be due to acid treatment prior to protein extraction (Blaicher et al., 1983).

CONCLUSIONS

Rapeseed products having low or high phytic acid content exhibit overall favorable functional properties compared with soybean products. This indicates that, irrespective of the phytic acid content of the final product, countercurrent extraction followed by isoelectric precipitation yields protein products with good functional properties. Phytic acid, in the range present in rapeseed products investigated, apparently has little influence on the functional properties with the exception of emulsifying capacity and emulsion stability (Table IV), which are generally better for products containing low levels of phytic acid. Removal of phytic acid by acid extraction prior to protein extraction was aimed at preparing a nutritionally favorable product (Blaicher et al., 1983). This treatment obviously does not influence most of the functional properties of the protein isolate adversely. However, some loss in foaming capacity and foam stability occurs. The overall good foaming properties of rapeseed products compared with soybean products indicate a good potential for their application in whipped food products.

Registry No. NaCl, 7647-14-5; phytic acid, 83-86-3; sucrose, 57-50-1.

LITERATURE CITED

Association of Official Agricutural Chemists *Official Methods of Analysis*, 12th ed.; AOAC: Washington, DC, 1975.
Blaicher, F. M.; Elstner, F.; Stein, W.; Mukherjee, K. D. *J. Agric. Food Chem.* **1983**, *31*, 358.
Coffmann, C. W.; Garcia, V. V. *J. Food Technol.* **1977**, *12*, 473.
Dench, J. E.; Rivas, N. R.; Caygill, J. C. *J. Sci. Food Agric.* **1981**, *32*, 557.
El Nockrashy, A. S.; Mukherjee, K. D.; Mangold, H. K. *J. Agric. Food Chem.* **1977**, *25*, 193.
Hagenmaier, R. *J. Food Sci.* **1972**, *37*, 965.
Kadagoda, L. P.; Nakai, S.; Powrie, W. D. *Can. Inst. Food Sci. Technol. J.* **1973**, *6*, 266.
Kinsella, J. E. *Food Chem.* **1981**, *7*, 273.
Lin, M. J. Y.; Humbert, E. S.; Sosulski, F. W. *J. Food Sci.* **1974**, *39*, 368.
Marshall, W. H.; Dutson, T. R.; Carpenter, Z. L.; Smith, G. *J. Food Sci.* **1975**, *40*, 896.
McWatters, K. H.; Holmes, M. R. *J. Food Sci.* **1979**, *44*, 771.
Mieth, G.; Brückner, J.; Kroll, J.; Pohl, J. *Nahrung* **1983**, *27*, 759.
Olson, R.; Anjou, K. *J. Am. Oil Chem. Soc.* **1979**, *56*, 431.
Quinn, J. R.; Paton, D. *Cereal Chem.* **1979**, *56*, 38.
Rutkowski, A. *Nahrung* **1975**, *19*, 941.
Sathe, S. K.; Deshpande, S. S.; Salunkhe, D. K. *J. Food Sci.* **1982**, *47*, 491.
Sosulski, F. W. *Cereal Chem.* **1962**, *39*, 344.
Sosulski, F. W. In *Food Colloids*; Graham, H. D., Ed.; Avi: Westport, CT, 1977; p 152.
Sosulski, F. W. In *Developments in Food Proteins—2*; Hudson, B. J. F., Ed.; Applied Science: London, 1983; p 109.
Sosulski, F. W.; Bakal, A. *Can. Inst. Food Sci. Technol. J.* **1969**, *2*, 28.
Sosulski, F. W.; Humbert, E. S.; Bui, K.; Jones, J. D. *J. Food Sci.* **1976**, *41*, 1349.
Thompson, L. U.; Lui, R. F. K.; Jones, J. D. *J. Food Sci.* **1982**, *47*, 1175.
Volkert, M. A.; Klein, B. P. *J. Food Sci.* **1979**, *44*, 93.
Wang, J. C.; Kinsella, J. E. *J. Food Sci.* **1976**, *41*, 286.
Yasumatsu, K.; Sawada, K.; Moritaka, S.; Misaki, M.; Toda, J.; Wada, T.; Ishii, K. *Agric. Biol. Chem.* **1972**, *36*, 719.

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Rapid Determination of Cell Wall Monosaccharides in Flaccidgrass[1]

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A two-stage trifluoroacetic acid (TFA) hydrolysis (2 N TFA followed by 0.25 N TFA each for 1 h at 121 °C) gave repeatable recoveries of fiber monomers and allowed detailed analyses of structural carbohydrates in a subtropical grass. The second hydrolysis was essential to cleave glycosyl trifluoroacetates, formed in the primary hydrolysis, to the free carbohydrate monomers and TFA. At optimal hydrolysis conditions cell wall preparations of *Pennisetum flaccidum* Griseb. (flaccidgrass), with neutral detergent fiber concentrations of 67.3%, yielded xylose, glucose, arabinose, and galactose monomers in the ratio of 24:11.5:5:1, respectively. Optimum monomer recovery occurred between 1- and 2-h hydrolyses in primary treatments with 2 N TFA. Under those conditions individual monomers differed in their survival, with some isomerization detected requiring correction for precise determination. This was accomplished by using an internal standard (sorbitol) added to samples either pre- or posthydrolysis.

