 0.99 between the observed iNDF from PROC NLIN and the predicted iNDF from the Vensim optimizations. The prediction of the lag resulted in low correlations ($r < 0.40$) with the observed values ($P < 0.05$) and consistently high RMSE (>1.5 h) for all combinations and is omitted from the tables. This inability to predict the lag was most likely due to the lack of time points that provide sufficient information to the optimizer to estimate the lag. However, imprecise prediction of the lag does not significantly affect the estimates for the other parameters if the lag is constrained within a reasonable range of observed values during the optimization, as previously shown (Raffrenato et al., 2009). In our laboratory procedure, the lag is generally less than 3 h and can average less than 1.5 h depending on the forage and rate of hydration at incubation.

Although analysis with these time-point combinations resulted in parameters describing the data equally well, our objective was the description of the kinetics of NDF digestion that could be used by commercial laboratories with a minimum of time points; therefore, the analysis of the bias in the actual values of the parameters obtained due to calculation method was important for proper application. Pool sizes and rates of digestion were evaluated for their prediction accuracy and biological

relevance, with the preferred prediction having a small regression bias and minimal unexplained variation. The prediction of the pool sizes resulted in RMSE equal or less than 4.2% of the total NDF and the lowest values were for the 48-120h combination (3%) (Table 6.3). The prediction of the rates resulted instead in RMSE equal or less than 0.0071 (1/h) with the lowest RMSE for the 48-96 h combination. Lastly, the prediction of the iNDF was characterized by a very low RMSE, between 1.3 and 2.1% of the total NDF fraction. The specific relationships between the predicted and observed parameter values are shown in Table 6.4, 6.5 and 6.6. All R^2 from regressions of observed on predicted parameters were consistently high, except for the k_2 of the 48-120 h combination (0.29) (Table 6.5). However, all other parameters resulted in R^2 always numerically higher than 0.76. Precision of the 48-120 h combination is therefore less than the other combinations for the prediction of k_2 only, and this was confirmed by the slope being different than 0 ($P < 0.05$) (Table 6.5). Both the 36-120 and 48-120 h combinations over-predicted the size of $pdNDF_1$ by about 10% of the total NDF, however slopes were not statistically different than 1 ($P > 0.05$) (Table 6.4 and 6.5). Predictions from the 48-96 h combination resulted instead in intercepts not different than zero and slopes not different than one for all parameters predicted, with R^2 between 0.76 and 0.98 (Table 6.6). The distribution of the MSPE for the three time point combinations are shown in Tables 6.7, 6.8 and 6.9. The distribution of the MSPE was similar among predicted parameters, with random variation among the regression coefficients providing the greatest contribution to the total MSPE (>50%), for all three combinations and all parameters. The rest of the MSPE was in general shared between bias and slope variation.

Table 6.4. Relationship between the predicted and observed model parameters when using 36 and 120 h as intermediate fermentation time points, and 240 h as the end point.

Parameter	Intercept	Slope	R^2
$pdNDF_1$	0.1199[a]	0.8483	0.83
$pdNDF_2$	0.0108	0.7499	0.84
k_1	0.0022	0.9240	0.87
k_2	0.0009	0.7551	0.77
iNDF	-0.0128	1.0228	0.98

[a]: Intercept significantly ($P < 0.05$) different from 0.
[b]: Slope significantly ($P < 0.05$) different from 1.

To test the effect of not using the measured iNDF (240 h), the optimizer was constrained to forage group-specific ranges for iNDF and this procedure resulted in an overall lower average RMS and higher R^2 for the 30-120 h and 36-120 h combinations. Under these conditions the 36 h time point did not provide the best solution as previously observed. The outcome demonstrated that the indigestible portion was efficiently estimated, even without the 240 h end point, and the optimization was able to explain 97% of the variation when using 30 and 120 h as time points (Table 6.10), and 90% when using 36 and 120 h (Table 6.11). Again, RMSE and MSPE were mainly the result of unexplained variation, for all predicted parameters (Table 6.12 and 6.13).

Table 6.5. Relationship between the predicted and observed model parameters when using 48 and 120 h as intermediate fermentation time points, and 240 h as end point.

Parameter	Intercept	Slope	R^2
$pdNDF_1$	0.1007[a]	0.8515	0.89
$pdNDF_2$	0.0160	0.8299	0.85
k_1	0.0022	1.0129	0.92
k_2	0.0032	0.4731[b]	0.29
iNDF	-0.0128	1.0197	0.98

[a]: Intercept significantly ($P < 0.05$) different from 0.
[b]: Slope significantly ($P < 0.05$) different from 1.

