# Neutral Monosaccharide Composition of Various Fibrous Substrates: A Comparison of Hydrolytic Procedures and Use of Anion-Exchange High-Performance Liquid Chromatography with Pulsed Amperometric Detection of Monosaccharides

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These experiments were to compare three carbohydrate hydrolysis procedures using various fibrous substrates and standards and to report an improved high-performance liquid chromatographic (HPLC) method to identify and quantify neutral monosaccharides. Procedure 1 used 2 N trifluoroacetic acid (TFA) as the hydrolytic agent. Procedures 2 and 3 used 72% sulfuric acid ($H_2SO_4$) as the hydrolytic agent but differed in temperature and duration of the secondary hydrolysis. The secondary hydrolysis occurred at 125 °C for 1 h for procedure 2 and 100 °C for 3 h for procedure 3. Two types of xylan and three types of microcrystalline cellulose, wheat bran, apples, wheat straw, and alfalfa were analyzed. The use of anion-exchange chromatography coupled with pulsed amperometric detection resulted in superior separation and detection of the neutral monosaccharides compared to previous HPLC techniques. Greater recoveries ($P < 0.05$) of glucose were obtained with procedures 2 and 3 compared to procedure 1 for alfalfa, apples, microcrystalline cellulose, wheat bran, and wheat straw. Greater quantities of the hemicellulosic monosaccharides, arabinose and xylose, were obtained with procedures 2 and 3 compared to procedure 1. More glucose ($P < 0.05$) was recovered from alfalfa and wheat straw following hydrolysis with procedure 2 compared to procedure 3. Data suggest that procedures 2 and 3, which use $H_2SO_4$ as the hydrolytic agent, are superior to procedure 1, which uses TFA. Our HPLC method allows for more precise quantification of neutral monosaccharides than has been reported previously with similar technologies.

Several procedures pertaining to the chemical hydrolysis of plant material with subsequent analysis of monosaccharides have been published (Neilson and Marlett, 1983; de Ruiter and Burns, 1986; Theander and Westerland, 1986; Morrison, 1988). These procedures differ in the hydrolytic agent used, the temperature at which the samples are hydrolyzed, and (or) the instrument used to separate and detect the monosaccharides.

The most commonly used hydrolytic agents are sulfuric acid ($H_2SO_4$; Neilson and Marlett, 1983; Theander and Westerland, 1986; Miron and Ben-Ghedalia, 1987) and trifluoroacetic acid (TFA; Albersheim et al., 1967; Talmadge et al., 1973; de Ruiter and Burns, 1987). Both hydrolytic agents have received criticism. For example, 2 N TFA apparently is unable to hydrolyze the glycosidic linkages of cellulose or the $\beta(1\rightarrow4)$ glycosyl linkages of the xyloglucan backbone (Talmadge et al., 1973). Morrison (1988) suggested that a major disadvantage of $H_2SO_4$ is the difficulty of removing the acid after sample hydrolysis. However, with the development of solid-phase resins such as Bio-Rad's AG 4×4, which removes mineral acids from solution, this is no longer a problem. Gas–liquid chromatography (GLC; Albersheim et al., 1967) and high-performance liquid chromatography (HPLC) both have been used to qualitatively and quantitatively measure monosaccharides (Barton et al., 1982). The major downfall of GLC is the necessity for derivatization of the monosaccharides prior to separation. This greatly increases the number of steps and, therefore, the time required for the assay. Also, the possibility of incomplete derivatization (reduction and acetylation) exists, which will reduce neutral sugar recovery. Major criticisms of the HPLC techniques are inadequate separation of the monosaccharides of interest and poor sensitivity.

The first objective of this study was to statistically compare three carbohydrate hydrolysis procedures using various fibrous substrates and standards. A second objective was to report an improved HPLC method to identify and quantify neutral monosaccharides in plant material.

## MATERIALS AND METHODS

**Samples and Sample Preparation.** Two types of xylan and three types of microcrystalline cellulose, wheat bran, apples, wheat straw, and alfalfa were independently obtained and analyzed in the experiment. Samples were as follows: xylan Lot 103298 and Lot 23262, ICN Biochemicals, Cleveland, OH 44128; microcrystalline cellulose Type 50 Lot 74F-0592, and cellulose CC-31 microgranular, Sigma Chemical Co., St. Louis, MO 63178; cotton, wheat bran, apples, wheat straw, and alfalfa, local suppliers. Apples were decored, blended, and lyophilized. Wheat bran, apples, wheat straw, and alfalfa were ground to pass a 40-mesh screen with a Wiley mill. Dry matter was determined on all samples by oven drying at 105 °C for 48 h (AOAC, 1984).

**Standard Preparation.** Monosaccharide standards, xylose, arabinose, glucose, galactose, mannose, rhamnose, and inositol, were purchased from Sigma and Aldrich Chemical Co., Inc., Milwaukee, WI 53233. Working standards in the range of 25 ppm were prepared by solubilization of monosaccharides in Millipore (Millipore Corp., Bedford, MA 01730) water.

**Determination of Neutral Monosaccharides by HPLC.** The procedures of de Ruiter and Burns (1986), Theander and Westerland (1986), and Neilson and Marlett (1983), referred to as procedures 1–3, respectively, were used to hydrolyze duplicate samples and monosaccharide standards. In procedure 1, a 50-mg sample was placed in a 50-mL screw-cap test tube. A 5-mL portion of 2 N TFA containing 104.8 mg of inositol/100 mL as an internal standard was added to each tube. Tubes were heated for 1 h at

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Table I. Percent Recovery of Monosaccharides[a] from a Standard Solution Using Various Chemical Hydrolysis Procedures

| procedure[b] | Ara | Xyl | Glc | Gal | Man | Rha | Ino |
|---|---|---|---|---|---|---|---|
| 1 | 96.7 | 94.2 | 97.4 | 93.6 | 94.6 | 98.3 | 102.0 |
| 2 | 92.8 | 82.3 | 92.7 | 93.7 | 86.8 | 96.4 | 101.0 |
| 3 | 94.8 | 91.9 | 92.4 | 91.2 | 88.0 | 96.9 | 103.0 |

[a] Key: Ara = arabinose, Xyl = xylose, Glc = glucose, Gal = galactose, Man = mannose, Rha = rhamnose, Ino = inositol. [b] Procedures: 1, de Ruiter and Burns (1986); 2, Theander and Westerland (1986); 3, Neilson and Marlett (1983).

121 °C in a block heater. Millipore water (35 mL) was added to each tube. Tubes were then heated for 1 h at 121 °C. In procedure 2, 50 mg of sample was placed in a 50-mL screw-cap test tube and 1 mL of 72% $H_2SO_4$ (w/w) containing 524 mg of inositol/100 mL as an internal standard was added to each tube. Samples were hydrolyzed for 1 h at 30 °C. Following hydrolysis, 28 mL of Millipore water was added to the 50-mL tube, which was heated for 1 h at 125 °C. In procedure 3, 50 mg of sample was placed in a 50-mL screw-cap tube and 1 mL of 72% $H_2SO_4$ (w/w) containing 524 mg of inositol/100 mL as an internal standard was added to each tube. Samples were hydrolyzed for 1 h at 25 °C. Following hydrolysis, 28 mL of Millipore water was added to the tubes, which were then heated for 3 h at 100 °C. After cooling, all hydrolysates were filtered through a Whatman GF/D glass filter (47 mm) and rinsed three times with 5 mL of Millipore water. The filtrate was neutralized as it passed through a preparatory column containing 15 g of Bio-Rad (Bio-Rad Laboratories, Richmond, CA 94804) AG 4×4 resin having a mesh size of 100–200 μm. The preparatory column was rinsed successively with 10, 60, and 60 mL of Millipore water. The effluent was collected and diluted to a final volume of 200 mL. Samples were stored at 4 °C until analyzed.

Monosaccharide composition was determined by HPLC. Forty microliters of sample previously filtered through a 0.2-μm filter was injected into a Dionex BioLC (Dionex, Sunnyvale, CA 94086) fitted with a 4 × 150 mm HPIC AS6A 5-μm ion-exchange column to separate the monosaccharides. Degassed mobile phase consisted of 0.1875 mM NaOH pumped at 0.8 mL/min for 25.5 min to elute the monosaccharides, 300 mM NaOH pumped at 0.8 mL/min for 8.0 min to clean the column, and 0.1875 mM NaOH pumped at 0.8 mL/min for 9.0 min to reequilibrate the column. A postcolumn delivery system was used to provide a constant flow (0.1 mL/min) of 400 mM NaOH to the electrochemical cell. Total run time per sample was 42.5 min. Mobile phase and column were maintained at room temperature. The Dionex Pulsed Amperometric Detector II (PAD II) equipped with an electrochemical cell containing a gold working electrode was used to detect the monosaccharides. The reference cell of the electrochemical cell was filled with 400 mM NaOH. The applied potentials E1, E2, and E3 were set at +0.1, +0.6, and −0.8 V, respectively. The pulse durations were 300, 120, and 300 ms for applied potentials E1, E2, and E3, respectively. The detector was set at a sensitivity of 3K.

**Statistical Analysis.** Data were analyzed within substrate as a completely randomized design (McClave and Dietrich, 1985) using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1982); treatment means were compared only within substrate.

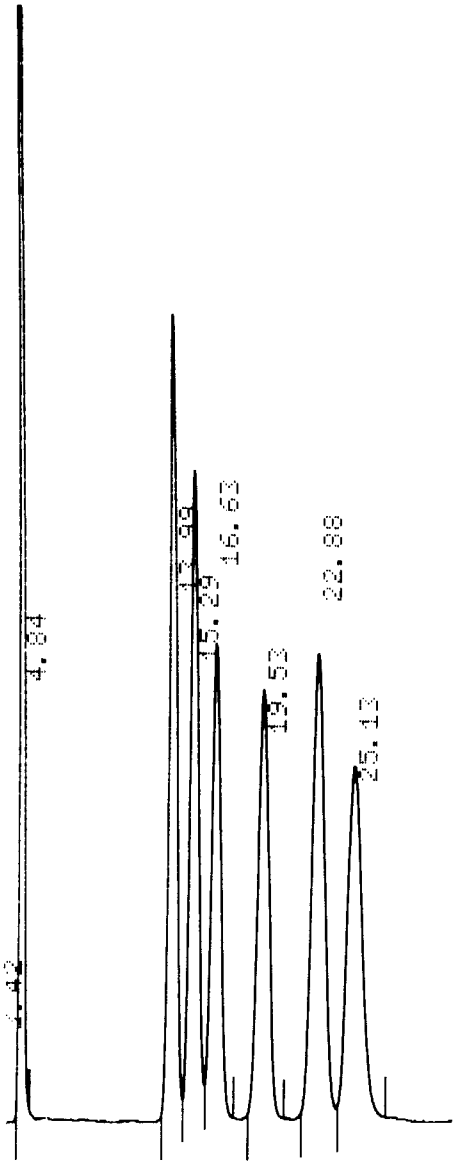


**Figure 1.** