VALIDATION OF THE 2.4 TIMES LIGNIN FACTOR FOR ULTIMATE EXTENT OF NDF DIGESTION, AND CURVE PEELING RATE OF FERMENTATION CURVES INTO POOLS

P. J. Van Soest, M. E. Van Amburgh,
J. B. Robertson and W. F. Knaus*
Department of Animal Science
Cornell University
and
*BOKU – University of Natural Resources and Applied Life Sciences
Department of Livestock Sciences
Vienna, Austria

INTRODUCTION

The factor 2.4 times lignin content of neutral detergent fiber (NDF) is used in the Cornell Net Carbohydrate and Protein System (CNCPS) to estimate the potentially degradable NDF pool. The amount of NDF estimated by 2.4 times lignin is assumed to be obligately undegradable and thus would have a rate of digestion of zero. The value 2.4 was based on long-term methane fermentations of 60-90 days on various waste materials (Chandler et al., 1980). However, its accuracy has not been evaluated for common forages, particularly alfalfa and corn silages which are the primary sources of fiber in dairy rations. The 2.4 factor is also needed in the calculation of rates of digestion of NDF.

Rates of digestion are important in evaluating forage NDF quality and not highly correlated with lignin contents. The best correlation between rate and extent of NDF digestion is at 24 hours. However, at 48 hours and longer the relationship is poor (Van Amburgh et al., 2003). Fermentation curves of NDF times are not linear on logarithmic or other bases (Van Soest 1994, Van Soest et al., 2000). Mertens (1973) indicated that there are at least two pools of degradable NDF, a fast and a slow pool. However, his data extended to only 96 hours and the NDF pool representing the slower phase was not well characterized. In the 2000 Cornell Nutrition Conference proceedings (Van Soest et al., 2000), we presented a second order model to deal with nonlinearity. However, there are two approaches to account for second-order behavior. The first is the true second order equation which has been difficult to apply (Van Soest et al., 2000). The other is to break the curve into first order components. To settle this question longer term fermentations were required. The objectives of this study are to evaluate the 2.4 factor times lignin content of NDF ($U_{2.4}$) and to clarify the nature of the slow digesting fraction. A model for fermentation curves is also presented.

FORAGES AND METHODS

This paper presents data on 21 forages with fermentation times to greater than 200 hours. The forages included in house standards, two alfalfas, two timothies and orchardgrass, guinea grass, and two wheat straws. An additional 13 corn silages were supplied by Van Amburgh et al. (2003).

Sample Handling

Forages were dried in a forced air oven (60° C) and ground through a 1mm screen in a Wiley Mill. Fermentations up to 96 hours were carried out in 125 ml Erlenmeyer flasks in 39° C water bath under constant carbon dioxide in Goering and Van Soest buffer (1970) and inoculated with rumen fluid from a cow fed hay and grain. Rumen inoculum was not blended and was strained through four layers of cheesecloth and filtered through glass wool to aid in removal of very small particles that would affect the recoveries of indigestible material. Blank samples were run for all time points and used to correct for any contamination from the rumen fluid inoculation. The same cow was used for all fermentations. Long term fermentations > 96 hours are analytically difficult because the amounts digested per unit time are small and have large analytical error. Filters and nylon bags are prone to loss of finely divided undigested matter. Filtration directly on sintered glass crucibles also involves loss of fines. Alternatives are filtration on sand, centrifugation or paper (Uden 2005). Fermentations from 96 to 240 hours were conducted in 250 ml centrifuge bottles using the procedure above, except that every 72 hours the samples were centrifuged, the supernatant was poured off, and the sample were re-inoculated. Overall, fermentations were carried out for 6, 12, 24, 30, 36, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hours for nine forages. Twelve others were fermented for 6, 12, 24, 30, 36, 48, 72, 96, 144 and 216 hours. Neutral detergent fiber analysis was conducted with amylase, but without the use of sodium sulfite (Van Soest et al., 1991). In addition, the ND residues were filtered in crucibles that contained approximately 4-5 grams of sea sand to provide a filtering aid and prevent the loss of very small particles. Lignin was determined from acid detergent residues hydrolyzed in 72% sulfuric acid for 3 hours. Results are expressed on a dry matter basis.

