hazard (Fitt et al., 1980). Further, the ion-exchange resins used in the heavy metal column are as rigid as silica but have an inert surface and are stable over a wide range of pH and thus, generally, last longer (Palmer, 1979) than the normal-phase carbohydrate columns (Hurst et al., 1979). However, operation of the heavy metal column at an elevated temperature is considered a disadvantage by some investigators (McGinniss and Fang, 1980). Another advantage of the heavy metal column is that sugars generally elute in the order of decreasing molecular weights, so deterioration products or large-chain carbohydrates elute first (Wong-Chong and Martin, 1979). High molecular weight compounds elute last from the normal-phase column and the peak broadening which occurs makes it difficult to know when the last peak has eluted (Palmer, 1979).

Registry No. G, 50-99-7; M, 3458-28-4; Ga, 59-23-4; R, 3615-41-6; A, 147-81-9; X, 58-86-6; cellobiose, 528-50-7; cellotriose, 33404-34-1.

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Received for review October 4, 1982. Accepted February 28, 1983. This work was supported in part by National Institutes of Health Grant AM-21712 and by the College of Agricultural and Life Sciences, University of Wisconsin—Madison.

High-Pressure Liquid Chromatographic Analysis of Component Sugars in Neutral Detergent Fiber for Representative Warm- and Cool-Season Grasses

William R. Windham,* Franklin E. Barton, II, and David S. Himmelsbach

Hemicelluloses are complex groups of cell wall polysaccharides which comprise significant proportions of the dry matter of many warm- and cool-season forages. Hemicellulose was obtained by acid (2 N trifluoroacetic acid) hydrolysis of the neutral detergent fiber (NDF) fraction and analyzed for monosaccharides by high-presure liquid chromatography (HPLC). Upon hydrolysis of the NDF, the acid-soluble portion was greater ($P < 0.05$) than the amount of hemicellulose as determined by the difference between NDF and acid detergent fiber (ADF). Xylose and arabinose were the major hemicellulosic sugars in the 1-h acid-soluble NDF of both warm- and cool-season grasses. During acid hydrolysis of NDF and subsequent HPLC analysis, residues were obtained which may be related to fiber digestibility. Preliminary investigations of the residues with NMR spectroscopy indicate the presence of an acid-resistant macromolecule such as a lignin–carbohydrate complex. Identification of these residues may provide information on the difference in digestibility of warm- and cool-season grasses.

The polysaccharide constituents of forages are a major source of energy to ruminants. As a result, numerous workers (Bailey and Ulyatt, 1970; Daughtry et al., 1978; Dehority, 1973) have investigated carbohydrate composition and its subsequent digestion. Hemicelluloses are cell wall polysaccharides which comprise 10–25% of the dry matter of many temperate grasses and 20–45% of the dry matter in tropical grasses. Previous methods used for determining the carbohydrate composition of any plant cell wall samples required elaborate and time-consuming fractionation to separate and measure the various sugars (Waite and Gorrod, 1959; Albersheim et al., 1967; Bailey, 1967).

Van Soest and Wine (1967) designed a two-detergent extraction procedure that removed soluble carbohydrates and left a fibrous carbohydrate residue. One of these

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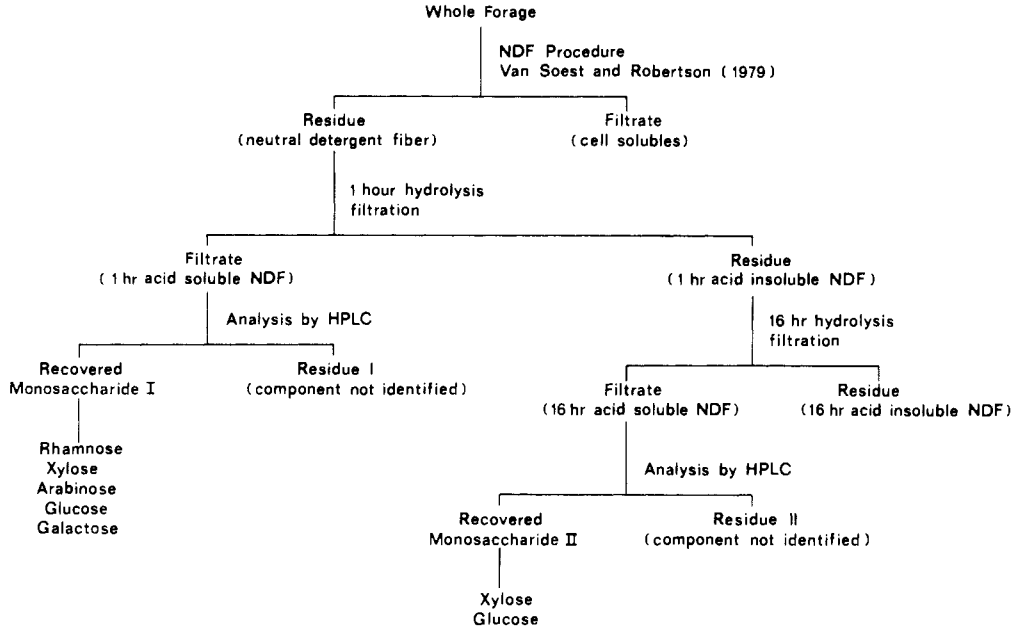