Table 6.6. Relationship between the predicted and observed model parameters when using 48 and 96 h as intermediate fermentation time points, and 240 h as end point.

Parameter	Intercept	Slope	R^2
$pdNDF_1$	0.0675	0.9213	0.76
$pdNDF_2$	0.0072	0.8129	0.86
k_1	0.0002	0.9499	0.92
k_2	0.0008	0.8222	0.92
iNDF	-0.0135	1.0360	0.98

[a]: Intercept significantly ($P < 0.05$) different from 0.
[b]: Slope significantly ($P < 0.05$) different from 1.

Table 6.7. Root mean squared error (RMSE), mean bias (observed – predicted), and distribution of the mean squared prediction error (MSPE), per parameter, when using 36 and 120 h as intermediate fermentation time points, and 240 h as end point.

			Distribution of MSPE*		
Parameter	RMSE	Mean bias	Bias	Slope	Random
$pdNDF_1$	0.0420	0.0278	0.0771	0.0175	0.1259
$pdNDF_2$	0.0401	-0.0210	0.0443	0.0500	0.0819
k_1	0.0071	-0.0039	0.0015	0.0002	0.0034
k_2	0.0034	-0.0017	0.0003	0.0002	0.0006
iNDF	0.0138	-0.0067	0.0045	0.0005	0.0141

*: $\times 10^3$

Table 6.8. Root mean square error (RMSE), mean bias (observed – predicted), and distribution of the mean squared prediction error (MSPE), per parameter, when using 48 and 120 h as intermediate fermentation time points, and 240 h as end point.

			Distribution of MSPE*		
Parameter	RMSE	Mean bias	Bias	Slope	Random
$pdNDF_1$	0.0304	0.0074	0.0055	0.0179	0.0693
$pdNDF_2$	0.0308	-0.0025	0.0006	0.0194	0.0747
k_1	0.0063	-0.0032	0.0010	0.0000	0.0019
k_2	0.0021	-0.0030	0.0009	0.0010	0.0020
iNDF	0.0136	-0.0049	0.0024	0.0004	0.0156

*: $\times 10^3$

Also, in this case as in previous estimations, the optimization of the lag values resulted in the lowest correlations, however these were still significant ($P < 0.05$), and the highest RMSE and MSPE, confirming a lack of information for the optimizer. As previously shown (Raffrenato et al., 2009), the estimation of iNDF was therefore more important than the estimation of the lag in the prediction of the other parameters of a decay model. This suggests that a better understanding and definition of ranges for iNDF within a forage type, or preferably by species, will result in a reliable estimation of the indigestible fraction through non-linear approaches and that would preclude the need to conduct long-term fermentations (240 h).

All other parameters resulted in very low bias, slope and random variation (Table 6.12 and 6.13). The outcome of this work suggests the optimization in Vensim can become a routine tool to better define fiber fractions in forages for a better decision making process for nutritionists.

Table 6.9. Root mean squared error (RMSE), mean bias (observed – predicted), and distribution of the mean squared prediction error (MSPE), per parameter, when using 48 and 96 h as intermediate fermentation time points, and 240 h as the end point.

			Distribution of MSPE*		
Parameter	RMSE	Mean bias	Bias	Slope	Random
$pdNDF_1$	0.0381	-0.0081	0.0066	0.0047	0.1560
$pdNDF_2$	0.0345	-0.0156	0.0242	0.0244	0.0702
k_1	0.0058	-0.0038	0.0014	0.0001	0.0019
k_2	0.0021	-0.0009	0.0001	0.0001	0.0002
iNDF	0.0148	-0.0041	0.0017	0.0012	0.0190

*: $\times 10^3$

Table 6.10. Relationship between the predicted and observed model parameters when using 30 and 120 h as fermentation time points, and constraining iNDF to forage-family-specific ranges.

Parameter	Intercept	Slope	R^2
$pdNDF_1$	0.0944	0.8562	0.77
$pdNDF_2$	0.0098	0.8445	0.73
k_1	0.0101	0.9010	0.76
k_2	0.0023	0.8485	0.75
iNDF	0.0157	0.9590	0.97

Table 6.11. Relationship between the predicted and observed model parameters when using 36 and 120 h as intermediate fermentation time points, and constraining iNDF to forage-family-specific ranges.

