Evaluation of High-Performance Liquid Chromatography for Measurement of the Neutral Saccharides in Neutral Detergent Fiber

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The ability of two high-performance liquid chromatographic columns to measure the six neutral monosaccharides, glucose, mannose, galactose, rhamnose, arabinose, and xylose, and cellobiose found in neutral detergent fiber (NDF) hydrolysates was evaluated by using saccharide standards and hydrolysates of NDF residues from a typical low-fiber diet and from a fecal sample collected during ingestion of the low-fiber diet. The normal-phase column did not adequately separate the hexoses, major saccharides in NDF, while galactose and rhamnose, present only in small quantities, coeluted from the heavy metal column. The heavy metal column was 2–3 times more sensitive than the normal-phase column and detected cellobiose. The detection capabilities and other operating properties of the heavy metal column make it the column of choice to separate saccharides commonly found in food and fecal NDF.

These experiments were conducted to evaluate high-performance liquid chromatography (HPLC) as a method to measure the neutral saccharides in neutral detergent fiber (NDF). The NDF method (Goering and Van Soest, 1970) has been criticized because it is a gravimetric procedure, assumes all of the recovered material is fiber, and yields no information about the chemical composition of the fiber residue. Further, the NDF fails to recover labile components of fiber although they generally account for only a small fraction of most foods (Theander and Aman, 1981). However, interpretation of the physiological function of fiber in the gut requires that food and fecal fiber be measured by similar methods. Only the NDF method, in contrast to most methods of food fiber analysis, recovers a fecal fiber fraction that is free of bacterial and endogenous mucopolysaccharides (Brauer et al., 1981). If the carbohydrate components of NDF could be quantitated, several of the limitations of the Van Soest procedure would be avoided and it would be possible to characterize a major fraction of food and fecal fiber by the same method.

In the experiments reported below, various mixtures of standards of the six neutral monosaccharides generally found in NDF, glucose (G), galactose (Ga), mannose (M), xylose (X), rhamnose (R), and arabinose (A), were separated by two HPLC columns. The NDF was hydrolyzed by the method of Saeman (Selvendren et al., 1979). Since it has been reported that the carbohydrate decomposition during secondary hydrolysis is at least 4 times that observed during primary hydrolysis (Saeman et al., 1954), the effect of the duration of the secondary hydrolysis on recovery of NDF as carbohydrate was determined as well.

MATERIALS AND METHODS

NDF Preparation. Food NDF residue was collected from a composite of a day's intake of a low fiber diet used in a previous metabolic study (Slavin and Marlett, 1980a). The food composite used for the present study contained the same amounts of canned fruits and vegetables, potatoes, and rice but the high-protein bread was not incorporated into the composite. Fecal NDF residue was extracted from a fecal composite representing 25 days of excretion by one subject (subject E) during ingestion of the same low-fiber diet (Slavin and Marlett, 1980b). Collection of NDF residues was modified to generate 200 mg of NDF residue for hydrolysis. Two samples of the food composite (2 g each) and of the fecal composite (1.5 g each) were treated separately with neutral detergent solution (1 h, 100 °C) (Goering and Van Soest, 1970). To improve filtration, samples were then centrifuged (10000g, 20 min) and the supernates discarded. Residues were washed twice with hot, deionized water and centrifuged

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after each wash. The food NDF residues were treated with hog amylase solution (EC 3.2.1.1, Sigma Chemical Co., St. Louis, MO) to remove residual starch (American Association of Cereal Chemists, 1978). The two samples of each residue were then filtered through a single crucible with glass wool (~2 g) as a filtering aid (Brauer et al., 1981). The residue was washed with approximately 1 L of hot, deionized water and dried overnight. After the crucible was weighed, all NDF residue and glass wool pads were carefully removed from the crucibles and placed in beakers. Crucibles were reweighed to correct for sample left on the crucible, which averaged less than 5 mg. The food composite was 4% NDF and the fecal composite 16% NDF. The starch content of the food NDF, which was very low, 1.3 ± 0.1%, was measured with glucose quantitation by glucose oxidase–peroxidase.

Hydrolysis. NDF residues were hydrolyzed by the Saeman procedure (Selvendran et al., 1979). A preliminary experiment was conducted to determine the optimum time for secondary hydrolysis of both NDF samples. Samples were covered with minimal (~10–15 g) 72% H_2SO_4 and left at 24 °C for 3 h (primary hydrolysis). Samples then were diluted with distilled water (70–100 g) to give 2 N H_2SO_4 and heated (100 °C) for 1, 2, or 3 h (secondary hydrolysis). Duplicate samples of both food and fecal NDF were subjected to the three secondary hydrolysis time points. Unhydrolyzed material and glass wool were recovered by filtering each sample through a tared, Gooch crucible. The unhydrolyzed material, which was assumed to be Klason lignin, was weighed. Glass wool was not affected by the hydrolysis procedure.

Hydrolysates were titrated with 0.25 M $Ba(OH)_2$ with constant stirring to pH 5–6 to precipitate barium sulfate (Selvendran et al., 1979) and allowed to stand for about 1 h before the pH was readjusted. Samples were centrifuged (1000g, 15 min) to precipitate the barium sulfate and the supernates were lyophilized. Lyophilized samples were dissolved in 5 mL of distilled, deionized water and excess barium ions removed with a strong cation-exchange resin (AG 50W-X8, 200–400 mesh, hydrogen form, Bio-Rad Laboratories, Richmond, CA). Samples were filtered through a reverse-phase C_{18} resin (Sep-PAK, Waters Associates, Milford, MA) to remove nonpolar contaminants and through a 0.22-μm Millipore filter (Millipore Corp., Bedford, MA) to remove particulates.

Standard monosaccharides, G, Ga, M, X, A, and R (100 mg of each sugar), also were hydrolyzed, neutralized, and analyzed by HPLC. Losses of standard monosaccharides, determined on the heavy metal, ion-exchange column, were (%) G, 33.5, X, 38.1, Ga/R, 32.9, A, 37.1, and M, 25.6. Losses during neutralization were assessed by washing the barium sulfate precipitate with water and measuring the carbohydrates in the supernate. Standard monosaccharides also were treated with the two resins used to purify the hydrolysate to check whether either resin bound carbohydrate. Neither neutralization nor treatment with either resin had any effect on carbohydrate recovery.

HPLC Analysis. The two HPLC columns used to separate the saccharides were: a μ Bondapak carbohydrate column, 4 mm i.d. × 30 cm (Waters Associates), with acetonitrile (Burdick and Jackson, Muskegon, MI) and water (Alltech Associates, Arlington Heights, IL) in a ratio of 85:15 as the mobile phase and an Aminex HPX-85 heavy metal cation-exchange carbohydrate column, 7.8 mm × 300 mm (Bio-Rad Laboratories, Richmond, CA). The mobile phase for the heavy metal column was distilled, deionized water which had been filtered through 0.45-μm Millipore filters and which was kept degassed by maintaining it at

85–90 °C with continuous stirring. The acetonitrile–water phase was filtered through 0.5-μm Fluoropore filters (Millipore Corp.) and degassed ultrasonically (30 s) (Bransonic ultrasonic cleaner, Shelton, CT). The heavy metal column was maintained at 85 °C by a water jacket (Model 9502, Alltech Associates) connected to a circulating water bath (Model FJ, Haake, Inc., Saddle Brook, NJ).

The HPLC system consisted of a 2-L solvent reservoir, a 6000-psi dual piston pump, (Constametric III, Laboratory Data Control, Riviera Beach, FL), a syringe-loading sample injector fitted with a 50-μL loop (Model 7125, Rheodyne, Berkeley, CA), a refractive index detector (Model R401, Waters Associates), and a strip chart recorder (Omniscribe B-5117, Houston Instruments, Austin, TX).

Evaluation of HPLC Analysis. Saccharide standards (Sigma Chemical Co., St. Louis, MO) were dried overnight in vacuo (60 °C) before being dissolved in distilled, deionized water. The six monosaccharides, G, Ga, M, X, A, and R, were included in a series of five standard solutions with each monosaccharide at a concentration of 0.1, 0.25, 0.5, 0.75, and 1.0%. Cellobiose (1%) was included in the 1% standard. All peaks from the 1.0% standard solution were collected and cochromatographed on paper (Whatman No. 1) (Saeman et al., 1963) with appropriate standards for positive identification of the peaks. Sugars were detected by dipping in aniline hydrogen phthalate followed by brief heating (10 min, 100 °C).

The detection limit of each sugar on each column was calculated by assuming that detector noise was excessive at an attenuation less than 8× and that 1 cm was the smallest peak height that could be accurately measured. The precision of each column was determined by calculating the coefficients of variation of each sugar from 10 replicate analyses of the 1.0% standard.

The capacities of the two HPLC systems to determine each monosaccharide in the presence of various proportions of the others were established by a dose–response experiment. Four different mixtures of the six monosaccharides were prepared; each of the four mixtures contained three sugars each at a concentration of 0.5%, while the other three were added at a concentration of 1.0%. The mixtures were as follows: (1) 0.5% X, A, and R and 1% G, Ga, and M; (2) 0.5% Ga, X, and R and 1% G, M, and A; (3) 0.5% G, M, and A and 1% Ga, X, and R; (4) 0.5% G, Ga, and M and 1% X, A, and R.

RESULTS

Significantly more of the carbohydrate from both the food and the fecal NDF was recovered when the duration of the secondary hydrolysis was 2 h (Table I). Therefore, NDF samples were hydrolyzed by a 3-h primary hydrolysis and a 2-h secondary hydrolysis.

Normal-Phase Column. The order of elution of the seven standard sugars from the normal-phase column, identified by paper chromatography, was R, X, A, M, G, Ga, and cellobiose (Figure 1). Three of the four hexoses were inadequately separated. Cellobiose was well separated from the monosaccharides but did not elute until 25 min. All monosaccharides eluted by 9 min. Increasing the acetonitrile content of the solvent to 90% did not improve resolution and hindered quantitation by increasing analysis time and broadening the peaks of the slower eluting sugars. A flow rate of 2.0 mL/min was optimum; peaks were not as distinct at lower flow rates, and excessive back-pressure developed without improving separation at flow rates greater than 2.0 mL/min.

Coefficients of variation of peak heights for 10 replicate analyses of the 1% monosaccharide standard on the normal-phase column were (%) as follows: R, 0.6; X, 1.1; A,

Table I. Effect of Time of Secondary Hydrolysis on Sugar Yield

	mg/100 mg of hydrolysate					
	food composite[a]			fecal composite[b,c]		
sugar	1 h	2 h	3 h	1 h	2 h	3 h
cellotriose	0.5	0.0	0.0	0.4	0.0	0.0
cellobiose	1.0	1.0	0.2	0.8	0.2	0.0
glucose	30.7	45.9	43.0	23.2	33.0	26.0
xylose	3.6	5.4	4.3	3.3	4.0	3.2
galactose/ rhamnose	1.3	2.0	1.8	1.0	1.0	0.8
arabinose	3.1	4.5	3.3	1.4	1.3	1.2
mannose	3.6	6.4	3.4	1.4	2.1	1.8
recovery, %	43.8	65.2	56.0	31.5	41.6	33.0

[a] Reversion product [height (cm) of peak]: 1 h, 9.1; 2 h, 5.8; 3 h, 2.5. [b] Reversion product [height (cm) of peak]: 1 h, 14.8; 2 h, 10.2; 3 h, 4.7. [c] Corrected for Klason lignin which represented the following amounts of NDF weight: 1 h, 39.5%; 2 h, 35.3%; 3 h, 38.0%.
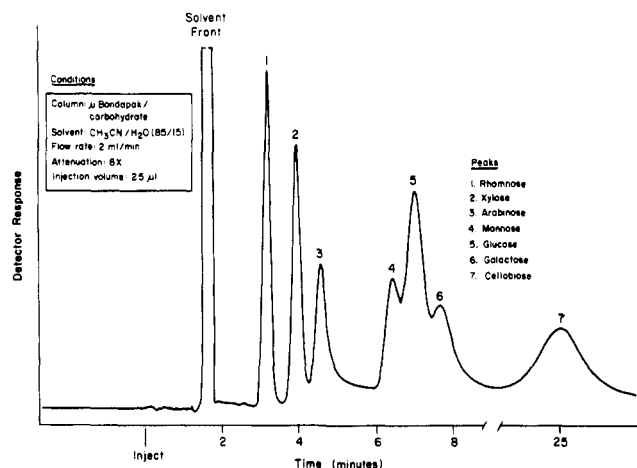

Figure 1. Separation of a mixed carbohydrate standard containing the six neutral monosaccharides and cellobiose found in dietary fiber on a normal-phase carbohydrate column.