Figure 1. Flow diagram for the fractionation of forage cell walls.

procedures used a neutral detergent solution which extracted the cell solubles and left a residue of hemicellulose, cellulose, and lignin. The second extraction was with an acid detergent solution and left a residue consisting of cellulose and lignin. Bailey and Ulyatt (1970) indicated that the neutral detergent residue represented a useful measure of the total slowly digestible plant carbohydrate constituents. The carbohydrate analyses of isolated hemicelluloses from different forages that appear in the literature are difficult to compare due to different isolation, hydrolysis, and detection methods. Therefore, the objective of this study was to compare the monosaccharide compositions of the neutral detergent fiber fractions, which include the hemicellulose, from selected tropical and temperate grasses.

MATERIALS AND METHODS

Description of Grass Samples. Coastal Bermuda grass (CBG) [*Cynodon dactylon* (L.) Pers.] was harvested after a 4-week regrowth and a spring growth in 1976 and 1978, respectively, while Pensacola Bahia grass (PBG) (*Paspalum notatum var. saurae Parodi*) was harvested in 1978 after a 4-week regrowth. Both grasses were grown at Tifton, GA, and fertilized at the rate of 56.2 kg of N, 19.7 kg of P_2O_5, and 37.5 kg of K_2O per h. Pangola digit grass (PANG) (*Digitaria dicumbens* stent.) was harvested after a 4-week regrowth from Gainesville, FL, and was fertilized with 60.3 kg of N, 20.1 kg of P_2O_5, and 40.2 kg of K_2O per ha. Boone orchard grass (OG) (*Dactylis glomerata* L.), Clair timothy (TIM) (*Phleum pratense* L.), and Kentucky-31 tall fescue (KY-31) (*Festuca arundinacea* Schreb.) were harvested in 1976 and 1977 after 4-week regrowths from Lexington, KY, after fertilization with 38.59 kg of N/h. Samples were handled and prepared prior to analysis as described by Barton et al. (1976).

Chemical Analysis. Neutral detergent fiber (NDF), acid detergent fiber (ADF), permanganate lignin (PML), and cellulose were determined as described by Van Soest and Robertson (1979). Hemicellulose was calculated as the difference between NDF and ADF; lignin was determined as weight loss due to potassium permanganate treatment of the ADF residue; cellulose was determined as the weight loss upon ashing the permanganate-treated ADF residue. Crude protein was determined by a macro-Kjeldahl procedure (Association of Official Analytical Chemists, 1970). The Tilley and Terry (1963) two-state in vitro dry matter digestibility (IVDMD) procedure was used to determine the digestibility of the grasses.

Hydrolysis and Fractionation of Forage Cell Walls. The flow diagram for the fractionation of forage cell walls is shown in Figure 1. Sugar analysis of the cell walls from the tropical grasses were conducted by weighing duplicate 200-mg samples of isolated NDF into 25-mL reaction flasks with Teflon seals, adding 20 mL of 2 N trifluoroacetic acid (TFA), and hydrolyzing at 121 °C for 60 min (Barton et al., 1982). Boone orchard grass was hydrolyzed for 15, 30, 45, 60, 120, 180, and 240 min to determine the optimum time of hydrolysis for the cool season forages. After hydrolysis, the hydrolysate was filtered through a 15-mL coarse Büchner funnel and washed with 100 mL of boiling distilled water to yield a filtrate (1-h acid-soluble NDF) and a residue (1-h acid-insoluble NDF). The residue was dried at 110 °C for 12 h to determine the amount of cell wall material hydrolyzed.