Parameter	Intercept	Slope	R^2
$pdNDF_1$	0.0937	0.7954	0.72
$pdNDF_2$	0.0122	0.9187	0.70
k_1	-0.0098	0.8821	0.69
k_2	0.0011	0.7819	0.71
iNDF	0.0132	0.9102	0.90

Table 6.12. Root mean squared error (RMSE), mean bias (observed – predicted), and distribution of the mean squared prediction error (MSPE), per parameter, when using 30 and 120 h as intermediate fermentation time points, and constraining iNDF to forage-family-specific ranges.

			Distribution of MSPE*		
Parameter	RMSE	Mean bias	Bias	Slope	Random
$pdNDF_1$	0.0474	0.0181	0.0821	0.0121	0.1342
$pdNDF_2$	0.0789	-0.0135	0.0212	0.0121	0.0821
k_1	0.0105	0.0028	0.0021	0.0004	0.0046
k_2	0.0026	-0.0014	0.0004	0.0007	0.0012
iNDF	0.0316	0.0054	0.0054	0.0032	0.0321

*: $\times 10^3$

Table 6.13. Root mean squared error (RMSE), mean bias (observed – predicted), and distribution of the mean squared prediction error (MSPE), per parameter, when using 36 and 120 h as intermediate fermentation time points, and constraining iNDF to forage-family-specific ranges.

			Distribution of MSPE*		
Parameter	RMSE	Mean bias	Bias	Slope	Random
$pdNDF_1$	0.0471	0.0199	0.0721	0.0341	0.1211
$pdNDF_2$	0.0654	-0.0099	0.0128	0.0099	0.0912
k_1	0.0096	0.0033	0.0043	0.0012	0.0045
k_2	0.0014	0.0009	0.0011	0.0012	0.0016
iNDF	0.0343	0.0044	0.0061	0.0021	0.0456

*: $\times 10^3$

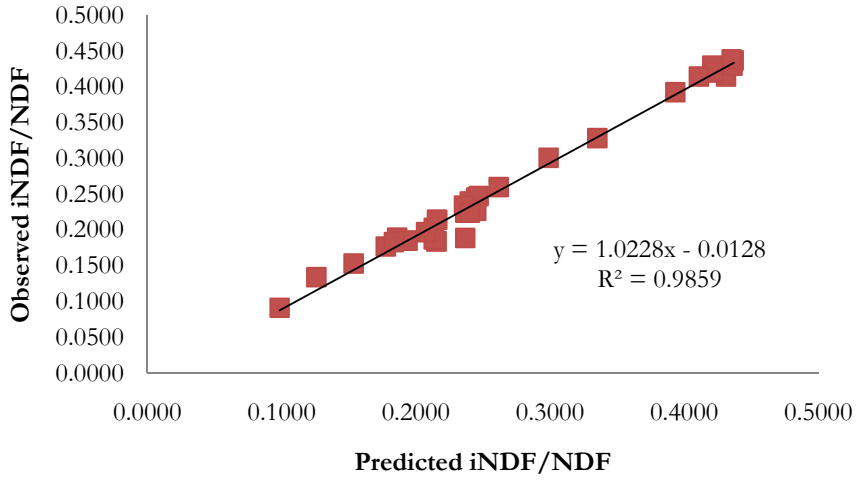

Figure 6.4. Regression of observed iNDF/NDF values from the non-linear estimation on predicted values from the 36-120 h optimization (using 240 h as end point).