Figure 2. Separation of the neutral carbohydrates in the food and fecal NDF samples by a normal-phase carbohydrate column.

Figure 3. Separation of a mixed carbohydrate standard containing the six neutral monosaccharides and cellobiose found in dietary fiber on a lead-form cation-exchange column.

1.1; M, 2.0; G, 1.1; Ga, 1.1; mean ± SD, 1.2 ± 0.4%. The detection limit (μg) of each sugar was as follows: R, 12.1; X, 13.2; A, 20.6; M, 22.2; G, 18.3; Ga, 32.0. Detection response of each sugar was linear within the range analyzed, from 25 to 250 μg. With one exception, sugars could be accurately measured in the presence of different proportions of the others; recoveries ranged from 98 to 102%. The peak height of galactose could not be measured when the concentration of galactose was less, i.e., 0.5%, than that of glucose, i.e., 1.0%.

R, A, X, and G were detected when both the food and fecal NDF residues were analyzed on the normal-phase column (Figure 2). Because the NDF hydrolysates were predominantly G, accounting for >75% of the detected sugars (Table II), neither M nor Ga was detected. No cellobiose was detected by the μBondapak column. Peak no. 1 was not identified.

Heavy Metal Column. Six peaks eluted when the seven standard sugars were applied to the heavy metal ion-exchange column (Figure 3). When the peaks were collected and chromatographed on paper, it was found that Ga and R coeluted as peak no. 3. Separation of all sugars was complete after 25 min. Increasing the flow rate to 0.6 mL/min decreased the analysis time to 18 min with only a slight decrease in resolution. When the flow rate was decreased to 0.3 mL/min, resolution was not notably better and analysis time was increased to 30 min.
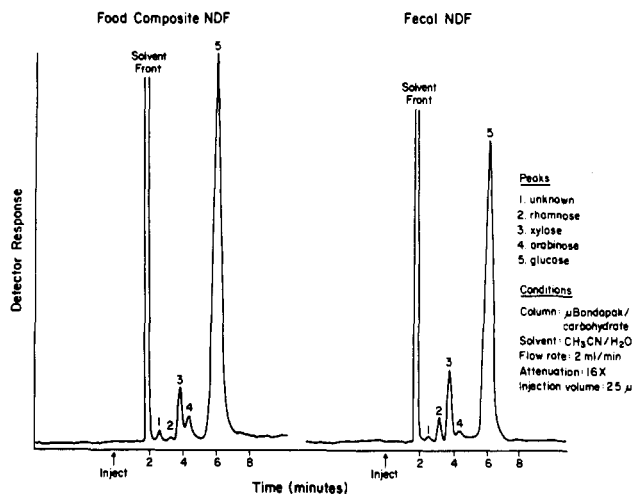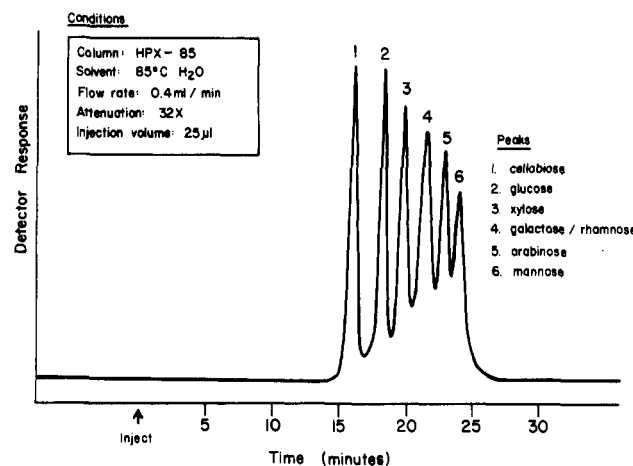
Table II. NDF Monosaccharides in Food and Feces As Measured with Two HPLC Systems