Mathematical Analysis

A way of estimating extent, rate of digestion and pool sizes, without conversion to natural logarithms, is derived from a modification of the first order differential equation involving integration for two available pools and an unavailable residue:

$$1.\quad S = be^{-k_1 t} + P_2 e^{-k_2 t} + U$$

Where S is residual NDF at times t, b is a composite involving lag and size of pool 1 (See equation 4). P_1 and P_2 are fast and slow digesting pools, respectively and U is the ultimate indegradable fraction. If only late fermentation times are used (48 hours and later), the fast first pool has largely decayed out and only pool two remains:

2. $S > 48\ h = P_2 e^{-k_2 t} + U$

If values of S with time are regressed on values of $e^{-k_2 t}$, the intercept of the regression estimates U, and the slope estimates the size of the slow digesting pool. Regressions are conducted iteratively varying the value of k_2. As sequential regressions are conducted with increasing values of k_2, R^2 rises and passes through a maximum and declines. The solutions for k_2, P_2 and U are taken at the point of maximum R^2. This analysis is consistent with that of Mertens' approach (1973) which subtracted a quantity assumed to be indigestible that would linearize the slope of ln S upon time. However, Mertens included earlier times to 96 hours in his analysis and thus confounded the fast and slow pools. His estimates of U were as a result are too high.

Analysis of the Fast Digesting Pool

Using the regressions obtained from this iterative application of equation 2, predicted values of the contribution of the slow pool plus U were generated for earlier times (6 to 48 hours) and subtracted from observed S to give estimates of the first pool. These values were analyzed iteratively according to equation 3:

3. $S_1 = be^{-k_1 t}$

Where the value of b involves lag (L) and size of first pool P_1 in equation 4:

4. $L = (1/-k_1) \ln (P_1/b)$, or $b = P_1 e^{-k_1 L}$

Size of P_1 is fixed by equation 5:

5. $P_1 + P_2 + U = 1$

As values of k_1 are iteratively increased, the intercept passes from negative through zero to positive. The iterative application of equations 3 and 4 assume a zero intercept, which in this case was used as the criterion of solution. The values of k_1 and b were taken at zero intercept. Values of P_1 from equation 5 are substituted into equation 4 to calculate lag.

RESULTS AND DISCUSSION

The value of U is a function of lignin content which has long been recognized as a primary factor limiting fiber digestion (Van Soest, 1994). However, the results shown here add new dimensions. The relationship between 2.4 times lignin content of NDF ($U_{2.4}$) and the iteratively determined value of ultimate extent (U_i) is shown in Figure 1. The relationship is not significantly different from unity. The average U_i is slightly higher than $U_{2.4}$ but not significantly so. The deviations between $U_{2.4}$ and U_i are not correlated with $U_{2.4}$. The coefficient of variation is about 10%. The use of the 2.4 factor is validated.