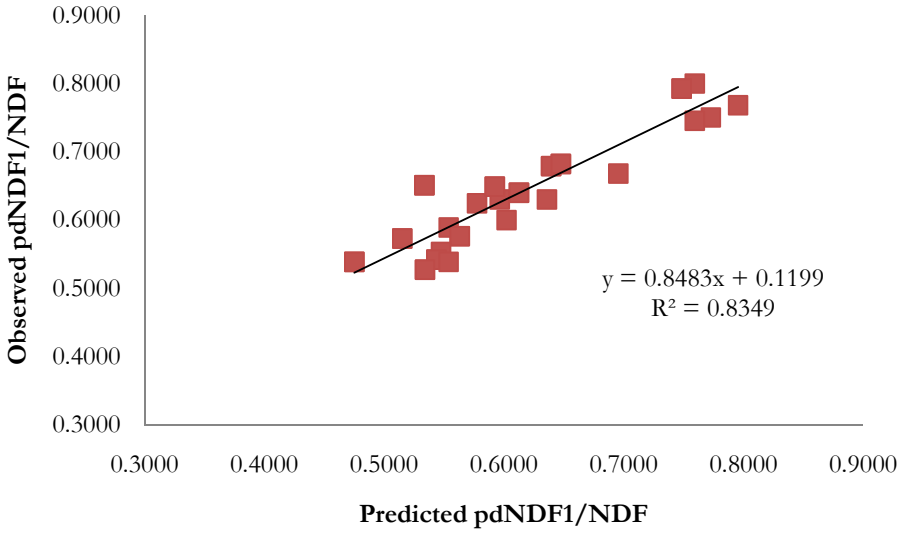

Figure 6.5. Regression of observed $pdNDF_1$/NDF values from the non-linear estimation on predicted values from the 36-120 h optimization (using 240 h as end point).

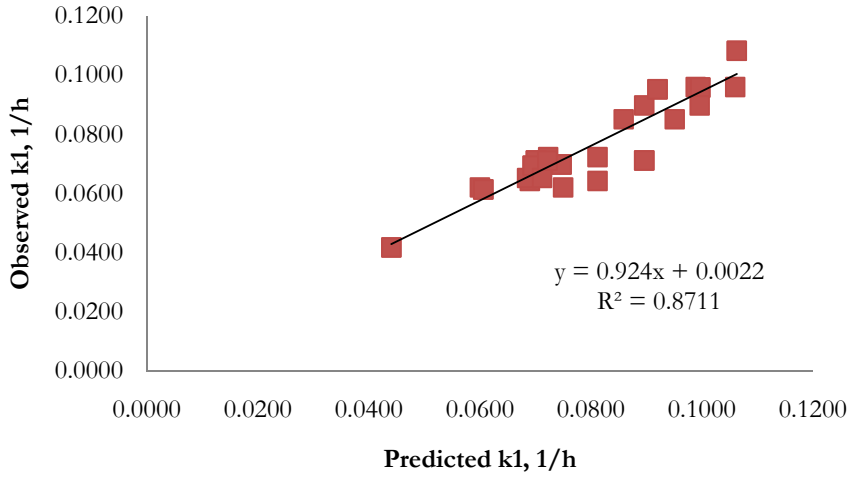

Figure 6.6. Regression of observed k_1 values (1/h) from the non-linear estimation on predicted values from the 36-120 h optimization (using 240 h as end point).

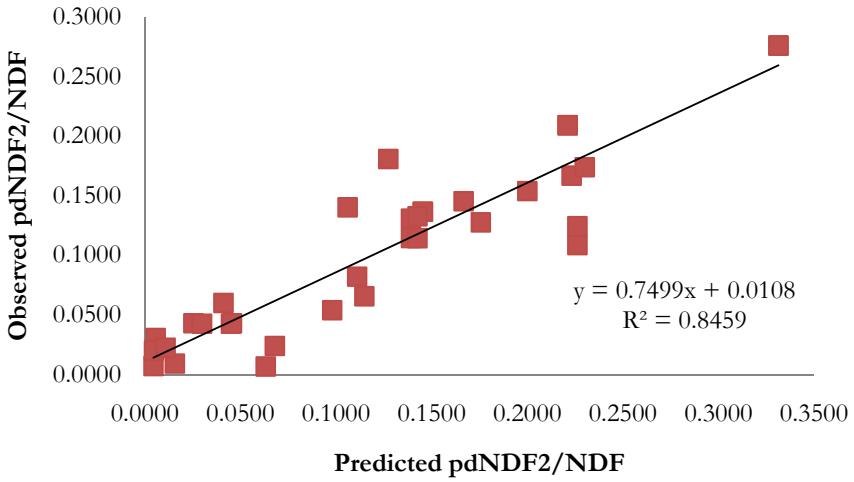

Figure 6.7. Regression of observed pdNDF2/NDF values from the non-linear estimation on predicted values from the 36-120 h optimization (using 240 h as end point).

