Rapid N-Methylimidazole-Catalyzed Acetylation of Plant Cell Wall Sugars

Allan S. Bittner,* Lorin E. Harris, and William F. Campbell[1]

Cell walls, prepared from reed canarygrass, *Phalaris arundinacea* L., were treated with 72% sulfuric acid followed by dilution to 2 N and hydrolysis for 3 h in a boiling water bath. The hydrolysates were neutralized with concentrated ammonia, reduced, and acetylated with acetic anhydride with *N*-methylimidazole as a catalyst. Acetylation was completed in 5 min. Selected hydrolysis times for plant material were 0.5 h with 72% sulfuric acid at 22 °C followed by hydrolysis for 3 h with dilute acid at 95 °C. Plant cell wall delignification prior to acid hydrolysis and the effects of delignification on cell wall carbohydrate and lignin content were examined. Delignification of the NDF residues with hot acid chlorite removed carbohydrate, lignin, and possibly protein. The maximum carbohydrate yields from the hydrolyzed samples were observed for the lignified cell wall preparations. The method is proposed as a means for rapid assessment of plant cell wall carbohydrate for the nutritional evaluation of feeds and forages.

The analysis of sugars in plant cell wall polysaccharides using gas–liquid chromatography is emerging as a useful tool for the nutritional evaluation of various feeds and forages. Sloneker (1971) adapted the methods of Albersheim et al. (1967) to the analysis of plant cell wall sugars using a two-step sulfuric acid hydrolysis. Collings and Yokoyama (1979) used 1 N trifluoroacetic acid to hydrolyze a delignified, ammonium oxalate insoluble cell wall preparation. They determined noncellulosic sugars with gas–liquid chromatography and determined cellulose gravimetrically as the acid-insoluble residue.

Accurate quantitation of sugars is complicated by losses during the hydrolysis and neutralization steps. Hough et al. (1972) presented an excellent account of the inaccuracies of sugar quantitation due to destruction by acid hydrolysis and losses in the neutralization process. They observed that neutralization of acid hyddrolysates with carbonate salts as used by Sloneker (1971) may result in underestimation of the various sugars found in plant cell walls. Trifluoroacetic acid hydrolysis (Albersheim et al., 1967) eliminates the neutralization step, but it is limited to analysis for sugars other than those derived from cellulose and oligosaccharides derived from the β-(1,4)-glycosyl linkages of xyloglucan (Bauer et al., 1973). Consequently, trifluoroacetic acid hydrolysis of cell walls, as applied by Collings and Yokoyama (1979), may underestimate noncellulosic sugars and overestimate cellulose.

Methods for the determination of total plant cell wall sugars by gas chromatography are generally time consuming and are not always practical for application to routine feed analysis. The derivatization procedure of Albersheim et al. (1967) required 3 h for acetylation at 120 °C. Collings and Yokoyama (1979) shortened the acetylation time to 1 h without any apparent loss in sensitivity. Pyridine has been used as a nucleophilic catalyst for the acetylation of various sugars while the reaction time was shortened considerably. Since pyridine is unpleasant, its use in acetylation reactions is generally avoided. Recently, Wachowiak and Connors (1979) introduced *N*-methylimidazole as a catalyst for the rapid acetylation of hydroxy compounds. Acetylation time was reduced to 5 min.

Delignification of plant cell wall preparations with acid chlorite has been used as a preparatory step for carbohydrate analysis by several researchers (Morrison, 1975; Bailey and Pickmire, 1975; Collings and Yokoyama, 1979). Apparently the method for plant cell wall isolation determines the components removed by hot acid chlorite treatment. Morrison (1975) summarized previous work which indicated that cell wall carbohydrate was lost as a result of delignification and that not all lignin was removed by acid chlorite treatment. Ely et al. (1956) concluded that acid chlorite removed greater percentages of protein than of lignin when holocellulose fractions were prepared from forages high in protein. Collings and Yokoyama (1979) proposed the use of acid chlorite for the gravimetric determination of lignin in feeds and forages prior to determination of noncellulosic sugars with gas chromatography. Bailey and Pickmire (1975) suggested that delignification prior to analysis of noncellulosic sugars was unnecessary for immature plants low in lignin.