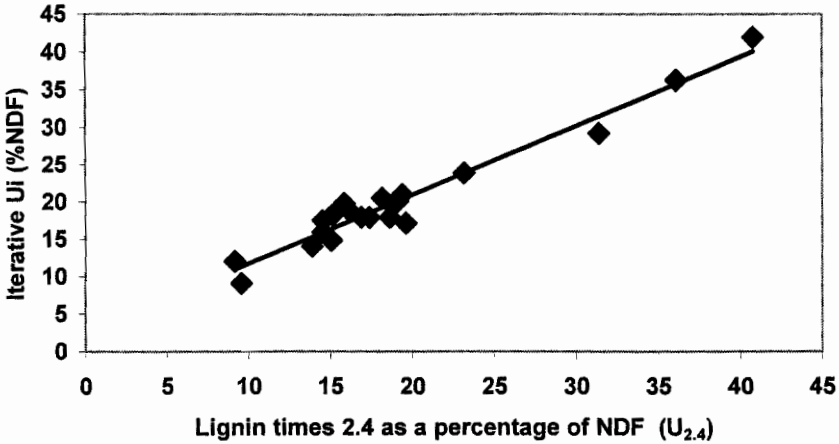
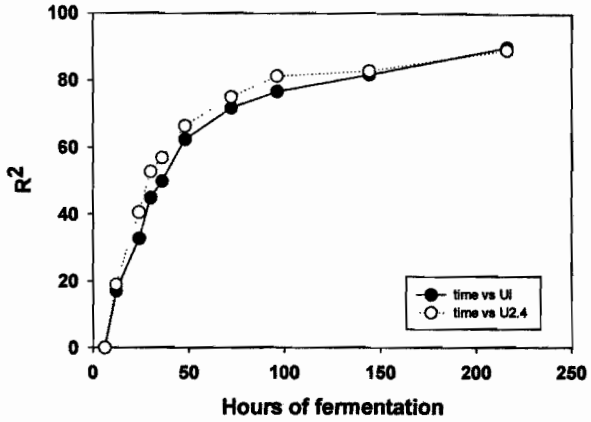
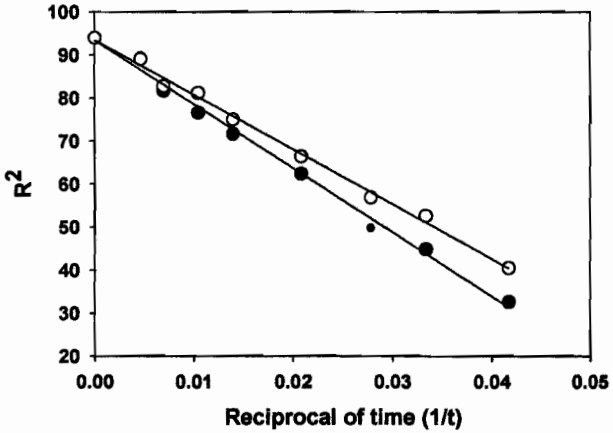


Figure 1. The relationship between the iterative extrapolation (Ui) and the lignin content of NDF times 2.4. The regression equation is: y = 2.56 + 92.4 x. R2 = 94.0, Sdy.x = 1.89.

Further understanding of the effect of lignin on digestibility is disclosed by examining the correlations between U and the extents of digestion at different fermentation times. The R^2's for the respective time sets rise with time of fermentation (Figure 2a) and are strikingly linear when plotted against the reciprocal of time (Figure 2b). The regressions for $U_{2.4}$ and U_i intersect at the same R^2 (93.5%) at zero which is the reciprocal of infinite time. This value is close to the R^2 observed between U_i and $U_{2.4}$ (94.0%) in Figure 1. These very high R^2's are not significantly different from each other. The values of U_i are the extrapolated extents of the respective 21 forages regressed to infinite time and are statistically in order with extents from earlier finite fermentation times. From this it can be concluded that acid detergent lignin quantitatively accounts for NDF indigestibility within the limits of analytical error.

The low R^2 associated with lignification at early fermentation times has engendered a complaint about the inadequacy of lignin measurement; however, these low relationships are the result of other factors such as rates of digestion and size of carbohydrate pools not particularly associated with lignification. As these components decay and digest out, the relationship with lignin (and U) rises.





Figures 2a and 2b. Upper figure: Correlations between ultimate indigestibility , (U_I and $U_{2.4}$) and with digestibilities observed at measured times. Data sets for NDF digestibilities of 21 forages at 6, 12, 24, 30, 36, 48, 72, 96, 144 and 216 hours are calculated for their respective correlations between extent of digestion and values of U_I and $U_{2.4}$ at each time point.