Chemical solubilization of cell wall polysaccharides generally yields a heterogeneous mixture of components that gives little information about the in situ carbohydrate polymers. Hemicellulose hydrolysis in acid or base is often incomplete while cellulose, prepared by the removal of other polysaccharide constituents, invariably contains small amounts of glycosyl residues other than glucose. These may be chain terminator monomers (Darvill et al., 1980) or other hemicellulosic sugars bound tightly to the β 1-4 glucan chains (Mühlethaler, 1967). The quantitative recovery of sugars in acid hydrolysates of cell wall preparations is, therefore, difficult. Traditional methods of hydrolysis of cell wall polysaccharides to their component monomers involve hot, dilute mineral acid treatment. Monosaccharide dehydration and the differential ease of glycosidic bond hydrolysis prevent quantitative recovery of monomers in acid hydrolysates. Bailey (1973) found

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that bond resistance to hydrolysis increased in the order 1-2, 1-3, 1-4, 1-6. Links involving the C-1 of a furanose are very acid labile, while those involving uronic acid C-1-linked residues, as found in acidic pectic polysaccharides and the glucuronoarabinoxylans, are very acid resistant. Conditions sufficiently harsh to hydrolyze the uronosidic bonds often result in degradation of uronic acid as well as the dehydration and degradation of the neutral sugars (Darvill et al., 1980).

The hydrolytic yield of more resistant polysaccharides such as cellulose, xylans, and mannans can be improved by pretreatment with cold 72% H_2SO_4 before hydrolysis with 1 N acid (Bailey, 1973). Traditional methods for hydrolysis of wood polysaccharides (Adams, 1965; Jones and Penny, 1963) with H_2SO_4, for alditol acetate preparation and subsequent gas–liquid chromatographic analysis, involves laborious preparative steps. Moreover, an undefined fraction of the hemicellulosic and cellulosic polymers are hydrolyzed, making quantitative analysis difficult. Incomplete hydrolysis and dehydration to furfurals and (hydroxymethyl)furfural products are processes that interfere with accurate estimation of monosaccharide composition of polysaccharide polymers. An alternative procedure consists of the hydrolysis of cell wall with trifluoroacetic acid (TFA) (Jones and Albersheim, 1972; Sloneker, 1971; Fengel et al., 1978; Ford et al., 1979). Albersheim et al. (1967) used 2 N TFA in attempts to hydrolyze plant cell wall polysaccharides and reported difficulties in quantifying the level of hydrolysis products released from xylans and pectic substances. Quantitative recovery of sugars with their technique was poor, ranging from 11 to 60%, indicating that without improvement the use of TFA as a hydrolyzing agent would be of little value in precise chemical characterization of cell wall hydrolysates. Gas–liquid chromatography of the trimethylsilyl (Me_3Si) ether derivatives and their application to quantitative analysis of monosaccharide and polysaccharide hydrolysates have been reported (Sweeley et al., 1963; Bradbury et al., 1981).

Hydrolysis in 2 N TFA for 1 h at 121 °C was proposed to remove a hemicellulosic fraction (Morrison, I. M., personal communication). At concentrations of 90% at 100 °C, cellulose was hydrolyzed but there was considerable degradation of the hemicellulosic component. Hydrolysis with 2 N TFA also solubilizes lignin–carbohydrate complexes (Windham et al., 1983; Barton et al., 1982). Talmadge et al. (1973) noted that the glycosidic linkages of cellulose were not hydrolyzed, nor were β 1-4 glycosyl linkages of xyloglucans (Bauer et al., 1973) when treated with 2 N TFA for 1 h at 121 °C. Neutral sugars present in cell wall polymers in aldobiuronic linkage are released intact, and uronosyl residues of the wall polymer are degraded (Jones and Albersheim, 1972). This is contrary to the reported resistance of aldobiuronic acid C-1 linkages to acid hydrolysis (Darvill et al., 1980).

A rapid technique for cell wall compositional analysis is important for determining the causes of incomplete in vivo rumen digestion of forages, particularly in the poorly digested, mature subtropical grasses. A rapid technique for estimating the composition of indigestible cell walls would also be useful in selection programs with the objective of improving the digestibility of species having heritable cell wall characteristics. A major advantage of TFA use is its ease of removal from hydrolysates due to its volatility (bp) = 72.4 °C). Also, TFA appears to be a versatile solubilizing and hydrolyzing agent for structural polysaccharide analysis of noncellulosic neutral sugars. However, rigidly controlled hydrolysis conditions may be required for quantitative sugar recoveries.