This paper reports a procedure for the rapid analysis of the sugars in lignified plant cell walls using gas–liquid chromatography. The effects of acid chlorite delignification of cell wall carbohydrate, lignin, and dry matter loss were also examined.

MATERIALS AND METHODS

Preparation of Plant Samples. The neutral detergent fiber (NDF) method of Goering and Van Soest (1970) was selected as a standard procedure for the illustration of delignification phenomena as well as for the measurement of total sugars using gas chromatography. Mature reed canarygrass, *Phalaris arundinacea* L., samples were ground in a wiley mill (40-mesh screen). Dry matter was determined after placing samples overnight in a 60 °C vacuum oven.

Preparation of Alditol Acetates. A 30–40-mg sample of NDF residue was transferred to a 13-mm tube with a Teflon-lined screw cap. A 0.5-mL portion of a 72% sulfuric acid solution was then added to the tube, and the mixture was stirred with a small glass rod. The reaction was allowed to proceed for 0.5 h at 25 °C while the mixture was occasially stirred with a glass rod. The mixture was then diluted to 2 N sulfuric acid (0.5 mL of acid and 5 mL of water) with water and degassed in a desiccator by using a water aspirator. The desiccator was then filled with nitrogen and the tube capped and placed in a boiling water bath (95 °C) for 3 h.

Following the hydrolysis, a 100-μL aliquot of the dilute acid solution was added to a second screw-capped tube. The aliquot was neutralized with a few drops of concentrated ammonia. At this time the myoinositol internal

Department of Animal, Dairy and Veterinary Science, Utah State University, Logan, Utah 84322.
[1]Professor of Crop Physiology, Plant Science Deparment, Utah State University, Logan, Utah 84322.

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standard was added followed by addition of a 10 mg/mL solution of sodium borohydride in 1 N ammonia (Albersheim et al., 1967). The reduction reaction was allowed to proceed for 1 h. Neutralization with ammonia eliminates the need for neutralizing with salts that interfere with quantative recovery of the sugars (Hough et al., 1972). The reduction reaction was stopped by dropwise addition of glacial acetic acid until the evolution of hydrogen gas ceased. Acidified methanol (0.5 mL of 9:1 methanol–acetic acid) was then added and the samples were evaporated to dryness in a 40 °C water bath with a stream of filtered air. Acidified methanol was added twice more and the sample was evaporated to dryness after each addition. The acidified methanol additions were followed by three 0.25-mL additions of methanol and the samples were evaporated to dryness after each methanol addition.

Following the methanol additions to remove the volatile methyl borate esters (Albersheim et al., 1967), 250 μL of *N*-methylimidazole (Sigma) was added to the dry sample followed immediately by the addition of 200 μL of acetic anhydride (Wachowiak and Connors, 1979). The solution was thoroughly mixed and allowed to sit for 5 min at room temperature. Wachowiak and Connors (1979) injected this mixture containing the acetylated derivatives directly into the gas chromatograph. Our experience with this mixture was that it interfered with sample resolution and shortened column life. To alleviate this problem, 1 mL of water was added to the imidazole–acetic anhydride mixture and the contents were mixed. The addition of water did not appear to affect the stability of the acetylated alditols. Chloroform was then added and the contents of the tube throughly mixed. The volume of chloroform added depended on the sample size and the sugar yield and was either 100 or 200 μL. The tubes were then centrifuged at 1000 rpm for 2 min to enhance the settling of the chloroform layer on the bottom of the tube. A 0.5–2.0-μL portion of the chloroform was then injected into the gas chromatograph. Acetylated sugar standards remained stable for several months when stored at 0 °C.