Lower figure (2b): Same data plotted against reciprocal of time. Regressions between R^2 and reciprocal of time are extrapolated to zero, which is infinite time. Six and 12 hour fermentations have low R^2's and are not in line with those of 24 hours and later and are not included in the regression statistics and the figure 2b. Regressions for U_I, y = 93.4 – 1489/time; R^2 = 99.31%. For $U_{2.4}$, y = 93.5 – 1272/time; R^2 = 99.53%.

Evaluation of Pools and Rates

The subtraction of the value of $U_{2.4}$ from the residual substrate at late fermentation times (48 hours and later) allows estimates of pool 2 and its rate of digestion k_2. Subtraction of the estimated amounts of residual pool 2 from early time residues (6 to 36 hours) provides data for pool 1 which are in turn iteratively analyzed for its rate k_1 according to equations 3-5. The result of this analysis resolves the entire fermentation curve into its components P_1, P_2, and U. Example for a bmr corn silage is shown in Figure 3, which is representative of all the forages in this study. When plotted on a semilogarithmic basis, an inflection is observed near 48 hours which is the time when the first pool has decayed out. The rate of digestion of this first phase is a composite of the disappearance of pools 1 and 2 and yields a mean rate k_d which is the value used in the CNCPS. It does not have validity beyond 36 hours. This defect is not serious for lactating dairy cattle where rumen retention is on the order of 30 hours. However, application to animals at lower feed intakes may require adjustment for slower rates of digestion.

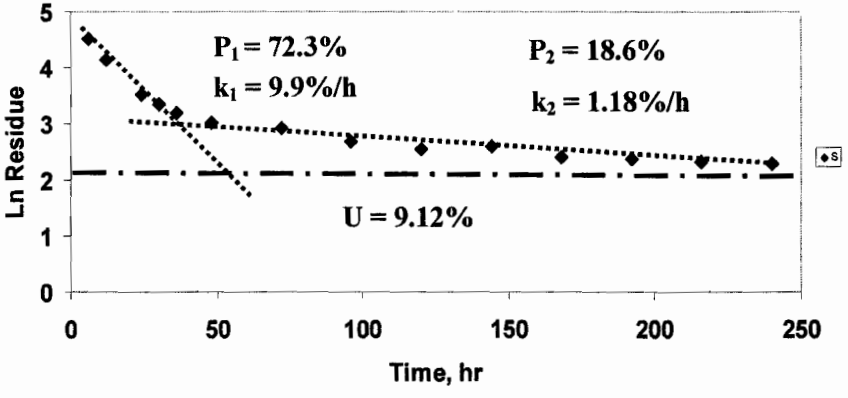


Figure 3. Example showing partition of corn silage (bmr) NDF into pools and rates. Pool 1 is exhausted by 48 hours and produces an inflection in the curve.

The sum of pools 1 and 2 and $U_{2.4}$ effectively reproduce fermentation curves with an average accuracy of 98% for the 21 forages. Average mean deviation for 21 forages between observed and predicted values of S is 1.2% with a bias of -0.2.

The average values for $U_{2.4}$, P_1, P_2, k_d, k_1 and k_2 are shown in Table 1. Alfalfas and straws have the highest lignification while brown midrib corn silages are lowest. Alfalfas have the lowest content of pools 1 and 2, while grass species (including corn silages) are higher in pool 2. Rates of digestion are highest for brown midrib corn silages. Alfalfas have a high k_1 rate (pool 1) but lowest k_2 (pool 2). Rates of digestion vary markedly with corn varieties and with maturity in perennial forages. The size of pool 2 is greatest for grass family plants and is negatively associated with pool 1 (Table 2).

Table 1. Mean values of $U_{2.4}$, pool sizes and rates of digestion.

	N	$U_{2.4}$	P_1[a]	P_2[b]	k_d[c]	K_1[d]	k_2[d]
			% NDF			%/h	
Corn silages (normal)	11	16.4	61.7	21.9	4.5	8.2	0.7
Corn silages (brown midrib)	2	9.4	72.4	18.2	6.2	10.7	1.2
Alfalfa	2	38.4	48.9	12.7	5.8	10.0	0.4
Grasses	4	19.4	55.8	24.8	3.9	7.4	0.6
Wheat straw	2	25.1	48.4	26.5	1.7	4.1	0.4

[a]Fast digesting pool 1.
[b]Slow digesting pool 2.
[c]k_d is a mean rate of digestion using the time point calculation from 24 hour extents (Van Amburgh et al., 2003)
[d]Estimated rates of digestion for pool 1 (k_1) and for pool 2 (k_2).

Table 2. Correlations among parameters.[a]

Parameter	k_1	k_2	P_1	P_2	$U_{2.4}$
k_d	+0.89	+0.51	+0.61	-0.76	-0.12
k_1		+0.53	+0.41	-0.45	-0.04
k_2			+0.73	-0.14	-0.02
P_1				-0.45	-0.72
P_2					-0.27

[a]Nineteen degrees of freedom: p 0.05 > 0.43; p 0.01> 0.55

Table 2 shows correlations between the parameters of pools and rates. The poorer correlations indicate large interactions between forage groups. For example, the correlation between $U_{2.4}$ and k_1 is -0.76 for grasses and corn silages, but the inclusion of alfalfa reduces it to -0.04. Mean rate k_d is a better predictor of pools and their rates than is $U_{2.4}$. The $U_{2.4}$ and the k_d calculated from the 24 hour time point (Van Amburgh et al., 2003) are the available predictors that may be available from general laboratory analyses, and tentative equations are provided below. Rate of digestion of the first pool is highly correlated with k_d (Figure 4). This occurs because both k_d and k_1 are logarithmic and form a linear relationship, whereas that between 24 hour extent and k_1 is nonlinear.

The correlations between pools 1 and 2 with k_d are +0.61 and -0.76, (R^2's 37 and 58%) respectively. However, because the sum of P_1, P_2 plus $U_{2.4}$ equals unity, values are separated by only two degrees of freedom. If values of P_1 and P_2 are unitized, i.e. the sum of P_1 and P_2 equal unity, the correlations of these unitized values with k_d are much improved (Figure 5) The regression suffices to predict both P_1 and P_2 which are inversions of each other, and have identical R^2's (69%).

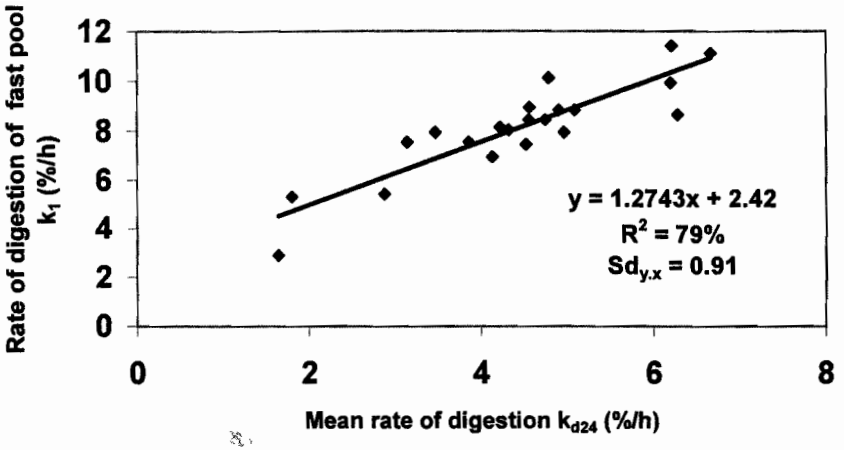


Figure 4. Relationship between mean rate of digestion (6 to 36 h), kd and rate of digestion of the fast first pool k1.