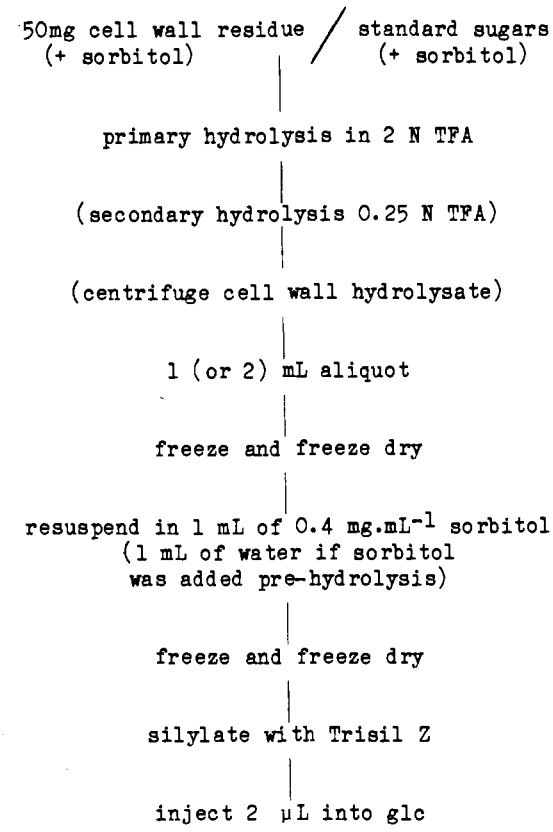

Figure 1. Procedure for determination of Me_3Si derivatives of standard sugars or cell wall monosaccharides by gas–liquid chromatography (GLC). Procedural alternatives are given in parentheses.

The objective of this study was to improve the acid hydrolysis yield from the indigestible cell walls of the subtropical species *Pennisetum flaccidum* Griseb. (flaccidgrass) by various TFA treatments to establish hydrolysis conditions for the quantitative recovery of cell wall derived monosaccharides.

MATERIALS AND METHODS

Materials. Pure sugar standards used in the calibration experiments were L-(+)-arabinose (Aldrich Chemical Co.) and D-xylose, D-galactose, D-glucose, and D-sorbitol (Fisher Scientific Co.). Except where stated, a cell wall sample used for technique evaluation was a neutral detergent fiber residue (NDF) obtained from a whole plant (tops only) of *P. flaccidum* (flaccidgrass) harvested at early maturity (NDF = 67.3%). The residue was collected in tared Gooch crucibles following the traditional NDF (Goering and Van Soest, 1970) extraction, freeze-dried, weighed, and scraped into screw-capped vials for storage at −10 °C.

Experiments were conducted to characterize the hydrolysis procedures by determining the monosaccharide composition of trifluoroacetic acid (TFA) hydrolysates. The general procedure is outlined in Figure 1. All derivatization and gas–liquid chromatography (GLC) procedures were the same throughout. A 0.5-mL portion of the silylating solution Trisil Z [the use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service, nor USDA, ARS, of the products named nor criticism of similar ones not mentioned] (Pierce Chemical Co.), with an active silylating compound (trimethysilyl)imidazole (TMSI) in pyridine, was added to a 3.5-mL septum vial containing a freeze-dried sample for monosaccharide determination. The residue was solubilized by shaking thoroughly on a Vortex mixer for 30 s and allowed to stand for at least 30 min prior

to analysis. A 2100 Varian Aerograph gas–liquid chromatograph was fitted with 61 × 4 mm i. d. dual glass columns packed with 3% SE-30 (Applied Science Laboratories) liquid phase on 100/120 CW-HP Chromasorb W solid support. An Autolab (Spectra Physics) System I computing integrator was used to integrate and record the chromatograms. A 2-μL portion of each sample was injected onto the column and component monosaccharide anomer separation achieved with a programmed column temperature that increased from 160 to 220 °C at 2 °C min^{-1} from injection. Injector and detector temperatures were maintained at 230 and 250 °C, respectively. The integrator was programmed to resolve peaks to a base line or with a last fused peak trapezoidal base-line correction in the case of fused peaks.

TFA Hydrolysis Conditions. *Acid Strength.* A 50-mg sample of flaccidgrass cell wall (NDF) residue, together with 4 mg of sorbitol (internal standard), was hydrolyzed in 3 mL of TFA at concentrations of 0.5, 1, 2, 3, 4, 5, 6, and 10 N. Samples in triplicate were hydrolyzed in 45-mL screw-cap tubes in an autoclave for 1 h at 121 °C. A 100% survival of sorbitol was assumed in the 0.5 N TFA treatment. The samples were cooled and centrifuged at 1000*g* for 5 min. A 1-mL aliquot of the supernatant was transferred to a 3.5-mL reacti vial and covered with perforated parafilm. The samples were then frozen and freeze-dried overnight. A 1-mL portion of water was added, the solution was thoroughly mixed, the vials were centrifuged to remove sediment, and a 0.5-mL aliquot was transferred to another 3.5-mL reacti vial. The samples were again frozen and freeze-dried prior to silylation and GLC analysis.

Hydrolysis Time. Triplicate 50-mg samples of ND-treated cell wall residue were hydrolyzed in 2.0 N TFA for 0, 5, 15, 30, 60, 120, and 480 min. Subsamples (1 mL) were taken from centrifuged hydrolysates. Subsequent cleanup steps and derivatization were as previously described except that the sample was redissolved in 1 mL of a sorbitol standard (0.4 mg mL^{-1}). Residues following hydrolysis were saved, and weight difference used to determine percent hydrolysis.

Effect of Primary and Secondary Hydrolysis on Monosaccharide Yield. Three treatments, designated A–C, all containing a primary hydrolysis step using 2 N TFA, but varying secondary hydrolysis procedures, were used to test their effect on monosaccharide recovery. Four replicates of 50-mg samples of cell wall residues from a whole-plant sample of flaccidgrass were each weighed into 45-mL screw-cap tubes. A 4-mL portion of a 2.5 N TFA and 1 mL containing 4.0 mg of sorbitol were added to each tube of treatments A–C. Primary hydrolysis in 2.0 N TFA occurred for 1 h at 121 °C. The samples were cooled, and either 35 mL of 0.1 N TFA (treatment A) or 35 mL of distilled water (treatment B) was added to each tube, bringing the TFA concentration to 0.35 and 0.25 N, respectively. The secondary hydrolysis was also for 1 h at 121 °C. Treatment C samples were brought to a 40-mL volume with distilled water, and 2-mL aliquots were prepared for GLC analysis.

In addition to sugar dry weight recovery from GLC analysis, a percentage hydrolysis value was determined by residue dry weight difference. Combined replicate residues were collected in tared Gooch crucibles that had equilibrated in a desiccator for 4 h. Samples were washed into the crucible with two rinses of hot distilled water, then washed two times with acetone, freeze-dried, and weighed after equilibration in a desiccator.

Hydrolysis Survival. Monomer survival during hydrolysis, relative to an internal standard, was determined using a single-stage hydrolysis in 2 N TFA for either 1 (A) or 2 (B) h. Monomer survival was calculated with reference to 100% recovery of sorbitol added after hydrolysis. Alternatively (C), the internal standard was added prior to hydrolysis and correction made for the survival of each hydrolysate monosaccharide including the internal standard in a two-stage hydrolysis (2 N TFA followed by 0.25 N TFA for 1 h at each step).

Table I. Retention Time and Anomeric Proportion of Standard Sugar Me_3Si Derivatives Determined by Gas–Liquid Chromatography

anomer	retention time, s	anomer proportion in aq equil.,[a] % of isomer
β-arabinose	486	42.2
α-arabinose	530	53.0
γ-arabinose	575	4.8
α-xylose	637	42.1
β-xylose	737	57.9
γ-galactose	872	5.3
α-galactose	953	34.5
α-glucose	1016	43.3
β-galactose	1049	60.1
sorbitol	1158	
β-glucose	1229	56.7

[a] After hydrolysis in 2 N followed by 0.25 N TFA.

Calculations. Calculations were based on chromatogram peak area per milligram sugar relative to sorbitol peak area per milligram of sorbitol, and adjustments were made to account for variability in sample injection volume. Sorbitol was used as the internal standard; however, glucose was used as the control isomer to determine survival of sorbitol through the two-stage hydrolysis. The stability of standard sugars for these hydrolysis conditions was examined to aid in the selection of appropriate procedures for future use.

RESULTS AND DISCUSSION

Silylated carbohydrates are easily prepared and are quite stable. Their volatility at temperatures greater than 230 °C makes them ideal for gas-phase analysis. TMSI appears to be one of the most rapid and powerful silylating agents reported for hydroxyl groups. Silylation occurs with less anomerization than HMDS–TMDS–pyridine (Kim et al., 1967) and will proceed in the presence of small amounts of water (Pierce, 1968). Linear detector responses occurred relative to the concentration of all standard sugars (data not shown). All anomers of component isomers known to occur in cell walls of subtropical grasses were detected without interference (Table I). The α and β anomer silylated derivatives represent the normal mutarotated forms. In addition, γ anomers of arabinose and galactose were detected; however, their structures were uncertain. The γ component of galactose has been found to mutarotate in pyridine (solvent in Trisil Z) to form α and β anomers of 2,3,5,6-tetrakis-*O*-(trimethylsilyl)-D-galactofuranoside (Shallenberger and Acree, 1966; Acree et al., 1968) and presumably cochromatograph at the γ anomer retention time. It is possible that arabinose forms a similar furanoside derivative in pyridine. Interconversion of monosaccharide anomers in aqueous solution occurs through formation of intermediate acyclic aldehydes or their hydrates (White et al., 1978). Interconversion occurs in aqueous solution where the aldehyde usually exists as a minor component of the equilibrium mixture.