The filtrate was evaporated almost to dryness on a rotary evaporator at 40 °C under reduced pressure (30 mmHg) and washed 3 times with distilled water (150 mL each wash). The filtrate was then diluted to 4 mL with distilled water and passed through a Waters C_{18} Sep-PAK cartridge. The cartridge was charged with 2 mL of acetonitrile followed by a 4-mL water wash. The filtrate was eluted through the cartride followed by a 2-mL water wash and evaporated to dryness. The filtrate was subsequently dissolved in 1 mL of methanol–water (60:40 v/v) for component sugar analysis. Total recovery of component sugars (recovered monosaccharide I) was calculated following HPLC analysis and expressed as a percent of the 1-h acid-soluble NDF. The difference (residue I) between 1-h acid-soluble NDF and total recovered monosaccharide I was not identified.

The 1-h acid-insoluble NDF was hydrolyzed for 16 h in 20 mL of 2 N TFA (Morrison, 1973). The hydrolysate was filtered and washed as described previously to obtain a filtrate (i.e., 16-h acid-soluble NDF) and a residue (i.e., 16-h acid-insoluble NDF). The filtrate and residue were handled as previously described. Upon analysis by HPLC, total component sugars were expressed as a percent of the 16-h acid-soluble NDF as recovered monosaccharide II. Components not identified (residue II) were determined as the difference between the 16-h acid-soluble NDF and

Table I. Compositional Analysis of Grasses

forage	% of whole grass dry wt						
	CP[a]	NDF[b]	ADF[c]	PML[d]	cellulose	hemicellulose	IVDMD[e]
warm season							
Coastal Bermuda grass	8.5	71.9	33.8	4.9	28.9	38.1	55.4
pangola grass	7.0	80.4	44.3	8.2	36.1	36.1	47.8
pensacola Bahia grass	15.7	66.1	34.2	3.5	30.7	31.9	59.6
cool season							
Boone orchard grass	11.8	54.9	31.4	3.5	27.9	23.5	62.1
Kentucky-31 tall fescue	13.1	53.2	27.4	3.0	24.4	25.8	71.3
Clair timothy	12.4	50.8	26.5	3.6	22.9	24.3	67.5

[a] Crude protein ($N \times 6.25$). [b] Neutral detergent fiber. [c] Acid detergent fiber. [d] Permanganate lignin. [e] In vitro dry matter digestibility.

Table II. Monosaccharide Composition of 1-h Acid-Soluble NDF Fraction

forage	% of whole grass dry wt					
	rhamnose	xylose	arabinose	glucose	galactose	recovered monosaccharide I
Coastal Bermuda grass	0.07[a,b]	14.8[b]	4.2[b]	5.2[b]	trace	24.3[b]
pangola grass	0.09[b]	15.2[b]	5.1[c]	5.1[b]	trace	25.5[b]
pensacola Bahia grass	0.09[b]	12.7[c]	5.4[c]	3.7[c]	0.85[b]	22.7[c]
Boone orchard grass	0.13[b]	11.1[d]	4.1[b]	2.4[d]	0.82[b]	18.5[d]
Kentucky-31 tall fescue	0.10[b]	13.0[c]	4.3[b]	1.1[e]	0.63[c]	19.1[d]
Clair timothy	0.22[c]	10.8[d]	4.2[b]	2.5[d]	0.84[b]	18.6[d]

[a] Each value is the mean of six observations (i.e., three injections of duplicate hydrolysates). [b-e] Means within columns with common superscripts do not differ ($P < 0.05$).

recovered monosaccharide II.

Sugar Standards. Working standards in the range of 10–20 mg/mL (1–2%) were prepared by dilution in methanol–water (60:40 v/v) solution. A concentration of 10 mg/mL yielded the optimum resolution for the sugar standards and was used as an external standard to determine the concentration of the sugars in the hydrolysates.

Liquid Chromatography: Equipment and Procedures. HPLC was performed with a Waters Associates ALC 201 chromatograph equipped with a U6K injector, a Model 600 solvent delivery system, and a differential refractometer detector having a sensitivity of 1×10^{-7} refractive index units and attenuation $64 \times {}^1/_4$. The HPLC was connected to a Hewlett-Packard 3390A recording integrator. Sugars were separated on a 30 cm × 5 mm i.d. stainless steel column packed with Micromeritics Microsil-NH_2 and a Waters Associates guard column packed with Lichrosorb-NH_2. Operating conditions were as follows: column temperature, ambient; refractometer detector temperature, 4 °C below ambient; eluent, water–acetonitrile (25:75 v/v) with a flow rate of 2.5 mL/min at pressures from 1600 to 2000 psi. Samples (usually 10 μL) were injected via the U6K injector.