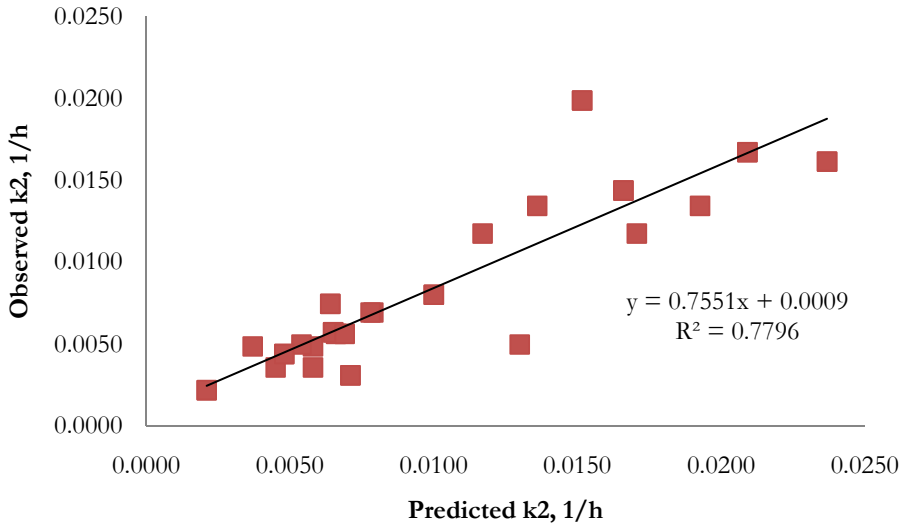

Figure 6.8. Regression of observed k_2 values (1/h) from the non-linear estimation on predicted values from the 36-120 h optimization (using 240 h as end point).

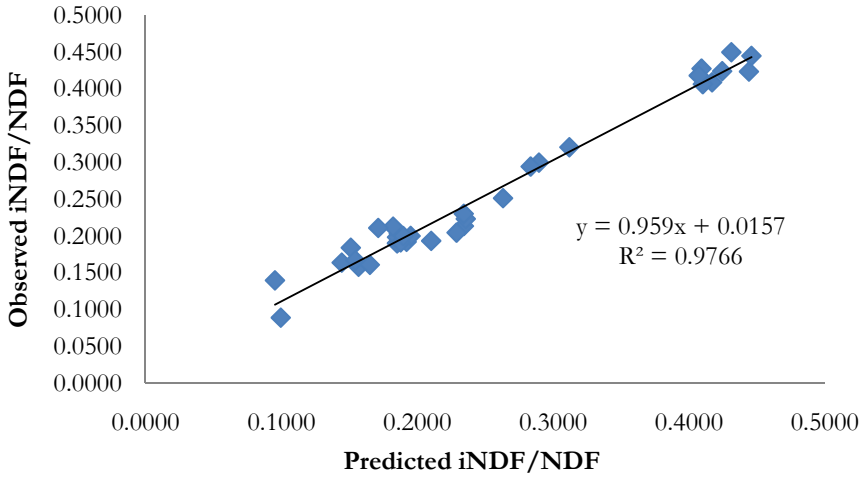

Figure 6.9. Regression of observed iNDF/NDF values from the non-linear estimation on predicted values from the 36-120 h optimization (using forage group-specific iNDF ranges).

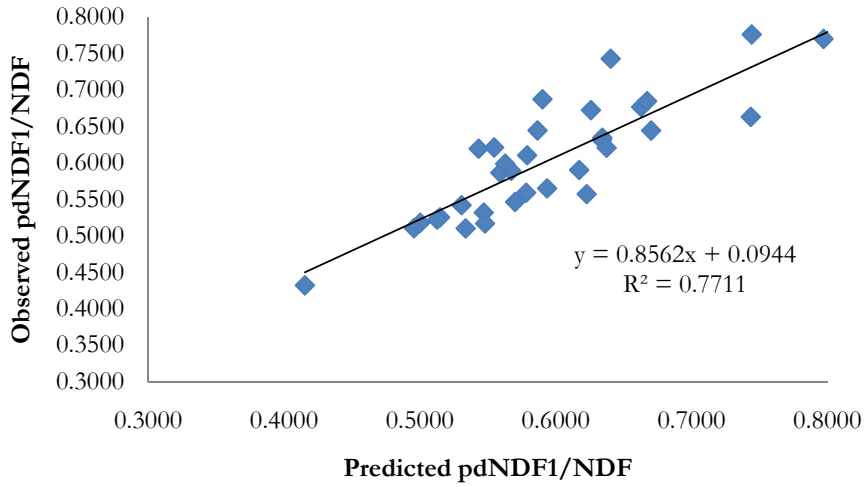

Figure 6.10. Regression of observed $pdNDF_1$/NDF values from the non-linear estimation on predicted values from the 36-120 h optimization (using forage group-specific iNDF ranges).