TFA Hydrolysis Conditions. Degradation of monosaccharides in hydrolysates under severe hydrolyzing conditions may contribute to a significant reduction in GLC-determined yield. Significant losses in apparent yield

Table II. Relative Neutral Sugar Recovery[a] during TFA Hydrolysis of a Flaccidgrass Neutral Detergent Residue (NDF = 67.3%) at Various Acid Concentrations

TFA concn,[b] N	neutral sugar,[c] %				
	arabinose	xylose	galactose	glucose	total
0.5	8.0 (1.3)[d]	32.5 (3.4)	1.4 (0.11)	11.6 (1.1)	53.5
1	8.8 (0.4)	34.7 (1.0)	1.5 (0.02)	13.0 (0.3)	58.0
2	7.6 (0.4)	30.4 (0.5)	1.3 (0.06)	11.4 (0.2)	51.1
3	8.5 (0.7)	29.7 (3.9)	1.4 (0.14)	12.6 (1.4)	52.5
4	8.2 (0.2)	28.7 (2.1)	1.4 (0.15)	13.2 (0.7)	51.5
5	7.8 (0.9)	25.4 (4.3)	1.3 (0.20)	12.8 (2.1)	47.3
6	6.0 (1.2)	13.8 (6.5)	0.9 (0.28)	11.1 (0.1)	31.8
10	4.5 (1.4)	10.4 (10.8)	0.8 (0.30)	9.6 (0.8)	25.3
SED[e]	0.7	3.6	0.15	0.9	

[a] Monosaccharide yield recoveries were adjusted to account for reduced survival of the internal standard (sorbitol) at increasing TFA concentrations. [b] A 15% loss of sorbitol was found in the 10 N TFA hydrolysis relative to the 0.5 N TFA treatment. [c] Values were based on an assumed 100% survival of sorbitol at 0.5 N TFA. [d] Values in parentheses are standard deviations. [e] Standard error of difference between treatment means.

of cell wall hydrolysates were found to occur at TFA concentrations of 5 N and greater (Table II). Relative monosaccharide yield was halved when hydrolysis was with 10 N TFA compared with 0.5 N TFA. In dilute concentrations TFA is not a particularly strong dehydrating agent; however, at the high concentrations losses were probably caused by this effect. Lower recoveries were associated with increased variability in estimates at the higher concentrations. Higher standard errors also occur for the 0.5 N TFA hydrolysis treatment. A 1–2 N TFA concentration appeared best for hydrolysis, giving repeatable yields of the monosaccharides. However, relative yields were overestimates of the true neutral sugar recovery because of hydrolytic losses of sorbitol not accounted for in the 0.5 N TFA treatment.

Although apparent neutral sugar yield was shown to decline with increasing TFA concentrations, the actual dry matter loss during hydrolysis would be expected to increase with increasing acid concentration. Dark coloration of hydrolysates indicated charring of sugars at concentrations of 5 N and greater.

A more accurate hydrolysis survival estimate for each isomer, based on unhydrolyzed sugar standards, was necessary for a more precise monosaccharide compositional analysis of cell wall residues. An alternative method was to correct for losses of the internal standard rather than the inclusion of an internal standard throughout hydrolysis. The internal standard could be added to samples in an aqueous equilibration step after hydrolysis and the initial hydrolysate lyophilization.

Optimum hydrolysis time is dependent on a balance between the rate of release of hydrolyzable polysaccharides and the degradation of monosaccharides that occurs during prolonged treatment in acid at 121 °C. On the basis of residue weight loss during hydrolysis, a large proportion of hydrolyzed products was released within a relatively short time (Figure 2). Over 90% of the hydrolyzable monosaccharides was released within 1 h, and very little release occurred thereafter. The quantitative recovery of monosaccharides by GLC was severely affected at longer hydrolysis times. No hydrolysis products were found in either distilled water or in 2 N TFA (unheated control). For further quantitative analysis it was proposed that a 2 N TFA hydrolysis for 1 h would give the most desirable balance over time, allowing for both the increasing residue hydrolysis and increasing degradation of free monosaccharides in the hydrolysates.

Crystalline cellulose is known to resist hydrolysis in 2.0 N TFA (Talmadge et al., 1973). It is, therefore, reasonable to assume the hydrolysis products are primarily hemicellulosic and the residue mainly cellulose. The sugar

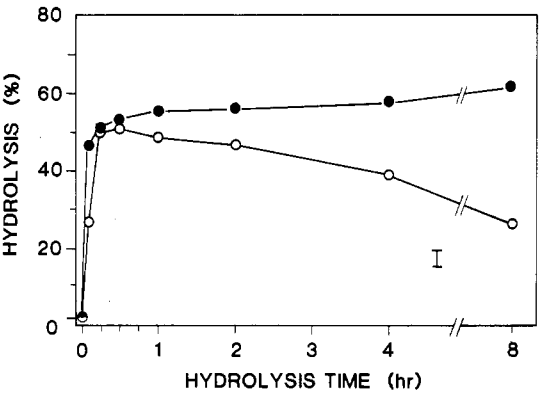

Figure 2. Hydrolysis percentages over time determined on an undigested neutral detergent residue (NDF = 67.3%) of whole early-maturity *P. flaccidum* Griseb. Values were estimated by residue weight difference before and after hydrolysis (●) or by total monosaccharide composition of the hydrolysate (○). Percent hydrolysis (residue difference) of unheated distilled water and TFA controls was 1.24 and 1.31%, respectively. Vertical bar is standard error for total monosaccharide composition only.

composition of 2.0 N TFA hydrolysates contained a high proportion of nonglucosyl monomers, which are characteristic of hemicellulosic polymers (Table II). Aldobiuronic residues, which occur in concentrations of up to 7% in some grass species (Gaillard, 1962), could also contribute to the hydrolyzed monomers. However, there is conjecture about the stability of aldobiuronic acids in 2.0 N TFA (Darvill et al., 1980; Jones and Albersheim, 1973).

Effect of Primary and Secondary Hydrolysis on Monosaccharide Yield and Hydrolysis Survival. The major products of TFA hydrolysis of cell wall polysaccharides are free monomers. However, the extent of esterification losses due to the formation of pyranosyl and furanosyl trifluoroacetates during hydrolysis using moderate strength TFA is not known. In strong mineral acid, dehydration occurs with the formation of furfurals and (hydroxymethyl)furfurals. These degradation products or fluoromethyl glycosides rarely form during hydrolysis using moderate strength TFA since TFA is not a strong dehydrating agent. However, a significant loss of monosaccharide yield may occur through esterification during glycosidic bond hydrolysis (Morrison and Boyd, 1979). Trifluoroacetate esters form mainly at the anomeric carbon atom. The presence of strong electron-withdrawing fluorine atoms of TFA facilitates an esterification reaction. The reaction mechanism for the addition of fluoroacetate to the C-1 position of the monosaccharide is shown in Figure 3. The esterified products can be hydrolyzed by a similar reaction in reverse (Morrison and Boyd, 1979).

Table III. Mean Composition[a] of Whole Flaccidgrass Cell Wall Neutral Sugars from TFA Hydrolysates with Various Primary and Secondary Hydrolysis Treatments

hydrol treatment[b]	neutral sugar, %					total hydrol[c]
	arabinose	xylose	galactose	glucose	total	
A	5.72	28.39	1.16	13.63	48.90	50.7
B	5.99	28.62	1.17	13.51	49.29	50.7
C	5.35	26.41	1.04	12.65	45.45	50.0
SED[d]	0.16	0.93	0.07	0.44	1.53	
contrasts[e]						
A vs. B	NS	NS	NS	NS	NS	
C vs. A, B	NS	*	NS	*	*	

[a] Values given are based on an assumed 100% survival of the internal standard through hydrolysis. [b] Key: A = primary hydrolysis in 2 N TFA followed by secondary hydrolysis in 0.35 N TFA; B = as for A, except secondary hydrolysis in 0.25 N TFA; C = primary hydrolysis in 2 N TFA and no secondary hydrolysis. [c] Hydrolysis determined by residue weight difference before and after treatment. [d] Standard error of difference between treatment means. [e] Single degree of freedom contrasts; NS and * = nonsignificant and significant at $p < 0.05$, respectively.

Table IV. Hydrolysis Survival of Standard Sugars for Various Hydrolysis Procedures

hydrol procedure	monosaccharide, %				
	arabinose	xylose	galactose	glucose	sorbitol
A[a] [2 N TFA, 1 h (n = 2)]	63.4	61.6	79.9	86.0	
B[a] [2 N TFA, 2 h (n = 4)]	63.0	49.1	71.2	79.6	
C[b] [2–0.25 N TFA for 1 h each step (n = 5)]	85.0	77.1	85.3	89.0	71.6
SED[c]	5.6	2.8	6.4	5.5	

[a] Posthydrolysis addition of internal standard (IS). [b] Prehydrolysis addition of IS. [c] Standard error of the difference between hydrolysis procedure means.

This occurs in a secondary hydrolysis at a lower TFA concentration by a nucleophilic substitution reaction involving a water molecule. Hydrogen ions catalyze both the esterification and ester hydrolysis reactions by making the carbonyl carbon more susceptible to attack.

Xylose, glucose and total neutral sugar percentages from flaccidgrass cell wall hydrolysates showed that a secondary hydrolysis step significantly improves their recoveries (Table III) An approximate 4% difference was found between primary hydrolysis only and the dual primary and secondary hydrolysis procedure. This difference was attributed to hydrolysis of esterified monosaccharides that accumulated during primary hydrolysis. Secondary hydrolysis in either 0.35 or 0.25 N TFA (A vs. B contrast) had no effect on individual or total monosaccharide yield improvement (Table III). The yield of arabinose and galactose, which comprised minor components, was not significantly altered.

Sugar concentrations from treatments A–C would all be subject to error if the relative differences in survival of the internal standard, or if the survival of individual isomers, were varied by the hydrolytic treatments. Sorbitol was probably not as susceptible to esterification as were the ring-structured monosaccharides but were equally susceptible to degradation during hydrolysis. Consequently, the relative survival of each isomer, including the internal standard for quantitative analysis, required determination. The dual hydrolysis improved hydrolytic survival of the standard sugars (Table IV), indicating reversion of bound neutral sugars to free monomers in the second (ester hydrolysis) step. The hexose sugars appeared least likely to undergo esterification in 2 N TFA. An improvement of between 3% (glucose) and 21.6% (arabinose) was observed with the inclusion of the weakly acidic secondary hydrolysis. The fraction of each monosaccharide degraded in each treatment (Table V) was not known, nor was the extent of the ester hydrolysis reaction. There was, as expected, greater variability in survival data when the internal standard was carried through the hydrolytic steps. Mean standard errors for individual neutral sugars within treatments A–C (Table V) were 2.1, 4.3, and 5.9%, respectively.