GLC Analysis of the Alditol Acetates. Samples were injected into a Varian Model 1700 gas chromatograph. A liquid phase of 0.2% poly(ethylene glycol) succinate, 0.2% poly(ethylene glycol adipate, and 0.4% silicone XF-1150 was coated on a silanized Gas Chrom Q support (100–200 mesh) and packed into a silanized glass column (120 × 0.3 cm). Column temperature was programmed immediately following injection at 1 °C/min from 140 to 180 °C. Injector and detector temperatures were 225 °C and the nitrogen carrier flow was 35 mL/min. Detector response was found to be linear from 0.25 to 2.5 μg of carbohydrate.

Precision of the derivatization procedure was measured using seven standard solutions of sugars found in plant cell walls with myoinositol added as the internal standard. Peak areas were determined by multiplying peak height by the peak width at half-height. The peak areas of each sugar were then divided by the peak area of the added internal standard.

Sugar concentrations of unknown plant samples were calculated from peak areas by using the response factor method as outlined by Grob (1977). Response factors were calculated using the average of three standard injections.

Delignification. Crucibles containing sample NDF residue were placed in a beaker containing a 1% acetic acid solution at 75 °C. The solution was allowed to percolate through the filter of the crucible until the entire residue was suspended in the hot acetic acid solution. Once the solution inside the crucible reached 75 °C (about 10 min), 10 mL of a 100 mg/mL sodium chlorite solution was pipetted into the crucible. The beakers were covered with a watchglass for the duration of the reaction. The reactions were stopped by rapid filtering with a vacuum from a water aspirator followed by five washes with hot water to remove the excess chlorite. The samples were dried overnight in a 60 °C vacuum oven. Dry matter loss, total sugars, and insolubility in 72% sulfuric acid were measured on samples exposed to the delignification reagent for 15, 30, 45, 60 and 120 min. Total sugars were determined using the phenol–sulfuric acid method of DuBois et al. (1956) on samples acid hydrolyzed with the two-step procedure. D-Glucose was used as the reference standard.

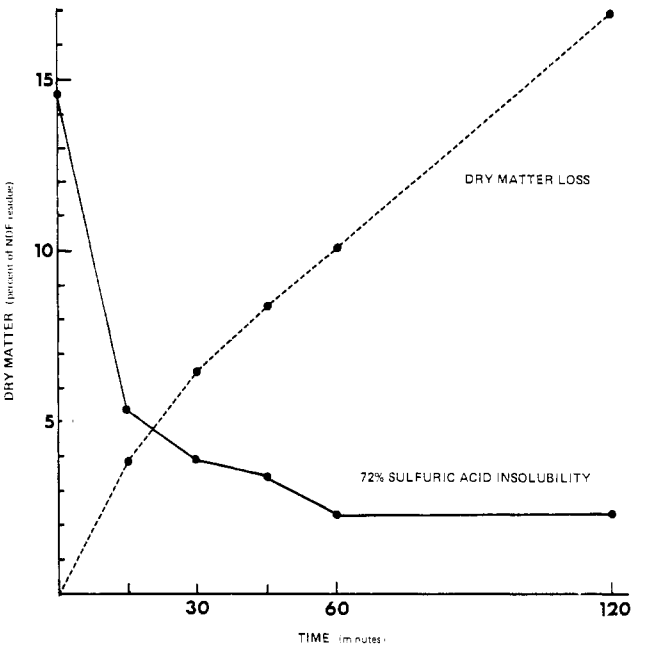

Figure 1. Dry matter loss and 72% sulfuric acid insolubility of reed canarygrass NDF residue as influenced by exposure time of residue to acid chlorite solution at 75 °C.

Acid Hydrolysis. Sugar yields from 72% sulfuric acid hydrolysis of sample NDF residue were measured at 0.5, 1, 2, 3, 4, and 5 h by using GLC, and total sugar yield from the two-step hydrolysis of sample of NDF residue was measured at 15-, 20-, 45- or 60-min exposure time to 72% sulfuric acid by using the method of DuBois et al. (1956). This was done to observe the rate of sugar destruction as well as to determine optimum exposure time to 72% sulfuric acid prior to hydrolysis with dilute acid.

Sugar yields from hydrolysis of the sample NDF residue with dilute acid were measured at 0.5, 1, 2, 3, or 4 h by using GLC. The dilute acid solution was degassed only after the initial dilution of the concentrated acid and not after each aliquot was taken (Hough et al., 1972).

RESULTS AND DISCUSSION

The effects of acid chlorite delignification on sample NDF residue were measured as dry matter loss and 72% sulfuric acid insolubility of the residual at five intervals over 2 h (Figure 1). Dry matter loss from the NDF residue increased with duration of delignification while the acid-insoluble portion of the residue decreased rapidly after the first 30 min of the acid chlorite treatment. The acid-insoluble residue remained constant after 60 min of delignification. Morrison (1975) reported similar results for grasses and legumes. The substantial decrease in dry matter of the sulfuric acid insoluble fraction during the first 15 min of acid chlorite treatment may reflect a rapid solubilization of protein by the delignification reagent. The dry matter residue of the 72% sulfuric acid insoluble

Table I. Carbohydrate Recovered from Delignified NDF Residue

delignification time, min	total sugars, mg/ 100 mg delignified	total sugars mg/ 100 mg NDF residue
0[a]	82.70	81.57
15	83.54	80.27
30	84.25	78.71
45	85.91	78.62
60	87.34	78.49
120	91.45	76.06

[a] Heated in 1% acetic acid solution for 120 min at 75 °C.

Table II. Carbohydrate Yield from Two-Step Acid Hydrolysis of Sample Residue As Affected by Exposure Time to 72% Sulfuric Acid[a]

exposure time, min	total sugars,[b] %
15	72.64
30	73.53
45	73.27
60	73.25

[a] Samples hydrolyzed with 72% H_2SO_4 for time indicated followed by dilution to 2 N and hydrolysis at 95 °C for 3 h. [b] Expressed as percent reed canarygrass NDF residue.

fraction at 15-min delignification time was slightly less than the lignin values of mature reed canarygrass reported by Collings and Yokoyama (1979). This also suggests the acid chlorite is a protein solubilization reagent as reported by Ely et al. (1956).

Total sugars were determined as a percent of the delignified residue and of the original NDF residue (Table I). Total sugar yield from the two-step acid hydrolysate increased with delignification time as the carbohydrate in the delignified residue was concentrated due to dry matter loss. Total sugar yield expressed as a percent of the original NDF residue indicated a loss of carbohydrate during the 2-h delignification period.

These observations indicated that treatment of the sample NDF residue with acid chlorite removed carbohydrate and some lignin and may have removed protein from the NDF prepared cell walls. The highest yield of total sugars, when expressed as percent of the original NDF residue, was measured in the lignified, untreated residue. These results suggest that delignification is an unnecessary step for preparation of plant cell walls low in lignin prior to hydrolysis with sulfuric acid.

The rapid decrease in the 72% sulfuric acid insoluble residue during the first 15 min of delignification may have been due to the rapid solubilization of non-cell-wall protein and nucleic acids that were not removed by the neutral detergent solution. This phenomenon may also occur with similar preparatory methods for isolating plant cell walls. Treatment with acid chlorite for brief periods of time may be a satisfactory method for removing protein from cell wall preparations prior to isolation of various cell wall carbohydrate polymers and lignin–carbohydrate complexes for methylation studies.

Acid Hydrolysis. Preliminary hydrolysis of cell walls with 72% sulfuric acid is necessary for degradation of the cellulose structure as dilute acid alone will not penetrate the crystalline structure of this polymer. Optimum hydrolysis time in concentrated acid was chosen to minimize pentose destruction and maximize total sugar yield.

The yield of individual sugars released from sample residue by 72% sulfuric acid hydrolysis was measured at various time intervals, by using GLC (Figure 2). Maximum xylose yield was observed at 30 min while arabinose yield remained relatively constant for the duration of the acid hydrolysis.

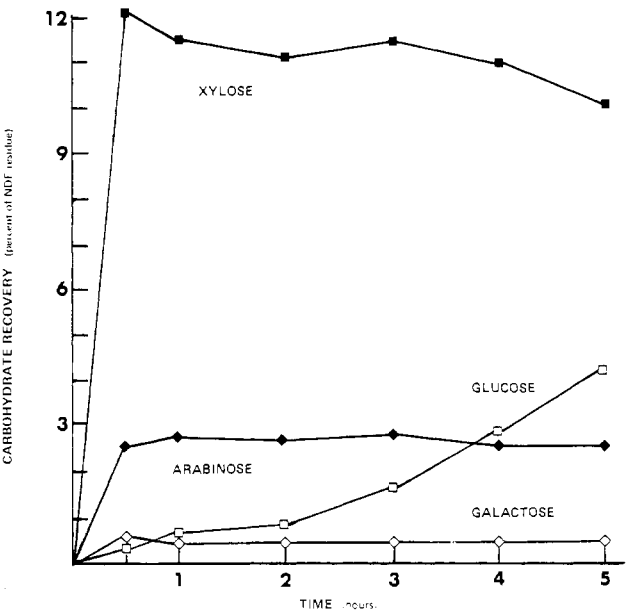

Figure 2. Sugar yield of reed canarygrass NDF residue as influenced by hydrolysis time in 72% sulfuric acid at 22 °C.

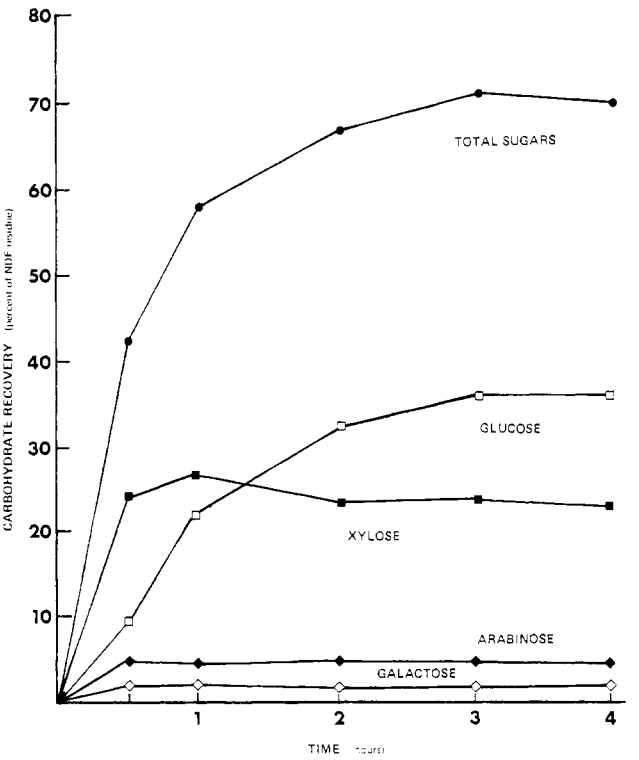

Figure 3. Sugar yield of reed canarygrass NDF residue as influenced by hydrolysis time in 2 N sulfuric acid at 95 °C.

Total sugar yield from the two-step acid-hydrolyzed sample was not affected significantly by exposure time to 72% sulfuric acid (Table II). A 30-min concentrated acid hydrolysis time was chosen on the basis of xylose yield in Figure 2 and total sugar yield in Table II. Optimum exposure time to concentrated acid may differ because of variations in cell wall structure, lignin content, and particle size.

Optimum hydrolysis time of sample residue in dilute acid at 95 °C was measured after dilution of the 72% sulfuric acid solution to 2 N. Aliquots were taken at the various times indicated (Figure 3). Glucose recovery was maximum at 3 h while xylose yield declined after 1 h. These results show that more accurate assessment of

Table III. Precision of Derivatization Procedure

	relative peak area of carbohydrate components to internal standard peak area						
standard no.	L-rhamnose	D-fucose	L-arabinose	D-xylose	D-mannose	D-galactose	D-glucose
1	0.339	0.420	0.413	0.393	0.412	0.387	0.397
2	0.324	0.390	0.420	0.387	0.425	0.398	0.416
3	0.340	0.417	0.433	0.399	0.436	0.412	0.419
4	0.337	0.406	0.409	0.383	0.401	0.368	0.385
5	0.314	0.373	0.420	0.386	0.417	0.386	0.412
6	0.306	0.364	0.405	0.364	0.415	0.386	0.410
7	0.304	0.414	0.419	0.375	0.419	0.404	0.418
mean	0.323	0.398	0.417	0.384	0.418	0.392	0.408
std dev	0.016	0.020	0.011	0.009	0.010	0.016	0.012

xylose concentration may be made by taking an aliquot at 1 h or by introducing correction factors for sugars measured at 3-h hydrolysis time. Correction factors allow for more accurate quantitation of individual sugars but do not account for all sugars originally present in the plant cell walls as destruction by acid and release of individual sugars occur simultaneously.

The destruction of sugars by the acid as well as the inability of the GLC method to determine uronic acids may account for the differences between sugar yield as measured by GLC and the sugar yield as measured by the method of DuBois et al. (1956). The method of DuBois et al. (1956) also tends to overestimate total sugars, particularly when a sample high in pentose is measured against a hexose standard.

Precision of the Derivatization Procedure. Precision of the procedure was satisfactory, as indicated by the values in Table III. Sugar recovery from the acid hydrolysates was not measured, as this will vary depending on acid volume, sample size, and the amount of air present in the reaction mixture. similar precision was observed for plant cell wall samples that were hydrolyzed and acetylated.

Although uronic acids in the hydrolysates were not quantitated in these experiments, they can be determined by using the methods of Jones and Albersheim (1972). Sloneker (1971) used the acetic–nitric acid reagent to determine cellulose and substracted glucose equivalents from total glucose to determine hemicellulosic glucose. Collings and Yokoyama (1979) attributed all glucose released by trifluoroacetic acid hydrolysis to hemicellulosic polymers originally present in the ammonium oxalate insoluble, delignified preparations. The acid-insoluble residue was proposed as a method for the gravimetric determination of cellulose.

Bauer et al. (1973) observed that trifluoroacetic acid hydrolysis of plant cell walls released all neutral sugars except those comprising cellulose and the oligosaccharides fragments of the β-(1,4)-glycosyl-linked xyloglucans. As a result, trifluoroacetic acid hydrolysis, as adapted to the determination of hemicellulose (Collings and Yokoyama, 1979), may have no advantage over the detergent system or dilute mineral acids for the quantitation of noncellulosic sugars. Starch present in the cell wall preparations of plants may further complicate the determination of noncellulosic and cellulosic polymers using glucose (MacRae 1971). We have found variable amounts of starch in cell wall preparations that appear to be a result of species differences as well as the method for cell wall isolation (Bittner, 1979).

The methylation techniques of Hakomori (1964) as used by Talmadge et al. (1973) and Sanford and Conrad (1966) can be adapted to the quantitation of noncellulosic polymers in feeds and forages. The use of cell wall polymeric composition in conjuction with total cell wall sugars would be more descriptive of cell walls than the heretofore empirical and semiempirical feed analysis methods. When the methods of Talmadge et al. (1973) are utilized, "marker" sugars such as xylose, mannose, rhamnose, and galacturonic acid could be used for rapid assessment of cell wall composition of plants of known polymeric composition. Plant cell wall could be described in terms of specific polymer nomenclature rather than with empirical or semiempirical categories.

Talmadge et al. (1973) and Sanford and Conrad (1966) suggested that polymer composition of plant carbohydrates may be less complex in structure than previously assumed. The use of total carbohydrate composition in conjunction with polymeric structure for quantitating plant carbohydrates will be the subject of future research in this laboratory.

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