	mg/100 mg of NDF			
	food composite		fecal composite	
mono-saccharide	heavy metal	normal phase	heavy metal	normal phase
cellobiose	1.0		2.0	
glucose	47.7	46.1	24.1	23.3
xylose	3.7	3.8	4.3	4.6
galactose	1.7[a]	[b]	1.4[a]	[b]
rhamnose		0.3		1.4
arabinose	2.8	2.6	1.0	0.8
mannose	2.8	[b]	1.3	[b]

[a] Rhamnose and galactose coelute. [b] None detected.

Coefficients of variation (%) of peak heights of 10 replicate analyses of the 1.0% standard on the heavy metal column were as follows: G, 0.2; X, 0.4; Ga/R, 1.0; A, 0.7; M, 0.8. Detection response was linear within the range analyzed, from 25 to 500 μg of injected sugar. Recovery of the monosaccharides in the presence of various proportions of the other monosaccharides ranged from 98 to 105%. When standards containing either 1% R or 1% Ga were applied separately to the heavy metal column, Ga eluted slightly before R. Therefore, the detector response of the Ga/R peak did not equal the sum of these two sugars.
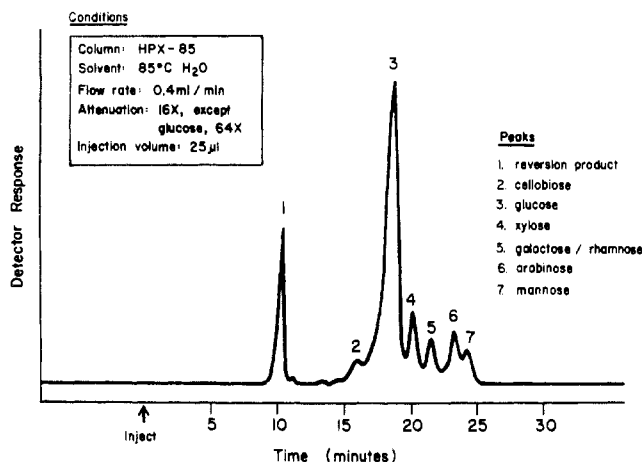
Figure 4. Separation of the neutral carbohydrates in the food NDF sample by a lead-form cation-exchange column.

Cellobiose, G, X, A, M, and a Ga/R peak were detected when the food and fecal NDF hydrolysates were analyzed by the heavy metal column (Figure 4; Table II). Peak no. 1 was not identified.

DISCUSSION

Neither of the HPLC columns that we tested was ideal for analyzing the monosaccharides in food and fecal NDF residues. Satisfactory separation of the six mono-saccharides and cellobiose found in the NDF hydrolysates could not be achieved even though both the flow rates and solvent characteristics of the columns were varied.

Comparable amounts of G, X, and A were detected by the two columns. However, because the heavy metal column also detected mannose and cellobiose, more of the total NDF carbohydrate was measured by this column, compared to the normal-phase column. R, but not Ga, could be quantitated on the chromatogram of the nor-mal-phase column, while these monosaccharides coeluted on the heavy metal column. When the fecal NDF hy-drolysate was analyzed, the heavy metal column detected the same amount of sugar in the Ga/R peaks as the nor-mal-phase column eluted as R. A considerably greater portion of the Ga/R peak, when the food NDF hydrolysate was chromatographed by the heavy metal column, ap-peared to be Ga and not R, since only trace amounts of R were detected by the normal-phase column, which does not detect Ga in NDF. Thus, while the heavy metal col-umn Ga/R peak may not precisely represent the sum of Ga and R, it may detect more of the total sugar applied to the column than the normal-phase column. Finally, analyses of either NDF hydrolysate by both columns in-dicate that Ga and R together account for only small portions of the total carbohydrate in NDF, in our samples, 2%. If Ga and R were significant carbohydrates in a sample, the Ga/R peak could be collected and the two sugars could be separated on the normal-phase column.