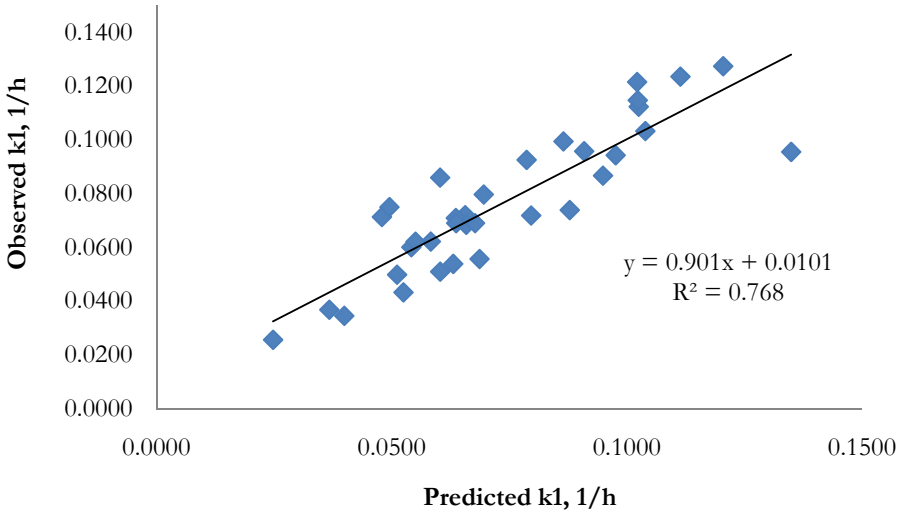

Figure 6.11. Regression of observed k_1 values (1/h) from the non-linear estimation on predicted values from the 36-120 h optimization (using forage group-specific iNDF ranges).

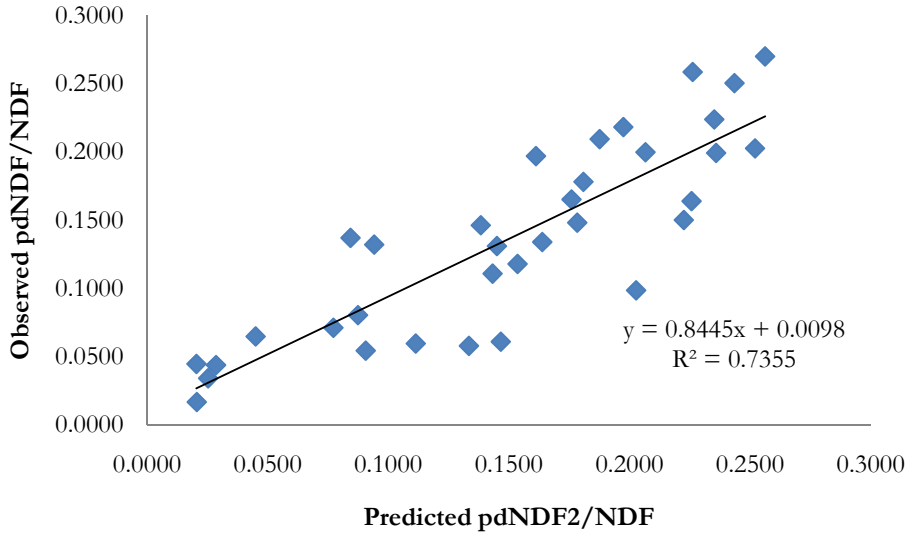

Figure 6.12. Regression of observed $pdNDF_2$/NDF values from the non-linear estimation on predicted values from the 36-120 h optimization (using forage group-specific iNDF ranges).