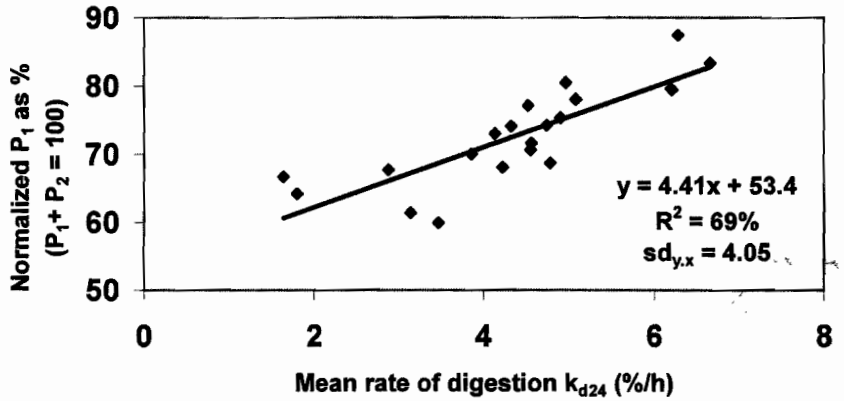


Figure 5. Relationship between mean rate of digestion and normalized pool 1. The relationship for pool 2 is the inverse image of this figure.

Equations for Estimating K_1, K_2, P_1, and P_2 from K_d at 24 Hours and $U_{2.4}$

For rate of digestion of the fast pool k_1:

6. $k_1 = 2.42 + 1.275\ k_{d24\,h}$ (percent per hour)

where k_d is the rate calculated from the time point procedure (Van Amburgh et al. 2003), R^2 = 79.1% with a standard deviation of 0.9%.

For the rate of digestion of the slow pool k_2, equation 7:

7. $k_2 = 0.739 - 0.02088\ U_{2.4} + 0.0795\ k_{d24h}$ as percent per hour

where $U_{2.4}$ and k_d are percents. R^2 is 70% and standard deviation 0.14%

Predicted value of pools 1 and 2 for this calculation have been normalized so that $P_1 + P_2 = 1$ and calculated in equation 8:

8. Normalized $P_1 = 53.39 + 4.41\ k_{d24h}$

Values of normalized P_2 are by difference: $P_2 = 1 - P_1$. Values of P_1 and P_2 on an NDF basis are obtained by multiplying by $(100 - U_{2.4})/100$; R^2 is 69% and standard deviation 3.67% (Figure 5).

The mean rate of digestion, k_d is calculated from a 24 hour extent of NDF digestion, where S_{24h} is the undigested residue as percent of NDF in equation 9:

9. $k_{d24h} = \ln A/(24 - L)$; where $A = (S_{24h} - U_{2.4})/(100 - U_{2.4})$

The value of lag (L) is usually 3 – 4 hours. A six hour fermentation would make lag estimation more precise, see Van Amburgh et al. (2003). A four hour lag was used throughout this paper. The number obtained in equation 9 is a negative coefficient indicating the proportion of the substrate disappearing per hour. This number needs to be multiplied by 100 to obtain percent per hour which is used in equations 6, 7 and 8.

Calculation of Extents per Time of Fermentation with a Validation Set

A validation set of 31 forages, including 10 alfalfas, 8 perennial grasses (timothy and orchardgrass) from Mertens (1973) and 13 new corn silages from Van Amburgh et al., (2003) were evaluated using 24 hour time-point calculations for k_d and $U_{2.4}$ values to calculate k_1, k_2, P_1, P_2 according to equations 6, 7 and 8. Curves were evaluated to 96 hours. Residual amounts of pool 1 were calculated by substitution of coefficients (%/100) of P_1 and k_1 into $P_1e^{-k_1(t-L)}$ and integrated at respective fermentation times. Similarly, residual amounts of pool 2 plus $U_{2.4}$ were calculated according to equation 2. Lag was ignored. The summed values of pools 1, 2 and $U_{2.4}$ at respective fermentation times were taken as the net remaining undegraded NDF. A program is available for these calculations from Van Amburgh, including k_d from 24 hour extents.