The minor sugars in the NDF residues were difficult to quantitate, in part, because refractive index detectors in-herently lack sensitivity. Between 15 and 30 μg of sugar were necessary for accurate detection with the normal-phase column and refractive index detector. Similar de-tection limits of 10 μg (Johncock and Wegstaffe, 1980) and 20 μg (McGinness and Fang, 1980) have been reported. The heavy metal column was more sensitive than the normal-phase column and could detect as little as 5–10 μg of each sugar. Palmer and Brandes (1974) have suggested that this increase in sensitivity may be due to the de-creased flow rate or to differences in the refractive index of the solvent. Improved temperature control of the de-

tector (Johncock and Wegstaffe, 1980) and the use of low-wavelength UV detection (Binder, 1980) have been suggested as methods to improve the sensitivity of HPLC analysis of carbohydrates.

When standards were injected, the precision of both columns was excellent. The mean precisions which we obtained with the normal-phase column, 1.2%, and with the heavy metal column, 0.5%, are comparable to those reported by other laboratories (Palmer and Brandes, 1974; Palmer, 1975; Dunmire and Otto, 1979).

It is difficult to measure the accuracy of HPLC methods because the various methods of measuring carbohydrates, paper, gas, and high-performance liquid chromatographies, or colorimetric methods, have different strengths and weaknesses (Dunmire and Otto, 1979). We did find good agreement between the amounts of sugars detected by both of the HPLC columns, however, which is indirect evidence of the accuracy of the method. Results obtained with the normal-phase column have been compared to the AOAC chemical methods (DeVries et al., 1979) and to enzymatic methods (Hunt et al., 1977) and found to be similar. Recoveries also appear to be essentially quantitative when various standard sugars are added to samples prior to analysis (Palmer, 1975; Dunmire and Otto, 1979).

In the present study, the influence of the duration of the secondary hydrolysis step of the Saeman procedure on hydrolysis losses was evaluated since earlier data (Saeman et al., 1954) indicated that the saccharides were most susceptible to degradation during this step. A variety of procedures to hydrolyze biological materials have been published (Saeman et al., 1954; Albersheim et al., 1967; Selvendran et al., 1979), although none has been applied specifically to the NDF residue. The Saeman procedure using a 3-h primary hydrolysis (20 °C) and a 2-h secondary hydrolysis (100 °C), however, has been recommended as the best method for hydrolysis of vegetable cell wall ma-terial, because it was accompanied by maximum release of all neutral sugars except rhamnose, with minimum degradation (Selvendran et al., 1979). We did not examine the effect of varying the length of the primary hydrolysis, since Saeman's data suggest that most losses occurred during the secondary hydrolysis step (Saeman et al., 1954). Recently, though, a 3-h primary hydrolysis has been shown to cause a 15% loss of the pentoses in defatted wheat bran fiber (Rasper et al., 1981).

Our results indicate that losses of standard carbohy-drates on a glass wool pad with a 2-h secondary hydrolysis range from 25% (mannose) to 38% (xylose). These losses were less than what was observed with either a 1-h or a 3-h secondary hydrolysis step. Other laboratories report substantially lower losses, from 5 (Sloneker, 1971) to 10% (Saeman et al., 1954) with the exception of xylose losses which were 14% (Saeman et al., 1954) and 20% (Sloneker, 1971). Other researchers using the same hydrolysis pro-cedure have not reported hydrolysis losses (Selvendran et al., 1979; Rasper et al., 1981). Small differences in acid strength and temperature also have been reported to in-fluence losses (Sloneker, 1971). We have not been able to identify the reasons for these extensive hydrolysis losses, although it is possible that the relatively large amounts of sulfuric acid needed to cover the glass wool pad in which the NDF was embedded was, in part, responsible.

Several other properties of the heavy metal column, in addition to its ability to detect more of the sugars found in NDF, make it preferable to the normal-phase column for quantitating NDF carbohydrates. The heavy metal column uses only water as the mobile phase, which is less expensive than acetonitrile and poses no environmental

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

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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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