Statistical Analysis. Data were analyzed by analysis of variance, linear regression, and orthogonal polynomials for unequally spaced treatments (i.e., time of hydrolysis) according to Steel and Torrie (1960). Differences between means were determined by using Scheffe's multiple comparison procedure as described by Kleinbaum and Kupper (1978).

RESULTS AND DISCUSSION

The amount of NDF and 1-h acid-soluble and insoluble NDF from samples of CBG and the temperate grasses harvested in different years was not different ($P < 0.05$). Thus, only the pooled means over years will be discussed.

Chemical analysis of the forages are given in Table I. The tropical grasses contain more NDF than temperate grasses (tropical average 72.8% vs. temperate average 52.9%), agreeing with data of Van Soest (1973) and Barton et al. (1976). The percent ADF was higher in the tropical grasses than temperate ones but contained about equal amounts of lignin with the exception of pangola grass. A major difference in the chemical composition of temperate and tropical grasses was the greater amounts of hemicellulose found in the tropical species which are consistent with the data of Daughtry et al. (1978).

Table III. Monosaccharide Composition of the 16-h Acid-Soluble NDF Fraction

forage	% of whole grass dry wt		
	xylose	glucose	recovered monosaccharide II
Coastal Bermuda grass	trace	4.7[a]	4.7[a]
pangola grass	0.53[a]	7.3[b]	7.8[b]
pensacola Bahia grass	0.11[b]	5.7[c]	5.8[c]
Boone orchard grass	0.09[b]	5.2[c]	5.3[c]
Kentucky-31 tall fescue	0.12[b]	6.5[d]	6.6[d]
Clair timothy	0.06[b]	4.6[a]	4.7[a]

[a-d] Means within columns with common superscripts do not differ ($P < 0.05$).

Barton et al. (1982) found that hydrolysis of NDF from tropical grasses with 2 N TFA at 121 °C was optimum at 1 h based on the recovery of component sugars. This time of hydrolysis is in agreement with Albersheim et al. (1967) for cell walls of "pinto bean" hypocotyls, and after 1 h the recovery of each sugar began to decrease. Boone orchard grass was hydrolyzed for 15, 30, 45, 60, 120, 180, and 240 min to determine the optimal time of hydrolysis for cool-season forages. Analysis of variance indicated a difference ($P < 0.05$) in the milligrams of carbohydrates recovered due to the time of hydrolysis. The recovery of xylose, rhamnose, and galactose indicated a curvilinear effect ($P < 0.001$) with increasing time of hydrolysis. Linearity appears to be associated with recovery at 30, 45, and 60 min hydrolysates for xylose and galactose. There was a linear increase ($P < 0.05$) in the recovery of glucose with increased time of hydrolysis, which was probably due to the hydrolysis of cellulose from the cell wall. The recovery of arabinose also indicated a curvilinear effect ($P < 0.001$) with 30 min being the optimum time of hydrolysis.

Table IV. Components in the Fractionation of Forage Cell Walls

forage	% of whole grass dry wt: 1-h acid-soluble NDF	recovered monosaccharide I	1-h acid-soluble NDF	16-h acid-soluble NDF	recovered monosaccharide II
Coastal Bermuda grass	40.2[a]	24.3[a]	31.7[a]	12.9[a]	4.7[a]
pangola grass	36.9[b]	25.5[a]	43.5[b]	16.6[b]	7.8[b]
pensacola Bahia grass	33.4[c]	22.7[b]	32.7[a]	14.7[c]	5.8[c]
Boone orchard grass	25.7[d]	18.5[c]	29.2[c]	13.2[a]	5.3[c]
Kentucky-31 tall fescue	26.9[d]	19.1[c]	26.3[d]	13.2[a]	6.6[d]
Clair timothy	25.6[d]	18.6[c]	25.2[d]	11.7[d]	4.7[a]

[a-d] Means within columns with common superscripts do not differ ($P < 0.05$).