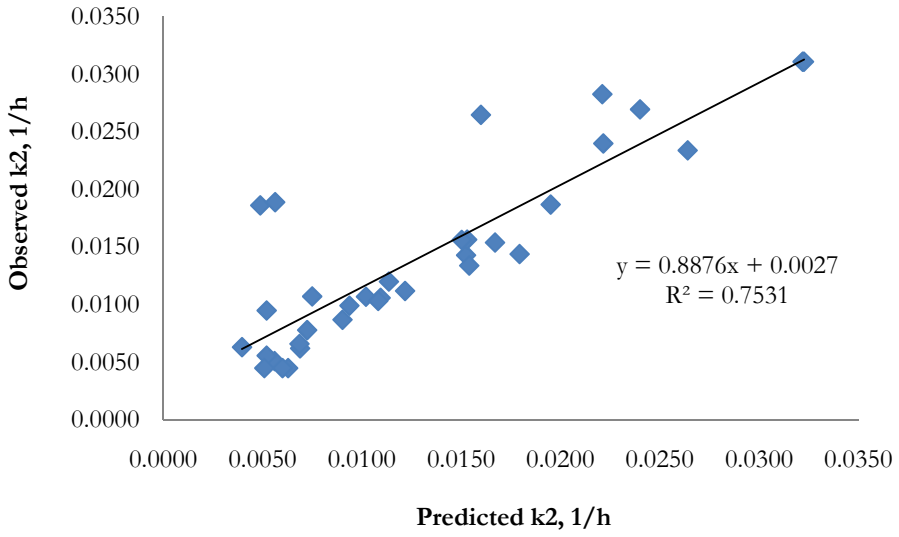

Figure 6.13. Regression of observed k2 values (1/h) from the non-linear estimation on predicted values from the 36-120 h optimization (using forage group-specific iNDF ranges).

After evaluating the global data set, it became apparent that due to the more variable nature of the iNDF and the apparent differences in the size and rate of the $pdNDF_1$ pool, it would be more efficient and practical to run forage group-specific solutions. Therefore we optimized all the same combinations of time points for each of the forage group, pooling forages within each group only, to determine if improvements in efficiency and accuracy could be obtained. We recognize the small number of forages per group, but the solutions appear to be very robust and were lower in RMS than previous solutions. The optimal combination of time points for each forage group using either 240 h as end point or when constraining iNDF to forage groups specific ranges, when ranked based on average R^2 and RMS, are shown in Tables 6.12 and 6.13.

Table 6.14. Optimal combinations of time points when using two time points in the curve plus 240 h as endpoint and respective average R^2 and RMS across forages within group (n is number of forages per group).

Group	n	Combination	R^2	RMS
Conventional C.S.	7	36-120	0.92	0.0012
BMR C.S.	6	30-120	0.93	0.0032
Grasses	6	36-96	0.89	0.0041
Straws and hays	4	48-120	0.88	0.0022
Alfalfas	7	24-96	0.94	0.0010

Table 6.15. Optimal combinations of time points when using two time points in the curve and constraining iNDF to forage group-specific ranges and respective average R^2 and RMS across forages within group (n is number of forages per group).

Group	n	Combination	R^2	RMS
Conventional C.S.	7	36-120	0.88	0.0012
BMR C.S.	6	30-120	0.81	0.0032
Grasses	6	36-96	0.73	0.0041
Straws and hays	4	48-120	0.82	0.0022
Alfalfas	7	24-96	0.93	0.0010

Overall the results indicate that 36 (or 48) and 120 h is the optimal combination of time points for most forage groups. Both bmr corn silages and alfalfas resulted in better goodness of fit when using 24-96 h and 30-120 h, respectively, most likely because of the time point where the $pdNDF_1$ is exhausted. Grass hays and straws instead resulted in better estimations of pools and rates when residual at 36-96 h or 48-96 h were used for the optimization, respectively. The prediction results appear to be dependent on when the fast pool is exhausted most likely due to the determination of the inflection point.

If a number of time point digestions are available, means and standard deviations of the respective lag and rate values can be calculated and their uniformity examined. An improvement of the lag estimation is possible using another time point early in the fermentation, thus providing more

information to the optimization. However the average value of the lag estimated by the non-linear composite decay, per group, was between 1.7 and 3.1 h, with the lowest values for alfalfas and the highest values for straws and hays, respectively. Constraining the lag to be the average per forage group during the optimization may improve the optimization. However, lag ranges can however vary within and among laboratories, depending on the in-vitro procedure and on the rumen fluid handling. Results show that an unknown lag (within the normal range) will not bias the final estimation of pools sizes and rates, but still means that the laboratory know and understand the average lag time and if there is a varation by method or forage type.

According to Ellis et al. (1999) determination of iNDF should be included in all basic feedstuff analysis because it has a predictable digestibility; it can be used for the estimation of the pdNDF as NDF-iNDF and it has an important role in contributing to rumen digesta load. Furthermore, a close empirical relationship between silage iNDF and OM digestibility (Nousiainen et al., 2003) indicates that iNDF is a useful entity for the prediction of the nutritive value of forages. We demonstrated that prediction of the indigestible fraction is possible if longer time points are not available, by using forage group-specific ratios of iNDF/(ADL/NDF) (Tables 6.10 and 6.11).