Difference values between observed and calculated extents are shown in Table 3, and the correlation between observed and calculated values for the 31 forages at each time point. The mean differences represent bias, negative values indicating underprediction of digestibility and positive ones over-prediction. These values average about one unit

of NDF digestibility with a standard deviation of about 2. The deviations are larger at early and late times with a lower R^2. However, the results show that if adequate values of 24 hours in vitro digestion with lignin and NDF analyses are available, digestibility of NDF can be estimated within a standard error of 2-3 units, which corresponds to 1-2 units of dry matter digestibility. This variation is well within the limits of the error in digestion trials and could be applied to estimation of 48 hour values from 24 hour values of digestion. The construction of the net fermentation curve is also possible. Poor correlation at early times reflects inadequacy of lag estimation, while variation at 48 to 96 hours is due to error in lignin determination. Adequate application of this calculation of net extents and pools would be helped by the availability of standard forage samples.

Table 3. Mean differences and correlations between observed and calculated extents per time point of digestion for 31 forages.

Time point (h)	Mean difference % initial NDF	Standard deviation	R^2 %
6	-0.99	2.49	48.52
12	+1.15	3.42	82.37
24	+1.39	1.31	98.81
48	+1.18	2.36	96.19
72	+1.35	2.37	96.49
96	+1.50	2.56	96.07

CONCLUSIONS

Fermentation curves of NDF can be partitioned into three components: a fast digesting pool, a slow digesting pool, and an indigestible fraction. The indegradable fraction is quantitatively associated with acid-detergent lignin. The factor 2.4 times lignin content of NDF is validated for the CNCPS.

The rate of digestion k_d is an average of fast and slow digesting pools and is valid up to 36 hours after which the fast pool has decayed out and rate of digestion at 48 hours and later is dominated by the slow second pool. Estimates of pool sizes and respective rates can be estimated from the lignin content of NDF x 2.4 and a 24 hour time point digestion. These estimates allow a complete construction of fermentation curves. From this it is possible to predict extent of digestion at any time of fermentation from a single fermentation at 24 hours and a determination of NDF and lignin. Analysis of forages for 24 hours of digestion, lignin, and NDF analyses with standard samples is recommended.

A lignin determination for the value of U is essential for the accurate calculation of digestion rates. Failure to correct for the indigestible pool will result in gross underestimation of rates, because then it is averaged over heterogeneous fractions including available and unavailable residues. In classical physical chemistry a kinetic rate is presumed to apply to a chemically uniform fraction. Accurate forage kinetics need to conform to this rule.

REFERENCES

Chandler, J. A., W. J. Jewell, J. M. Gossett, P. J. Van Soest and J. B. Robertson. 1980. Predicting methane fermentation biodegradability. In: J. Biotech. And Bioengineering Symp. No. 10, John Wiley & Sons. Pp. 93-107.

Goering, H. K. and P. J. Van Soest. 1970. Forage fiber analysis. Agric Handbook No. 379 USDA. Washington, DC.

Mertens, D. R. 1973. Application of theoretical mathematical models to cell wall digestion and forage intake in ruminants. Ph.D. Thesis, Cornell Univ., Ithaca, NY.

Uden, P. 2005. Loss of insoluble fibre fractions by filtration and centrifugation. Swedish University of Agricultural Sciences. Uppsala, Sweden. Anim Feed Sci Tech. Submitted.

Van Amburgh, M. E., P. J. Van Soest, J. B. Robertson and W. F. Knaus. 2003., Corn silage neutral detergent fiber: Refining a mathematical approach for in vitro rate of digestion. Cornell Nutrition Conf. Proceedings. Pp. 99-108.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. 2nd Edition. Cornell Univerisity Press. Ithaca, NY.

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

Van Soest, P. J., M. E. Van Amburgh and L. O. Tedeschi. 2000. Rumen balance and rates of fiber digestion. Cornell Nutrition Conf. Proceedings. P. 150-166.