Table V. Distribution of Unidentified Fractions of 2 N TFA Hydrolyzed NDF

forage	% of whole grass dry wt: residue I	residue II	AINDF[a]	total
Coastal Bermuda grass	15.9[b]	8.2[b]	18.8[b]	42.9[b]
pangola grass	11.4[c]	8.8[c]	26.9[c]	47.1[c]
pensacola Bahia grass	10.7[d]	8.9[c]	18.0[b]	37.6[d]
Boone orchard grass	7.2[e]	7.9[b]	16.0[d]	31.1[e]
Kentucky-31 tall fescue	7.8[e]	6.6[e]	13.1[e]	27.5[f]
Clair timothy	7.0[e]	7.0[d]	13.5[e]	27.5[f]

[a] 16-h acid-insoluble NDF. [b-f] Means within columns with common superscripts do not differ ($P < 0.05$).

However, 1 h was chosen as the time of hydrolysis for the cool-season forages since it was optimal for most of the sugars.

The monosaccharide composition of the 1-h acid-soluble NDF fraction of isolated NDF is shown in Table II. The data are expressed as the percentage of dry forage weight for each monosaccharide present in the recovered monosaccharide I. There was no difference ($P > 0.05$) in the duplicate hydrolysis and subsequent sugar analysis; therefore, the means of the analyses were reported. In addition, all data were corrected for silica content. Xylose and arabinose are the major components of the 1-h acid-soluble NDF for both warm- and cool-season forages. These data are in agreement with the findings of Gaillard (1965), Dehority (1973), Morrison and Williams (1978), Daughtry et al. (1978), and Collings and Yokoyama (1979). The tropical grasses contained 22.4 and 16.7% more xylose and arabinose, respectively, than the cool-season forages. There was little difference in the rhamnose content of the 1-h acid-soluble NDF with the exception of TIM which contained about twice as much as the other forages. The glucose concentration in 1-h acid-soluble NDF of the tropical grasses was greater ($P < 0.05$) than that present in the temperate grasses. On the average, the 1-h acid-soluble NDF of the tropical grasses contained 2.3 times more glucose than the temperate grasses. The amount of recovered monosaccharide I (Table II) was greater ($P < 0.05$) in the tropical than in temperate grasses, comprising 24.2 and 18.7% of the total dry matter of the grasses, respectively.

The 1-h acid-insoluble NDF (Figure 1) was hydrolyzed for 16 h in 2 N TFA for its constituent sugar content. The monosaccharide composition of the 16-h acid-soluble NDF fraction is shown in Table III. The monosaccharides are expressed as a percent of the recovered monosaccharide II (Figure 1) from 1 g of dry weight forage. Glucose was the major component in the 16-h acid-soluble NDF fraction. The hydrolyzed glucose (Tables II and III) from the recovered monosaccharide I and II fractions was 30.2% greater in the tropical grasses than in the temperate grasses. Pangola grass and Ky-31 had the greatest ($P < 0.05$) glucose content in the 16-h acid-soluble NDF fraction followed by PBG, OG, CBG, and TIM, respectively. The higher the percentage cellulose in the forage, the greater the glucose recovery. Xylose formed the remainder of the 16-h acid-soluble NDF.

The components in the fractionation of forage NDF are shown in Table IV. Upon hydrolysis, hemicellulose was assumed to be the 1-h acid-soluble portion of NDF. The amount of 1-h acid-soluble NDF was greater ($P < 0.05$) than the amount of hemicellulose as determined by the difference between NDF and ADF. The amount of 1-h acid-insoluble NDF was less ($P < 0.05$) than the amount of ADF. Bailey and Ulyatt (1970) reported that at least half of the pectic substances are not removed and much of the hemicellulose is not extracted with the 1-h ADF treatment, which would result in an overestimate of ADF and an underestimate of hemicellulose. Therefore, the 1-h acid-soluble and insoluble NDF data could possibly represent a value that is much nearer to hemicellulose and cellulose plus lignin, respectively. These data reflect the discrepancy in the estimation of hemicellulose as the difference between NDF and ADF. However, sequential detergent analysis has been shown to give higher estimates of hemicellulose compared to the estimate by difference

Table VI. Regressions of Various Residues from the Fractionation of Forage Cell Walls

component	regression	coeff of determination (r^2) with in vitro digestibility	SE[a]
acid detergent fiber	$y = 102.68 - 1.22$	0.86	0.17
1-h acid-insoluble NDF	$y = 98.87 - 1.19$	0.92	0.12
16-h acid-insoluble NDF	$y = 89.38 - 1.58$	0.94	0.14
residue I	$y = 72.67 - 1.08$	0.28	0.62
residue II	$y = 92.52 - 3.80$	0.36	1.78
AINDF,[b] RI[c]	$y = 89.62 - 1.54$ AINDF $- 0.08$ RI	0.94	0.22, 0.18
AINDF, RII, RI[c]	$y = 97.36 - 1.34$ AINDF $- 1.28$ RII $- 0.18$ RI	0.97	0.18, 0.16, 0.54
total (RI + RII + AINDF)	$y = 93.59 - 0.90$	0.85	0.13

[a] Standard error of regression coefficients. [b] 16-h acid-insoluble NDF. [c] Stepwise regression.

