one end of the starch polymer) and are unlikely to cause thinning at low levels of contamination.

Colorimetric assays, which merely measure the total reducing sugars liberated, are not able to provide this important type of detailed information. Furthermore, detailed action patterns might be useful in tracking down the source of the amylase contamination in food products.

Analytical Considerations. Bio-Rad product literature recommends operating the HPX-42A column at 85 °C. However, noticeable temperature-induced hydrolysis of the radioactive starch occurred when blank samples were analyzed at that temperature. The problem did not occur at temperatures below 70 °C.

HPX-42A columns are packed with a 4% cross-linked cation-exchange resin in the silver form. Bio-Rad recommends the use of deashing guard cartridges, which contain both cation- and anion-exchange resins, to protect the silver chloride salts. However, when in-line deashing precolumns were employed, there was a tendency for the amylases (especially the AMG 200L) to be retained on the column and to cause hydrolysis of radioactive starch in subsequent injections. As a result, the use of the precolumn was abandoned. Deashing was accomplished, instead, by the addition of Amberlite MB-1 ion-exchange resin to the samples prior to injection.

The HPX-42A column is capable of resolving oligosaccharides as large as dp 11. Combining two columns in series can separate oligosaccharides to dp 14, and by using several of these columns in series, separation of oligiosaccharides as large as dp 20 can be achieved.

Conclusion. The analytical approach using radioactive starch, HPLC, and a radioactive flow detector is sensitive and provides a more comprehensive picture of amylase-catalyzed hydrolysis than the commonly used colorimetric and viscometric amylase assay procedures. Futhermore, it is more useful than colorimetric assay methods for measuring small increases in α-amylase-induced starch degradation products in sample matrices that commonly contain high background levels of reducing sugars (e.g., corn syrups, milk, whey protein powders, and crude tissue extracts).

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Characterization of Trifluoroacetic Acid Hydrolyzed Subtropical Forage Grass Cell Walls[1]

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Hemicellulose determinations by trifluoroacetic acid (TFA) hydrolysis (2.0 N followed by 0.25 N TFA, 1 h at 121 °C each step) of forage grass [*Pennisetum flaccidum* Griseb. *Panicum amarum* (Elliot) var. *amarum*, *Panicum amarum* × var. *amarulum* (Hitchc. & Chase) P. G. Palmer, *Panicum virgatum* L.] cell walls were compared with acid (1 N H_2SO_4, 1 h at 121 °C), base (10% KOH, 90 min at 55 °C), and neutral detergent (ND) solubilization. Of these, TFA hydrolysis proved to be most useful for cell wall compositional analysis of subtropical grasses. Residue from TFA hydrolysis resembled a pectin-free cellulose fraction, and the hydrolysate contained stable neutral sugar monomers derived from hemicellulose. Weak acid (1 N H_2SO_4, 1 h at 55 °C) and weak base (1% KOH, 45 min at 55 °C) pretreatment of *Panicum* cell wall preparations for TFA hydrolysis resulted in minor (3%) yield increases, not considered sufficient for a subsequent modification of the TFA hydrolysis procedure.

Analytical estimation of structural polysaccharides in forages has been based on the differential solubilities of the various chemical components in acid or base. However, difficulties can occur in the chemical separation of plant cell walls into structural entities such as xylans (hemicellulose), β-glucan, cellulose, and pectin as some cleavage of covalent bonds may occur (Bailey et al., 1976). The hemicellulosic fraction of plant tissue remains the most difficult to quantify with many methods proposed (Blake and Richards, 1971). Traditionally, hemicellulose is the fraction insoluble in water and ammonium oxalate solution but soluble in acid or base under mild conditions. These methods include acidic fractionation after reflux with 0.5% ammonium oxalate (depectination) followed by reflux with 1 N H_2SO_4 (Bailey et al., 1978). Alternatively, hemicellulose is determined by alkaline fractionation in 5–10%

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KOH followed by stronger base treatment with KOH–H_3BO_4 (24–4%, v/v) to solubilize glucomannan and galactoglucomannan (Bailey et al., 1978; Reid and Wilkie, 1969a–c; Ford et al., 1979). Fractionation with sodium hydroxide solutions ranging in concentration from 10 to 17.5% is also recommended (Dehority, 1973). α-Cellulose is defined as the polysaccharide fraction insoluble in 17.5% NaOH. Another method of hemicellulose estimation proposed by Sullivan (1966) involves hydrolysis in yeast culture and soluble sugar determination of H_2SO_4-extracted hemicellulose. Lignin and crystalline cellulose remain after hemicellulose extraction by most methods. Tissue lignin content in excess of 6% is known to interfere with the extractability of base-soluble hemicellulose, and for this reason either delignification steps (Bailey and Pickmere, 1975; Bailey et al., 1976; Morrison, 1974a,b; Reid and Wilkie, 1969b) prior to hydrolysis or successive base extractions are recommended (Ford et al., 1979).

Van Soest (1963) proposed an empirical method of hemicellulose determination defined as the fraction soluble in acid detergent (AD) but insoluble in neutral detergent (ND). Cell walls insoluble in ND and termed neutral detergent fiber (NDF) comprise hemicellulose, cellulose, lignin, and cell wall protein. Acid detergent extraction serves primarily as a preparative step, giving acid detergent fiber (ADF) for determination of cellulose, lignin, silica, and ash (Van Soest and Wine, 1968). Cell wall protein causes inflated hemicellulose estimates, while the presence of biogenic silica, pectin, and tannins causes underestimation (Van Soest and Robertson, 1980). Pectin content in subtropical forages is generally low and therefore not a serious source of error in hemicellulose analysis. Use of trifluoroacetic acid (TFA) to hydrolyze cell wall preparations was reported by several workers (Albersheim et al., 1967; Barton et al., 1982; Fengel et al., 1978; Ford et al., 1979; Jones and Albersheim, 1972; Sloneker, 1971; Windham et al., 1983). Uronosyl residues are reported to be broken down during TFA hydrolysis (Jones and Albersheim, 1972). TFA hydrolysis of ND residues is, therefore, a convenient starting point for cell wall compositional analysis provided the pectin composition is of lesser interest. Quantitative recovery of the hydrolysate carbohydrate monomers was reported by de Ruiter and Burns (1986) using a modified TFA hydrolysis procedure, but it was unclear what cell wall fraction was solubilized. Some oligosaccharide fragments such as β-(1,4) glycosyl-linked xyloglucans are reported to be resistant to TFA hydrolysis (Talmadge et al., 1973).