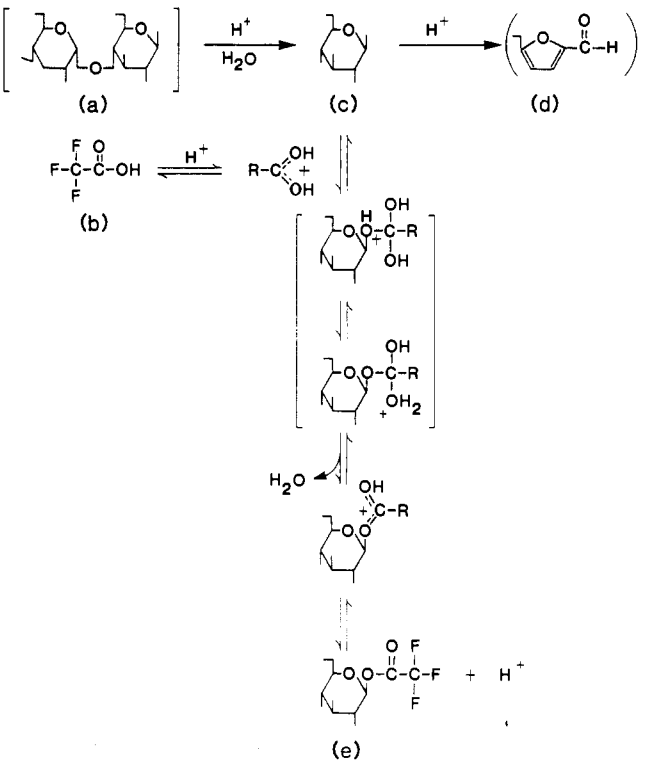

Figure 3. Reactions occurring during primary hydrolysis of polysaccharides in 2 N TFA, with the formation of free monosaccharides and pyranosyl/furanosyl trifluoroacetic esters: (a) polysaccharide with glucosyl residues; (b) trifluoroacetic acid, R = CF_3; (c) β-D-glucose; (d) (hydroxymethyl)furfural (rare); (e) trifluoroacetic ester derivative.

Some of the monosaccharide losses during hydrolysis could be accounted for by isomerization. Additional small peaks were observed at retention times equivalent to β-xylose and α-fructose in hydrolyzed sorbitol samples. Cyclization of the reduced straight-chain sorbitol at the C-1 carbon and C-5 hydroxyl forms a fructose molecule. Therefore, isomerization of the internal standard and standard sugars during hydrolysis could be an important

Table V. TFA (2.0–0.25 N) Hydrolysis Survival Percentages of Monosaccharide Standards and Isomerization Conversion Factors for Cell Wall Compositional Analysis

	factor		
sugar,[a] %	hydrol survival[b] const	sorbitol isomerizn to α-fructose	sugar isomerizn
arabinose	0.807	1 − α-Fru PKA/sorb PKA	−(0.0279 Xyl % + 0.0189 Gal %)[c]
xylose	0.889	1 − α-Fru PKA/sorb PKA	1.0279(1 − 0.0242 Sorb PKA/β-Xyl PKA)[d]
galactose	0.835	1 − α-Fru PKA/sorb PKA	1.089[e]
glucose	0.771	1 − α-Fru PKA/sorb PKA	

[a] Percent sugar from integrator output (combined anomer percentages). [b] (71.61[f] × 0.9576[g])/sugar survival (Table IV); f = sorbitol survival, g = correction for 4.24% conversion of sorbitol to β-xylose. [c] Isomerization of xylose to arabinose and galactose to arabinose. [d] Isomerization of sorbitol to β-xylose and isomerization loss of xylose to arabinose (PKA = peak area). [e] Isomerization loss of galactose to arabinose.

factor in determining the quantitative recovery of cell wall monosaccharides.

The extent of sugar standard isomerization was quantified, and correction factors are listed in Table V. Corrections based on these data should be applied to GLC output data in addition to correcting for relative hydrolysis survival differences between the internal standard and cell wall sugar components for quantitative determinations. Galactose and glucose isomers proved to be the most stable during 2 N TFA followed by 0.25 N TFA hydrolysis, and no isomeric corrections were necessary. A 2.70% and 1.89% conversion of xylose and galactose to arabinose, respectively, was observed. These sugars form both the α and β anomers of arabinose. Xylose correction for a 4.24% isomerization of sorbitol to β-xylose was also necessary. This proportion was assumed to be constant in all two-stage hydrolysis samples. A general correction for isomerization of sorbitol to α-fructose was proposed on the basis of relative peak areas on individual chromatograms as the extent of α-fructose formation was variable from sample to sample and perhaps dependent on small changes in hydrolytic conditions. It was assumed that the peak area of α-fructose (present only in hydrolyzed samples) occurred at the expense of sorbitol in equal peak proportions. Temperature of hydrolysis alone had little effect on sorbitol degradation, as distilled water controls treated at hydrolyzing temperatures resulted in complete recovery of sorbitol as a Me_3Si derivative. There was also no change in proportions of sorbitol isomers with primary alone vs. a two-stage hydrolysis. No detector response was produced in the monosaccharide range for Trisil-Z, acetone, pyridine, and TFA blanks. The sorbitol isomerization is proposed to have occurred during the primary hydrolysis step rather than during derivatization.