CONCLUSIONS

The calculation of k_1 and k_2 and the $pdNDF_1$ and $pdNDF_2$ presented here have the advantage of requiring minimum data to generate and thus might be more easily implemented by commercial laboratories. Since the calculations are direct and use more robust mathematical modeling procedures, they are simpler to implement because many observations are not required, although the user will need some understanding of the software and optimization procedures. The modeling approach described in this paper should provide nutritionists with better information about the heterogeneity and digestibility of NDF and the dynamic nature of the pool sizes that might influence feed intake and energy yield. A larger data set of long-term fermentations with intermediate time points are needed to build a data set able to explain the variation in NDF pools sizes and within forage group and this should be linked to the agronomic conditions the plants were grown under.

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CHAPTER SEVEN: CONCLUSIONS

The focus of this work was to improve the estimation and prediction of NDF digestibility and lignin methods to develop dynamic models of NDF digestibility for use in nutrition models. This goal was to provide information and data that would improve the use of forages by providing data that made formulation with forages more efficient. It was hypothesized that improvements of the methodologies for the estimation of rate and extent of NDF digestion were still possible and were related to heterogeneous behavior of NDF digestion. A better understanding of the extent of NDF digestion along with improved fiber chemistry would help clarify the variation associated with feeding larger volumes of NDF in both research and field application. This is especially true if these improvements are implemented in ration formulation systems and models.

We improved the ADL method through increased recovery of especially lower ADL forages, like bmr corn silages and immature grasses, and of forages and feeds with greater processing, such as feces, thus demonstrating the need for the additional filter. The use of a glass microfiber filter was implemented in the method for measuring in vitro NDF digestibility and we were able to obtain increased recoveries of NDF and the estimated iNDF at 240 h between 0 and 75% through the use of the filters. The improved recoveries of both ADL and iNDF resulted in range of the ratios iNDF/NDF to ADL/NDF between 1.73 and 7.59, showing the dynamic nature of the relationship. However, the prediction of iNDF from ADL still needs more information to better explain the observed range.

The study of the phenolic acids (ferulic and *p*-coumaric acids), variously linked with cell wall components, and their association with rate and extent of NDF digestion showed how these components appear to be distributed in the different cell wall fractions of the plant and not uniformly among forage families and therefore there is the need of consistency in reporting their respective amounts in plants. However, the quantification of these linkages with the associated lignin was able to explain up to 98% of the variation in NDF digestibility, but not uniformly across forage groups. Furthermore, the ADF content of phenolic acids and their respective linkages offer an opportunity to avoid dependence of different cell wall fraction measures, since results were more consistently associated to digestibility across forage groups, when compared to dry matter or NDF basis.

An additional objective was to create a better mathematical description of the digestion of NDF in vitro in pools of different size and rate of digestion. The improved fiber methodologies allowed for a clearer definition of a slow and a fast degrading NDF pool, and the iNDF fraction, using only two points in the fermentation curve and a forage group-specific range for iNDF according to the ADL/NDF content. This study further verifies the heterogeneous nature of NDF disappearance and provides approach for estimating the individual pool sizes and rates for application in diet formulation. This dynamic nature of NDF digestibility might influence feed intake, chewing and rumination activities and energy yield and could also be linked to growing season and agronomic conditions, in their effects of the varying NDF chemistry and DMI in ruminants. The multiple pool NDF approach could be used in the future to better calculate intake and

rumination and chewing activities, since these predictions and mechanisms have not been yet completely clarified with our knowledge. After better predictions are defined, the approach could be used, for example, to establish minimum pool size and rate for pool 1 (i.e. fast pool) to ensure higher levels of forage or NDF intake, rumen health and feed conversion efficiency.

All of the above results represent a step forward in understanding what fiber represents for a ruminant. On one hand, we now know that fiber from forage sources can bring into the diet a complex nutritional entity that can result in different outcomes, even with similar apparent chemical characteristics both from an animal perspective and from a nutritionist and farmer. However, on the other hand, we now have the chemical and mathematical knowledge of a forage to disentangle this complexity into pieces and study them individually to ultimately improve animal performance and health.