(Van Soest and Robertson, 1979). Sequential analysis of these forages could result in estimates of hemicellulose and ADF that more closely resemble that obtained by hydrolysis of NDF with trifluoroacetic acid.

The percentage of the NDF hydrolyzed by 1-h extraction with 2 N TFA that was recovered as monosaccharide I (Table IV) was greater ($P < 0.05$) in the cool-season forages, 71.9 vs. 65.8%, respectively. This trend is reflected in residue I (Table V) which represents the difference between 1-h acid-soluble NDF (Table IV) and recovered monosaccharide I (Table IV). The residue could be degradation products from the HPLC sample preparation, but preliminary investigations of residue I with NMR spectroscopy indicate the presence of a lignin–carbohydrate complex (LCC) (Barton et al., 1982). The C_{18} Sep-PAK used in sample preparation binds polar compounds. It appears that the sugars are bound to the lignin moiety as a true complex. If the sugars were free and not bound, they could be eluted through the Sep-PAK. When the complex is eluted from the Sep-PAK with either dioxane, acetone, or Me_2SO, the recovered dry matter is equal to residue I. Subsequent hydrolysis of the 1-h acid-insoluble NDF in 2 N TFA at 121 °C for 16 h yielded a 16-h acid-soluble NDF fraction (Table IV) and a 16-h acid-insoluble NDF residue (Table V). The 16-h acid-soluble NDF fraction represents on the average 12.7 and 14.7% of the temperate and tropical forage NDF, respectively (Table IV). In the temperate forages, 15.4% more material was hydrolyzed compared to the tropical forages. Thus, the tropical forages contained a greater ($P < 0.05$) amount of the 16-h acid-insoluble NDF residue (Table V). This trend is possibly due to the greater amount of lignin and its association with the cell wall (i.e., ADF). In general, the tropical grasses contain more lignin (Table I) which resulted in less of the 1-h acid-insoluble NDF hydrolyzed and a significantly greater ($P < 0.05$) amount of 16-h acid-insoluble NDF (AINDF, Table V). No definitive conclusions can be made in the comparison of the recovered monosaccharide II (Table IV) and residue II (Table V). However, only 41.4 and 43.5% of the tropical vs. temperate 16-h acid-soluble NDF was recovered as monosaccharide II, which indicates the presence of an acid-resistant macromolecule such as an LCC. On the average, the tropical forages contained 73.9, 50.2, and 19.4% more of the residues I, AINDF, and II, respectively, than the temperate forages. In addition, the total of the unidentified residues (Table V) was greater ($P < 0.05$) in the tropical forages, corresponding to decreased in vitro digestibility (Table I). Pangola grass had the lowest digestibility and the largest residual total whereas Ky-31 had the smallest total residue and the highest digestibility.

One criteria for evaluating the fractionation of forage NDF is the recovery of residues in NDF and their degree of correlation with IVDMD. Linear regression of various residues from the fractionation of forage cell walls is shown in Table VI. The coefficients of determination for 1-h and 16-h acid-insoluble fiber were greater ($P < 0.05$) than ADF; however, only 28 and 36% of the variation in IVDMD could be explained by residues I and II. Stepwise linear regression of the three residues was better than that of any residue alone. The addition of residue I in the three-pool model was not significant; therefore, residue II and AINDF provided the highest coefficient of determination with in vitro digestibility. The coefficient of determination for the total residue was not different from ADF but was less than the 1-h acid-insoluble NDF and stepwise regression of residue II and AINDF. The total of the residues corresponded to a −0.90% decrease in IVDMD for every unit increase in total residue. The identification of the residues of the NDF could provide insight into the differences in digestibility of tropical and temperate forages.

Registry No. Cellulose, 9004-34-6; hemicellulose, 9034-32-6; rhamnose, 3615-41-6; xylose, 58-86-6; arabinose, 147-81-9; glucose, 50-99-7; galactose, 59-23-4.

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Received for review April 27, 1982. Revised manuscript received October 22, 1982. Accepted January 13, 1983. Mention of a trade name or product does not imply endorsement by the U.S. Department of Agriculture over other products which may also be suitable.