CONCLUSIONS

Hydrolysis conditions prior to derivatization were studied, and methodology was developed that resulted in repeatable hydrolysis of cell wall polysaccharides residues. The methodology evaluated showed that hydrolysis of cell wall samples in two stages gave improved recoveries over a single-stage 2 N TFA hydrolysis. Increased recoveries were due to hydrolysis of pyranosyl or furanosyl trifluoroacetic esters in 0.25 N TFA to yield free monosaccharides. Both incubations were for 1 h at 121 °C. Primary hydrolysis at higher acid strengths and for longer periods resulted in degradation of hydrolysate monosaccharides. Centrifugation of hydrolysates before subsampling was necessary to remove fine sediment.

Accurate determination of survival was required for quantitative determinations. Correction for percent monosaccharide recovery in hydrolysates was required for galactose isomerization to arabinose and xylose to arabinose. Sorbitol was satisfactory as an internal standard; however, a correction constant was required to adjust for isomerization to β-xylose and cyclization to α-fructose during the sequential hydrolysis procedure if added prior to hydrolysis. Correction could be avoided by adding sorbitol posthydrolysis.

Registry No. L-Arabinose, 5328-37-0; D-xylose, 58-86-6; D-galactose, 59-23-4; D-glucose, 50-99-7.

LITERATURE CITED

Acree, T. E.; Shallenberger, R. S.; Mattick, L. R. *Carbohydr. Res.* **1968**, *6*, 498.

Adams, G. A. In *Methods of Carbohydrate Chemistry*; Whistler, R. L., Ed; Academic: New York, 1965; Vol. 5, p 269.

Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. *Carbohydr. Res.* **1967**, *5*, 340.

Bailey, R. W. In *Chemistry and Biochemistry of Herbage*; Butler, G. W., Bailey, R. W., Eds.; Academic: London and New York, 1973; Vol 1, Chapter 4.

Barton, F. E., II; Windham, W. R.; Himmelsbach, D. S. *J. Agric. Food Chem.* **1982**, *30*, 1119.

Bauer, W. D.; Talmadge, K. W.; Keegstra, K.; Albersheim, P. *Plant Physiol.* **1973**, *51*, 174.

Bradbury, A. G. W.; Halliday, O. J.; Medcalf, D. G. J. *J. Chromatr.* **1981**, *213*, 146.

Darvill, A.; McNeil, M; Albersheim, P.; Delmer, D. P. In *The Biochemistry of Plants—A Comprehensive Treatise*; Tolbert, N. E., Ed.; Academic: New York, 1980; Vol. 1, Chapter 3.

Fengel, D.; Wegener, A.; Heizmann, A.; Przyklenk, P. *Cellulose Chem. Technol.* **1978**, *12*, 31.

Ford, C. W.; Morrison, I. M.; Wilson, J. R. *Aust. J. Agric. Res.* **1979**, *30*, 621.

Gaillard, B. D. E. *J. Agric. Sci. (Cambridge)* **1962**, *59*, 369.

Goering, H. K.; Van Soest, P. J. *Agriculture Handbook No.* 379; ARS/USDA: Washington, DC, 1970.

Jones, T. M.; Albersheim, P. *Plant Physiol.* **1972**, *49*, 926.

Jones, J. K. M.; Penny, M. B. In *Technique for Organic Chemistry*; Weissinger A., Ed.; Wiley: New York, 1963; Vol. XV, Part 2, p 707.

Kim, S. M.; Bentley, R.; Sweeley, C. C. *Carbohydr. Res.* **1967**, *5*, 373.

Morrison, I. M. Hannah Research Institute, Ayr, Scotland KA6 5HL.

Morrison, R.; Boyd, R. In *Organic Chemistry*; Allyn and Bacon: Boston, 1979; Chapter 20.

Mühlethaler, K. *Annu. Rev. Plant Physiol.* **1967**, *18*, 1.

Pierce, A. E. In *A Technique for Gas-Phase Analysis*; Pierce Chemical Co.: Rockford, IL, 1968.

Shallenberger, R. S.; Acree, T. E. *Carbohydr. Res.* **1966**, *1*, 495.

Sloneker, J. H. *Anal. Biochem.* 1971, *43*, 539.

Sweeley, C. C.; Bentley, R.; Makita, M.; Wells, W. W. *J. Am. Chem. Soc.* **1963**, *85*, 2497.

Talmadge, K. W.; Keegstra, K.; Bauer, W. D.; Albersheim, P. *Plant Physiol.* **1973**, *51*, 158.

White, A.; Handler, P.; Smith, E. L.; Lill, R. L.; Lehman, I. R. In *Principles of Biochemistry*; McGraw-Hill: New York, 1978; Chapter 2.

Windham, W. R.; Barton, F. E., II; Himmelsbach, D. S. *J. Agric. Food Chem.* **1983**, *31*, 471.

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