Chemical pretreatment of fibrous residues has been widely used as a preparative technique to improve the extractability of desired cellular components. In particular, the effects of alkalies, ammonia, swelling agents, and oxidizing agents on microbial enzymatic hydrolysis in ruminants have been intensely studied (Han et al., 1983). Several modes of action for alkali on digestibility of cell walls have been proposed, such as the increased lability of lignin–carbohydrate ester bonds (Hartley and Jones, 1977) and hemicellulose linkages (Keys et al., 1969). Electron microscopic comparisons of 10% KOH-treated Coastal Bermuda grass *Cynodon dactylon* (L.) Pers. revealed a distortion of lignified and unlignified cell walls with fragmentation into individual cells and associated disruption of xylem tissue and degradation of sclerenchyma (Spencer and Akin, 1980). Following base treatment, the structural polysaccharides were therefore more available for microbial fermentation. In quantitative analysis of cell wall carbohydrates, Bailey (1973) proposed that recovery of the more resistant polysaccharides such as cellulose, xylans, and mannans could be improved by pretreatment in cold 72% H_2SO_4 before hydrolysis with 1 N H_2SO_4.

Table I. Selections of *Panicum* Taxa Used in Experiments 2 and 4 for Hemicellulose Content Evaluation

taxa	origin	part	IVDMD,[a] % H1	IVDMD,[a] % H2
		Experiment 2		
P. amarulum	Horn Island, MS	leaf blade	72.2	59.0
		leaf sheath	66.2	43.5
		stem	75.4	40.4
P. amarum	New Brunswick County, NC	leaf blade	73.5	62.3
		leaf sheath	63.1	53.3
		stem	60.3	30.5
P. virgatum	Beaufort County, SC	leaf blade	56.5	43.5
		leaf sheath	45.0	36.8
		stem	48.4	25.2
		Experiment 4		
P. amarulum	Onslow County, NC	whole	70.4	55.1
P. amarum	Bay County, FL	whole	65.5	50.1
P. virgatum	Suffolk County, NY	whole	54.7	41.2

[a] Two-stage in vitro dry matter disappearance (IVDMD) procedure. Values are means of two replicates with harvest 1 (H1) taken on June 2 and harvest 2 (H2) on July 6, 1982.

The objectives of this study were to compare several methods of hemicellulose analysis in subtropical grasses selected for differences in in vitro dry matter disappearance and to characterize the insoluble fraction remaining after TFA hydrolysis. In addition, acid and base pretreatments were evaluated for their effect on hydrolytic yield improvement of carbohydrate monomers by TFA.

MATERIALS AND METHODS

Plant Material. Flaccidgrass (*Pennisetum flaccidum* Griseb.) and three taxa in the Virgata group of *Panicum*, i.e. *Panicum amarum* (Elliot) var. *amarum*, *Panicum amarum* × var. *amarulum* (Hitchcock & Chase) P. G. Palmer, and *Panicum virgatum* L., were grown on an Appling sandy loam (typic Hapludults) in the upper coastal plain near Raleigh, NC. Flaccidgrass was sampled on four occasions at approximately 2-week intervals from May 20 through July 4, 1980. Accessions within the *Panicum* taxa were chosen to represent high, medium, and low digestibility on the basis of in vitro dry matter disappearance (Table I) and were harvested at two maturities (June 2, July 6). Whole plant and separated plant parts (leaf blade, leaf sheath, stem) were used for analysis. All samplings were taken from the original season's growth and ground after freeze-drying to pass a 1-mm screen.

Fiber Fractions. A generalized scheme for cell wall fiber and residue analysis is given in Figure 1. Cell walls were recovered from 0.5-g samples treated with neutral detergent (ND) (Van Soest and Wine, 1967). A pectin-free cellulose (PFC) residue was prepared by sequential extraction with ND and then acid detergent (AD), followed by $KMnO_4$ delignification. Detergent-soluble hemicellulose (HC) was determined by the difference between ND and AD extraction (Van Soest and Wine, 1967). Insoluble residues after detergent treatment and acid or base hydrolysis were recovered by filtration through Gooch crucibles followed by freeze-drying.

Acid and Base Fractionation. Direct chemical estimates of hemicellulose were made by solubilization in either 1 N H_2SO_4 (Ford et al., 1979) or 10% KOH (Fales et al., 1981) (Figure 1). Determinations were on 100-mg samples by a modified Haslemore and Roughan (1976) procedure. Glucose standards were prepared for a concentration range of 5–50% soluble sugars hydrolyzed by

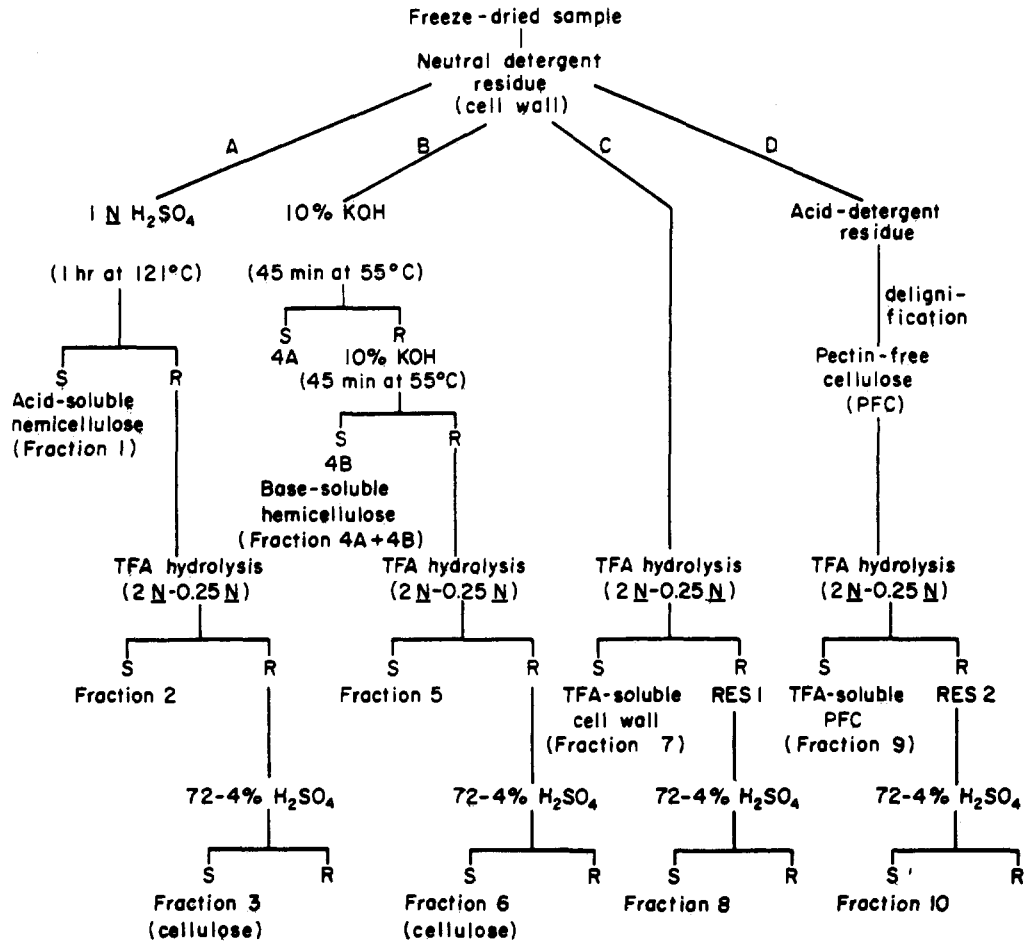

Figure 1. Hemicellulose and cellulose extraction of forage grass cell walls (R = residue; S = soluble fraction).

acid or base. Absorbance at 490 nm in a phenol–sulfuric acid procedure (Dubois et al., 1956) was used to determine soluble sugars. Lead acetate and chloroform cleanup steps had no effect on soluble sugar recovery and were consequently omitted (data not shown). Acid-soluble hemicellulose (HCA) hydrolysates were neutralized with an equal volume of 1 M NaOH. Base hydrolysate neutralization was with 1.78 M acetic acid. Base-soluble hemicellulose (HCB) was determined as the combined 4A + 4B fractions (Figure 1).

Neutral Sugar Determination. The composition of cell wall residues was determined either directly by two-step trifluoroacetic acid (2.0 N followed by 0.25 N for 1 h each step at 121 °C) hydrolysis (de Ruiter and Burns, 1986) or following an acid or base extraction. In the latter, both neutral sugars solubilized and the compositions of residues remaining after H_2SO_4 or NaOH treatment were determined by gas–liquid chromatography (GLC) as silylated derivatives (de Ruiter and Burns, 1986).

The cellulosic glucose composition of TFA-treated insoluble residues was determined by both silylation and GLC and by the phenol–sulfuric acid procedure. Initial solubilization was in 72% H_2SO_4 for 15 min followed by 4% H_2SO_4 hydrolysis (Ford et al., 1979).

Experiment 1. Comparison of Hemicellulose Determinations in Flaccidgrass. Four laboratory methods were compared by procedures previously outlined as follows: (1) Van Soest hemicellulose determined by difference (NDF − ADF); (2) dual-step TFA hydrolysis (2–0.25 N) of ND residues; (3) acid (1 N H_2SO_4) fractionation; (4) base (10% KOH) fractionation.

Cell wall residues in this experiment were isolated by sequential in vitro digestion followed by ND extraction. In vitro fermentation, yielding a range of hemicellulose concentrations in the residue, was a modification of the two-stage technique (Burns et al., 1976) with the omission of the second stage (pepsin digestion). Fermentation was halted at 0, 20, 40 and 72 h measured from inoculation to killing by the addition of 0.25 mL of a saturated (5%) $HgCl_2$ solution. After killing, the residues were washed with distilled water. The 0-h treatment was inoculated, killed immediately, and assumed to remove soluble cell contents. Residues from three field replicates were combined and analysis was performed on thoroughly mixed samples.

Experiment 2. Comparison of Methods of Hemicellulose Determination in Panicum Taxa. Three procedures yielding the soluble fractions 1, 4A + 4B, and 7 (Figure 1) were compared. These fractions represent alternative direct methods of hemicellulose analysis of ND-extracted cell wall. Plant material consisted of an accession from within each of three *Panicum* taxa that had been separated into plant parts (Table I).

Insoluble residue composition following hemicellulose extraction (fractions 2 and 5) was hydrolyzed by TFA and then compared with the TFA-soluble fraction 9 from PFC. These residues were proposed to be predominantly cellulosic with remnants of hemicellulosic carbohydrates. Following these treatments the cellulosic glucose composition of TFA-insoluble residues (fractions 3 and 6) was determined by 72–4% H_2SO_4 hydrolysis (Borchardt and Piper, 1970) followed by GLC determination of silylated derivatives and total carbohydrate determination by the phenol–sulfuric acid (Dubois et al., 1956) procedure. All fraction contents, unless otherwise stated, were calculated as a percent of original sample dry matter.

Table II. Gravimetric Determinations[a] of Hemicellulose Content in Undigested and Digested Flaccidgrass Cell Walls[b] by Four Laboratory Methods

incubn, h	detergent-sol fraction	TFA hydrolysis	fractionation	
			acid	base
undigested (control)	31.2	36.3 (29.6)[c]	36.4 (29.7)	30.4 (21.7)
0	31.2	33.7 (30.9)	36.0 (30.5)	29.5 (22.4)
20	19.8	23.3 (19.9)	23.4 (20.1)	20.4 (15.6)
40	13.9	16.3 (13.1)	16.1 (14.3)	14.5 (10.8)
72	10.8	13.1 (10.7)	12.8 (10.5)	11.7 (8.2)

[a] Values are given on a percent of original sample dry matter. [b] Samples had undergone prior ND extraction to isolate cell walls. [c] Values in parentheses are total neutral sugar recoveries by TFA hydrolysis and gas liquid chromatography.

Analysis of variance was used to test main effects due to taxa, harvest, and plant part. Main effect mean separation for taxa and plant part effects was by the Waller–Duncan's K ratio t-test (Waller and Kemp, 1976). Single degree of freedom contrasts were used to compare fraction composition for treatments A–C (Figure 1).

Experiment 3. TFA Residue Analysis. The relationship between pectin-free cellulose (PFC) and the residue remaining after the two-stage hydrolysis was examined by simple linear regression. The two hydrolysis residues involved were those recovered after ND extraction (RES1) or PFC preparation (RES2). Plant material selected was a high and low IVDMD plant genotype harvested at two maturities within two height classes (short and tall) in each of three *Panicum* taxa. A wide range of cell wall contents and digestibilities resulted and was enhanced by plant part separation.

Experiment 4. Weak-Acid and Weak-Base Pretreatment Effects on the Recovery of TFA-Hydrolyzed Monomers. Whole samples of three *Panicum* taxa (Table I) were treated with a modification of the procedure shown in Figure 1 to determine the effect of acid and base pretreatment on neutral sugar yield. The modificationtions involve the A and B treatments as follows: (A) 1 N H_2SO_4 pretreatment for 1 h at 55 °C giving an acid-soluble cell wall (fraction 1) and an insoluble residue; (B) 1% KOH pretreatment for 1 h at 55 °C. No dual-step base hydrolysis was involved. This resulted in a base-soluble cell wall (fraction 4) and an insoluble residue that was subjected to TFA hydrolysis.

RESULTS AND DISCUSSION

Hemicellulose Recovery by TFA Hydrolysis, Acid or Base Fractionation, and Detergent Solubilization. Two experiments, one with flaccidgrass cell wall residues at differing stages of fermentation (experiment 1) and the other with *Panicum* taxa of varying quality (experiment 2), were used to compare several analytical procedures for hemicellulose content with that of TFA hydrolysis. The analytical techniques for hemicellulose in these experiments are empirical ones. Comparisons of the hydrolytic yield can be made purely on a gravimetric basis; however,

Table III. Comparison of Soluble Fraction Composition of Acid, Base, and Untreated Cell Wall Residues of *Panicum* Taxa (Percent)

taxa	fraction[a]	GLC determination					phenol–sulfuric determin
		ara[b]	xyl	gal	glu	total	
P. amarulum	1	2.47 a[c]	16.45 b	0.57 a	2.07 b	21.56 b	21.96 c
P. amarum		2.71 a	19.12 ab	1.08 a	2.85 a	25.76 a	23.19 b
P. virgatum		3.07 a	21.28 a	0.70 a	2.19 b	27.24 a	25.52 a
harvest 1		2.77	18.17	0.76	2.51	24.21	23.01
harvest 2		2.74	19.73	0.81	2.24	25.51	24.10
part:[d] lf		2.77 a	16.04 b	0.77 a	2.89 a	22.46 b	21.92 c
sh		3.07 a	19.55 a	0.91 a	2.57 a	26.11 a	24.94 a
st		2.42 a	21.26 a	0.67 a	1.65 b	26.00 a	23.80 b
P. amarulum	4A + 4B[e]	2.30 c	17.56 b	0.43 a	1.01 b	20.90 b	18.87 b
P. amarum		2.80 b	17.93 b	0.35 b	1.35 a	22.43 b	21.45 a
P. virgatum		3.06 a	20.95 a	0.40 ab	0.73 c	25.13 a	21.73 a
harvest 1		2.80	18.05	0.38	1.19	22.42	20.52
harvest 2		2.64	19.58	0.41	0.87	23.22	20.79
		(NS)[f]	(*)	(NS)	(**)	(NS)	(NS)
part: lf		3.14 a	18.36 a	0.49 a	1.20 b	22.78 ab	21.11 b
sh		3.09 a	19.31 a	0.52 a	1.33 a	24.28 b	22.82 a
st		1.92 b	18.77 a	0.17 b	0.55 c	21.41 a	18.20 c
P. amarulum	7	2.81 b	20.06 c	0.78 b	2.58 b	26.23 c	
P. amarum		2.85 b	21.61 b	0.80 b	3.49 a	28.75 b	
P. virgatum		3.26 a	25.39 a	0.85 a	2.61 b	32.11 a	
harvest 1		2.90	21.23	0.75	2.97	27.84	
harvest 2		3.04	23.48	0.88	2.82	30.22	
		(*)	(**)	(**)	(NS)	(**)	
part: lf		3.24 a	18.94 c	0.92 b	3.58 a	26.68 c	
sh		3.27 a	23.31 b	1.06 a	3.16 b	30.80 a	
st		2.41 b	24.82 a	0.46 c	1.93 c	29.61 b	
contrasts on fractions[f]							
1 vs. 4A + 4B		NS	NS	**	**	**	
7 vs. 1, 4A + 4B		*	**	**	**	**	
1 vs. 7		NS	**	NS	**	**	
4A + 4B vs. 1, 7		NS	**	**	**	**	
4A + 4B vs. 7		*	**	**	**	**	
1 vs. 4A + 4B, 7		NS	**	*	**	*	

[a] Soluble fractions 1, 4A + 4B and 7 resulting from A–C treatments (Figure 1). [b] Key: ara = arabinose; xyl = xylose; gal = galactose; glu = glucose. [c] Means with the same letters are not significantly different by Waller's K ratio t-test. Comparisons apply within taxa, harvest, or part in the same column. [d] Key: lf = leaf blade; sh = leaf sheath; st = stem. [e] Base-soluble monosaccharides from two successive base (10% KOH) extractions. [f] NS, *, **,: nonsignificant, significant at $P \leq 0.05$, and significant at $P \leq 0.01$, respectively.

Table IV. Comparison of TFA-Soluble Fractions of Insoluble Residues of *Panicum* Taxa following Treatments A, B, and D (Acid and Base Pretreated and Pectin-Free Cellulose Residues) Using Gas-Liquid Chromatography (Percent)

taxa	fraction[a]	ara[b]	xyl	gal	glu	total
P. amarulum	2	0.17 b[c]	0.82 c	0.02 a	1.19 b	2.19 b
P. amarum		0.18 b	1.04 b	0.02 a	1.41 a	2.66 a
P. virgatum		0.22 a	1.19 a	0 b	1.25 ab	2.67 a
harvest 1		0.17 b	0.92 b	0.02 a	1.32 a	2.42 a
harvest 2		0.22 a	1.10 a	0.01 a	1.25 a	2.59 a
part:[d] lf		0.13 c	0.70 c	0.01 a	1.28 a	2.12 c
sh		0.21 b	0.96 b	0.01 a	1.33 a	2.51 b
st		0.24 a	1.38 a	0.01 a	1.24 a	2.88 a
P. amarulum	5	0.97 a	3.36 b	0.22 a	2.17 b	6.73 b
P. amarum		0.98 a	3.00 c	0.19 a	2.69 a	6.86 b
P. virgatum		1.00 a	4.63 a	0.19 a	2.31 b	8.14 a
harvest 1		0.86 b	2.99 b	0.15 b	2.27 b	6.31 b
harvest 2		1.08 a	4.33 a	0.25 a	2.51 a	8.17 a
part: lf		0.79 b	2.01 c	0.15 c	2.75 a	5.70 b
sh		1.18 b	3.94 b	0.28 a	2.42 b	7.82 a
st		0.98 a	5.03 a	0.18 b	2.00 c	8.20 a
P. amarulum	9	0.03 c	2.82 c	0	2.16 b	5.02 c
P. amarum		0.04 b	3.20 b	0	2.35 a	5.59 b
P. virgatum		0.06 a	4.02 a	0	2.32 a	6.40 a
harvest 1		0.04 b	3.17 b	0	2.30 a	5.15 a
harvest 2		0.05 a	3.52 a	0	2.26 a	5.83 a
part: lf		0.03 c	2.25 c	0	2.10 c	4.38 c
sh		0.04 b	3.21 b	0	2.44 a	5.70 b
st		0.06 a	4.56 a	0	2.30 b	6.93 a
contrasts on fractions[e]						
2 vs. 5		**	**	**	**	**
9 vs. 2, 5		**	**		**	*
2 vs. 9		NS	**		**	**
5 vs. 9		**	NS		NS	**
2 vs. 5, 9		**	**		**	**

[a] Fractions 2, 5, and 9 are described in Figure 1. [b] Key: ara = arabinose; xyl = xylose; gal = galactose; glu = glucose. [c] Means with the same letters are not significantly different by Waller's K ratio t-test. Comparisons apply within taxa, harvest, or part in the same column. [d] Key: lf = leaf blade; sh = leaf sheath; st = stem. [e] NS, *, **: nonsignificant, significant at $P \leq 0.05$, and significant at $P \leq 0.01$, respectively. All other comparisons were significant at $P \leq 0.01$.

useful quantitative information can be gained from a detailed analysis of the cell wall monomers in the hydrolysates. No methods are available that will remove the total hemicellulosic fraction from the cell walls of plants of widely differing composition. A chemical procedure that quantitatively hydrolyzes an identifiable hemicellulosic structural fraction would be of considerable use for cell wall research.

Experiment 1. The mean hemicellulose concentrations determined by several methods in this study are shown in Table II. The hemicellulose recovered from the cell walls of flaccidgrass declined to similar levels by nearly complete (72-h) digestion. Gravimetric hemicellulose determinations by base fractionation (HCB) and detergent solubilization (HC) were quite similar over the incubation range, but values were underestimated compared with those of acid fractionation (HCA) and TFA hydrolysis. The neutral sugar recoveries from residues of these latter acid treatments yielded values similar to those of the detergent-soluble hemicellulose (HC). Lower recoveries of base-treated cell wall residues may be due to the inability of base to hydrolyze the lignin–polysaccharide matrix as suggested by Bailey et al. (1976). It is known that phenolic components are partially solubilized in 2 N TFA (Barton et al., 1982; Windham et al., 1983). This may account for the greater hemicellulosic monomer yield by the acid treatments.

Experiment 2. The empirical detergent solubility technique of Van Soest (1963) is a useful standard for hemicellulose determination in forage grass tissue. de Ruiter (1983) found that only a small neutral sugar fraction could be hydrolyzed by 2 N TFA from AD residues. Therefore, the sugar composition hydrolyzed from the ND-soluble cell wall was considered to be predominantly hemicellulosic. The relationship between the TFA-soluble neutral sugar fraction and the more traditional "direct chemical" methods has not been documented. This experiment compares these methods using three *Panicum* taxa selected to cover a range of digestibilities. A detailed analysis of hydrolyzed cell wall monomer contents and cell wall fractions left unhydrolyzed was made on plant material that also differed in terms of IVDMD relative to taxa, maturity, and plant part (Table I).

The primary objective of the experiment was to utilize the variability in cell wall composition to evaluate the fractionation techniques. Three treatments (A–C) that removed hemicellulosic carbohydrates from cell wall preparations are shown in Figure 1 as fractions 1, 4A + 4B, and 7, respectively. Base-soluble hemicellulose (4A + 4B) was released slowly from the cell wall, necessitating a sequential extraction procedure. A small, but significant, monomer fraction was released during the second treatment with 10% KOH. It was assumed that no further extraction was necessary after the second step, although Ford et al. (1979) proposed three successive extractions to remove the readily available base-soluble sugars.

TFA hydrolyzed significantly ($P \leq 0.05$) more total hemicellulose than did either acid or base treatment (Table III). The acid and base hydrolyses differed little in their respective yields of arabinose and xylose cell wall monomers. Similarly, the two acid (H_2SO_4, TFA) treatments yielded equivalent concentrations of arabinose and galactose. There were large differences among all other treatment comparisons. Arabinose was readily hydrolyzed

Table V. Composition of TFA-Insoluble Residues[a] from Cell Walls of *Panicum* Taxa with Prior Treatment Sequence A and B[b]

taxa	harvest	method[c]					gravimetric hydrolysis	cellulose[d]
		phenol–sulfuric	direct GLC		TFA–GLC			
			xylose	glucose	xylose	glucose		
				Fraction 3				
P. amarulum	1	73.2 (2.4)[e]					78.4 (2.3)	19.7 (0.4)
	2	66.1 (4.2)	2.2 (0.2)	68.9 (3.6)	1.4 (1.1)	68.0 (7.2)	72.8 (2.4)	20.9 (1.0)
P. amarum	1	76.2 (1.8)					83.5 (0.2)	22.4 (2.3)
	2	73.8 (6.7)					79.8 (4.2)	24.9 (1.7)
P. virgatum	1	72.7 (1.9)					78.6 (2.4)	26.1 (4.5)
	2	70.9 (4.6)					76.1 (2.5)	27.5 (6.7)
				Fraction 6				
P. amarulum	1	85.7 (6.8)					88.5 (3.3)	20.0 (1.1)
	2	85.3 (3.4)	12.2 (4.3)	71.8 (4.8)	15.6 (5.4)	73.6 (7.8)	87.1 (2.7)	22.6 (2.3)
P. amarum	1	92.5 (1.8)					91.7 (2.3)	23.9 (3.2)
	2	92.5 (4.7)					90.5 (2.6)	27.5 (5.4)
P. virgatum	1	94.5 (7.0)					90.8 (0.5)	28.4 (3.4)
	2	80.8 (3.8)					90.2 (0.3)	26.8 (6.5)

[a] Residues were hydrolyzed in 72–4% H_2SO_4 according to Ford et al. (1979) and Borchardt and Piper (1970), and results are expressed as percent of TFA residue. [b] See Figure 1. [c] Phenol–sulfuric colorimetric procedure, silylation, and gas–liquid chromatography (GLC) of neutralized hydrolysate and TFA hydrolysis–silylation and GLC (TFA–GLC) of neutralized hydrolysate, respectively. [d] Percent of original sample DM based on the phenol–sulfuric determination and adjusted for DM losses in pretreatment. [e] Analysis conducted on combined replicate residues; values in parentheses standard deviations of means over plant parts.

from cell walls, irrespective of treatment, as indicated by the marginally significant comparisons between extraction methods. Significant ($P \leq 0.05$) difference between main effect means occurred more frequently in the TFA-hydrolyzed samples. This was particularly evident in the harvest comparisons. Hydrolysis of the cell wall with TFA was apparently more precise than acid or base fractionation and better suited for analytical detection of small differences in monomer composition.

Of the samples studied, the "TFA-soluble cell wall" determination can effectively substitute for acid (H_2SO_4) or base (KOH) fractionation of hemicellulose. Inaccuracies in the quantification of the cell wall neutral sugars in acid and base hydrolysis procedures is reported to occur because of losses at the neutralization step (Hough et al., 1972). Precipitates were formed during freeze-drying of neutralized hydrolysates, and some underestimation may have resulted. Soluble hemicellulose from *P. virgatum* was generally greater than from either *P. amarum* of *P. amarulum* (Table III). The arabinose and xylose components of *P. virgatum* were higher than in the other taxa, but the glucose content was similar or lower. These compositional characteristics are in agreement with observations linking high xylose and low glucose of hemicellulose with lower digestibilities (de Ruiter, 1984). The alkali-soluble glucose may be representative of the pentose-free β-(1,3)- or β-(1,4)-linked glucans which have been isolated by fractionation of hemicellulose from several temperate grasses (Buchala and Meier, 1973; Fraser and Wilkie, 1971). The GLC and phenol–sulfuric procedures compared favorably in terms of the total carbohydrate fraction detected in the hydrolysate (Table III).

The composition of residues after hemicellulose removal (fractions 2, 5, and 9) also differed depending on the prior treatment designed to remove the hemicellulose fraction (Table IV). Varying amounts of the cell wall remained bound during the TFA hydrolysis. Base-treated cell walls yielded a greater fraction than did acid-treated samples. Even after delignification of AD-treated cell walls, 5–6% of the cell wall could be recovered by TFA hydrolysis. Greater amounts were recovered from the samples of relative lower dry matter disappearance when analyzed for main effects of taxa, maturity, and plant part.

Table VI. Estimates of Linear Coefficients Relating Pectin-Free Cellulose (Y) to TFA-Treated Insoluble Fractions (X) in *Panicum* Taxa

coefficient[a]	RES1[b]		RES2	
	whole	parts	whole	parts
b_0 (SE)	8.71 (3.12)	3.54 (1.03)	−0.84 (1.53)	−1.14 (0.97)
b_1 (SE)	0.67 (0.09)	0.83 (0.03)	1.36 (0.06)	1.37 (0.04)
r^2	0.71	0.92	0.98	0.97
n	24	72	12	36
H_0: $b_0 = 0$	*[c]	**	NS	NS
$b_1 = 1$	**	**	**	**

[a] Model: $Y_i = b_0 + b_1X_i + \epsilon_i$. [b] RES1 = residue following ND and TFA hydrolysis; RES2 = residue following PFC and TFA hydrolysis (Figure 1); whole = intact plant; parts = leaf blade, leaf sheath, and stem. [c] NS, *, **: nonsignificant, significant at $P \leq 0.05$, and significant at $P \leq 0.01$, respectively.

Hydrolysis with 72–4% H_2SO_4 removed between 73 and 92% of the residue remaining after TFA hydrolysis (Table V). Glucose was the major monomer in hydrolysates, although a significant xylose fraction was recovered from residues pretreated in base (fraction 6). These noncrystalline carbohydrate components were left unhydrolyzed even after three prior treatments intended to remove this fraction. The complexity of cell wall structure and the strong covalent bonding between the polysaccharides are evident. A 1-h compared with the standard 15-min treatment in 72% H_2SO_4 had no effect on improving the gravimetric hydrolysis of TFA residues, but the monomer yield was reduced in both the phenol–sulfuric and GLC determinations. An inverse relationship was found between the cellulose composition and the dry matter disappearance (Table I) of the three *Panicum* taxa examined. Higher cellulose compositions were found in the more mature plants, which were associated with higher cellulose compositions of the stem fractions.

TFA Residue Analysis. *Experiment 3.* The procedures for cellulose determination advocated by Van Soest and Wine (1968) involve the delignification of acid detergent residues and calculation of cellulose remaining by

Table VII. Cell Wall Monosaccharide Components of Pretreated *Panicum* Forages (Percent)

treatments and effects	insoluble cell wall monosaccharides[a]					base-soluble cell wall monosaccharides[b]					total sugars[c]	soluble sugars[d] (acid/base pretr)
	ara[e]	xyl	gal	glu	total	ara	xyl	gal	glu	total		
pretreatment												
(A) ND + 1 N H_2SO_4	2.37 c[f]	21.73 a	0.65 b	2.88 c	27.64 a						27.64 b	0.50
(B) ND + 1% KOH	2.61 b	19.26 c	0.62 b	3.05 b	25.55 c	0.73	3.53	0.14	0.10	4.50	30.06 a	4.32
(C) ND (control)	2.86 a	20.07 d	0.76 a	3.35 a	27.04 b						27.04 c	
(D) ND + AD + delignification	0.08 d	3.84 d	0.02 c	2.06 d	6.01 d						6.01 d	
taxa												
P. amarulum	1.89 b	15.36 b	0.44 c	2.91 b	20.59 b	0.78 a	3.96 a	0.15 a	0.09 b	5.00 a	21.84 b	2.58 a
P. amarum	2.03 a	14.74 c	0.58 a	3.09 a	20.44 b	0.70 a	3.36 b	0.15 a	0.14 a	4.34 b	21.52 b	2.30 b
P. virgatum	2.03 a	18.59 a	0.52 b	2.51 c	23.65 a	0.70 a	3.29 b	0.13 a	0.08 b	4.20 b	24.70 a	2.35 ab
effects[g]												
pretreatment (P)	**	**	**	**	**						**	**
taxa (T)	**	**	**	**	**	NS	*	NS	**	*	**	NS
P × T	**	**	*	**	**						**	NS
harvest (H)	**	**	NS	**	**	*	**	*	**	*	**	NS
P × H	*	**	NS	*	**						NS	*
T × H	*	**	NS	*	**	NS	*	NS	*	*	NS	NS
P × T × H	NS	NS	NS	NS	*						NS	NS

[a] Insoluble component refers to cell wall fraction not removed in neutral or acid detergent (ND, AD) and acid or base treatment but hydrolyzed in TFA. [b] Cell wall monomers solubilized in 1% KOH pretreatment. No sugars were detected in acid (1 N H_2SO_4 pretreatment) hydrolysates by silylation and gas–liquid chromatography (GLC). [c] Combined TFA-soluble and 1% KOH-soluble cell wall determined by GLC. [d] Sugar fraction solubilized by acid or base pretreatment and determined by the phenol–sulfuric acid method. [e] Key: ara = arabinose; xyl = xylose; gal = galactose; glu = glucose. [f] Means with the same letter are not significantly different by Waller's K ratio t-test. Comparisons apply within pretreatment or taxa in the same column. [g] NS, *, **: nonsignificant, significant at $P \leq 0.05$, and significant at $P \leq 0.01$, respectively.

Table VIII. Carbohydrate Content (Percent) of Residues and Hydrolyzed Fractions of *Panicum* Taxa Pretreated with Acid (1 N H_2SO_4) or Base (1% KOH)

treatments and effect	fraction[a]			hydrolysis (gravimetric)	
	A	B	A + B	pretreatment (acid/base)	TFA
pretreatment					
(A) ND + 1 N H_2SO_4	30.5 b[b]	60.7 a	91.2 b	1.4	35.2 b
(B) ND + 1% KOH	39.2 c	58.6 b	97.8 a	10.1	27.2 c
(C) ND (control)	29.1 a	60.1 a	89.2 c		36.4 a
taxa					
P. amarulum	36.5 a	57.1 b	93.6 a	5.9 a	31.4 c
P. amarum	35.3 b	56.3 b	91.6 b	5.4 b	32.3 b
P. virgatum	27.2 c	65.9 a	93.1 a	5.9 a	35.2 a
effects[c]					
pretreatment (P)	**	**	**	**	**
taxa (T)	**	**	**	*	**
P × T	NS	NS	**	*	NS
harvest (H)	**	**	NS	NS	**
P × H	NS	NS	NS	NS	NS
T × H	*	NS	**	NS	**
P × T × H	NS	NS	NS	NS	NS

[a] Fraction of original sample solubilized in neutral detergent and acid or base pretreatment (A) and TFA-hydrolyzed insoluble cell wall sugar component plus pectin-free cellulose (B). [b] Means with the same letters are not signfiicantly different by Waller's K ratio t-test. Comparisons apply within pretreatment or taxa in the same column. [c] NS, *, **: nonsignificant, significant at $P \leq 0.05$, and significant at $P \leq 0.01$, respectively.

difference. Up to 15% of the pentosans and most of the cutin and silica present in the plant material survive this treatment, and therefore overestimates of cellulose composition often result. A PFC fraction described by Van Soest and Robertson (1980) as the residue remaining after sequential ND, AD, and delignification steps gave an improved estimate of the true cellulose component. Sequential extraction would be expected to remove an additional fraction of the pentosans of hemicellulosic polymers as well as pectin, tannins, and a variable amount of silica but not cutin. Crystalline cellulose is known to resist hydrolysis in 2.0 N TFA (Talmadge et al., 1973). It was hypothesized that the residue remaining after TFA hydrolysis would be an adequate predictor of the true cellulose content. Statistics for the relationship between the hydrolysis residues (RES1, RES2) and the PFC content for *Panicum* forages of varying digestibility (Table VI) showed the combined regression analysis on separated plant parts (leaf blades, leaf sheaths, stems) gave stronger relationships for the two residues than did whole samples alone. The hydrolysis residue (RES1) underestimated (slope estimates were significantly less than 1.0) the PFC content, suggesting that TFA treatment removes a greater cell wall fraction than what remains in the PFC residue. Nevertheless, the strong positive correlation between these fractions indicates their usefulness for predictive purposes. These data suggest that TFA hydrolysis may be too severe for quantitative yields of hemicellulose from the cell walls. Yet, the "pectin-free" cellulose obtained through sequential detergent extraction and delignification contained non-

glucose cell wall monomers, presumably of hemicellulosic nature, resulting in an overestimation of the PFC fraction (fraction 9). A deviation significantly ($P \leq 0.01$) greater than 1:1 was found for the relationship between PFC and RES2 (Table IV). This deviation was equivalent to a 5–10% TFA hydrolysis of the PFC residue by TFA. Release of chain-termination glucans or hemicellulosic xylose or glucose monomers within aggregated cellulose microfibrils could account for this deviation, bearing in mind the reported (Talmadge et al., 1973) resistance of β-(1,4)-linked cellulose to 2.0 N TFA hydrolysis. Complete hydrolysis of mature (harvest 2) RES1 and RES2 samples by 72–4% H_2SO_4 yielded an average of 7.0 and 9.5% xylose monomers, respectively, the remainder being glucose. There was no xylose detectable in immature (harvest 1) samples of RES1 or RES2.

Effect of Acid and Base Pretreatment on Recovery of Cell Wall Monosaccharides by TFA Hydrolysis. *Experiment 4.* The total monosaccharide recovery of soluble and insoluble components of pretreated cell walls differed among various pretreatments (Table VII). Base pretreatment (B) yielded a significantly larger ($P \leq 0.05$) total monosaccharide component than either the acid pretreated (A) or the control (C). A majority of this difference was attributed to fiber solubilization in the base pretreatment. Soluble sugar estimates made directly (phenol, sulfuric acid) or by GLC were similar. The H_2SO_4 pretreatment (under mild conditions) solubilized only a minor fraction of the cell wall, although the treatment did improve the total cell wall monosaccharide recovery of the insoluble cell wall component over that of fraction 7 (TFA-soluble cell wall). A small fraction of the pectin-free cellulose residue was hydrolyzed by TFA. Xylose and glucose were the major hydrolysate components. These were probably hemicellulosic remnants released from within crystalline cellulose microfibrils as mentioned previously. The effect of pretreatments, in terms of monomer recovery by sequential TFA hydrolysis (Table VII), was complex in view of the generally significant first-order interactions among pretreatment, taxa, and harvest. Pretreatment caused differential solubility among monomers and varying hydrolytic yields in samples of differing composition. For example, the yield of TFA-hydrolyzed sugars in *P. virgatum* was higher than for the other taxa. The difference was primarily due to greater recovery of xylose. In contrast, the glucose recovery from *P. virgatum* pretreatment residues was lower. Lignin content was similar among taxa (6.2%) but differed ($P \leq 0.01$) between harvests (harvest 1, 5.1; harvest 2, 7.3%).

The components accounted for by solubilization treatments and residue analysis (hydrolyzed sugars + PFC) were given as fractions A and B, respectively (Table VIII). The TFA residues were previously shown to consist predominantly of pectin-free cellulose. Samples with base pretreatment resulted in a 97.8% recovery, which was significantly greater than either acid pretreatment or control (no pretreatment). The improved recovery was due to a 10.1% partial hydrolysis of the cell wall by 1% KOH during pretreatment. This caused a lower TFA hydrolysis (27.2%) of the base-pretreated residue compared to acid-pretreated (35.2%) and control (36.4%) residues. Greater recovery from *P. virgatum* samples with low dry matter disappearance was mainly due to a greater TFA hydrolysis of pretreatment residues. A complete quantitative analysis of sample composition is desirable, and it was clear that base pretreatment gave the best results in terms of recovery. However, the additional information gained must be balanced against the time required for sample analysis with the additional procedural step.

ABBREVIATIONS

AD, acid detergent; ADF, acid detergent fiber; GLC, gas–liquid chromatography; HC, detergent-soluble hemicellulose; HCA, acid-soluble hemicellulose; HCB, base-soluble hemicellulose; IVDMD, in vitro dry matter disappearance; ND, neutral detergent; NDF, neutral detergent fiber; PFC, pectin-free cellulose; RES1, residue after ND extraction; RES2, residue after sequential ND, AD, and delignification steps; TFA, trifluoroacetic acid.

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Cobinding of Bile Acids to Carrot Fiber

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Cobinding of bile acids to carrot cell wall residue, an experimental model for fiber, was demonstrated by reversed-phase high-performance liquid chromatography. Binding ranged from 1 to 2 g of dihydroxy bile acid/100 g of cell wall residue from pH 8.0 to 6.0. The proton relaxation time of bound chenodeoxycholate, determined by solid-state CPMAS ^{13}C NMR spectroscopy, was similar to that for cell wall residue and indicates that binding is very tight. The binding order found was chenodeoxycholate > deoxycholate >> cholate. Protons are released during binding, and the extent of binding appears to be related to the calcium content of the cell wall residue. We propose a mechanism for binding that involves Ca^{2+} salt linkages between pectin in the cell wall residue and a bile acid.

Vegetable fiber in the diet can produce lowering of blood cholesterol levels (Kern et al., 1978; Jenkins et al., 1979). In vitro investigations have shown that certain vegetable fibers can absorb bile salt/acids under physiological conditions (Kern et al., 1978; Selvendran, 1978; Robertson et al., 1980). In vivo binding of bile salts/acids to dietary fiber blocks reabsorption. The body must draw upon its pool of cholesterol for synthesis of any bile acids lost through binding to dietary fiber. This process is presently thought to account for the hypercholesterolemic effect observed for vegetable dietary fiber. Birkner and Kern (1974) found that vegetable fiber exhibits binding for dihydroxy bile acids that is greater than that for the trihydroxy bile acid cholic acid and that binding is inversely related to pH. Material that is like dietary fiber from carrot can be prepared as an alcohol-insoluble residue (AIR) of walls of ruptured cells. Since carrot AIR has appreciable capacity to bind bile acids (Robertson, et al., 1980) and since its composition has been reported (Aspinall et al., 1983), we selected this material to investigate the chemical nature of such binding.

EXPERIMENTAL SECTION

Preparation of Carrot Alcohol-Insoluble Residue. Washed, truncated carrots were minced in a blender with water (100 mL; 200 g of carrot). The mixture was gradually frozen. After at least 24 h in the freezer, the material was thawed and washed sequentially with water and ethanol. Solid material was recovered by filtration through a medium sintered-glass filter and then stirred in refluxing ethanol (1 L; 200 g of original carrot) for 4 h. After recovery by filtration, the material was washed with acetone and then stirred in water (1 L; 200 g of original carrot). The pH was adjusted to between 7 and 8 with either dipotassium phosphate or dilute ammonium hydroxide. After the mixture was stirred for 1 h and the pH stabilized, the material was washed with water. A slurry, prepared with water (10 mL; 1 g of residue), was freeze-dried. The yield of alcohol-insoluble residue (AIR) was typically 3%. The sources of AIR's were as follows: commercial Canadian carrots, AIR I; laboratory greenhouse-grown Nantes Strong Top, AIR II; commercial Massachusettes carrots, AIR III.

High-Performance Liquid Chromatography (HPLC) of Bile Acids. A Du Pont Zorbex ODS, 4.6-mm i.d. × 15-cm length column was used with a mobile phase of pH 7.2, 0.02 M phosphate:acetonitrile = 67:35 (v/v) to separate the bile acids (Parris, 1977). A 1.0-mL flow rate was maintained with a Du Pont 870 pump and Series 8800 gradient controller in isocratic mode. Peaks were detected with a Waters R401 differential refractometer. Detector response was monitored with a Hewlett-Packard 3390A integrator. Standard bile acid solutions were made up in 0.05 M phosphate buffer, 2 mg each/mL. The final pH was obtained by microliter addition of acetic acid. The concentration of each bile acid was found to be directly related to either peak height or area, with negligible deviation only at low concentration (less than 0.2 mg/mL).

Measurement of Binding. The bottom of the barrel of a 10-mL glass syringe was covered with a disk of Whatman No. 2 filter paper cut from a sheet with a cork borer of appropriate diameter. The syringe was fitted with a Swinny adaptor containing a 13-mm, 22-μm Millipore filter. Carrot AIR, 0.25 g, was packed into the barrel and was then completely wetted with 2.5 mL of 0.05 M phosphate buffer containing bile acid(s), 2 mg/mL, at the desired pH. After 30 min of contact, some of the solution was expressed from the fiber by slowly applying pressure to the syringe plunger. The pH of the filtrate, collected in a 3-mL Reacti-Vial, was measured with a small- (6-mm) diameter combination electrode. The concentration of bile acid(s) before and after contact with AIR was then determined from peak heights and/or areas after HPLC. Percent binding (g bile acid/100 g of fiber) was calculated from the decrease in bile acid concentration in the test solution after exposure to the AIR.

Binding Equation. [1 − (Ht/Hs)] × [g BA/mL solution] × [mL solution/g AIR] × 100 = % binding (g BA/100 g fiber). Key: Ht = peak height, bile acid test

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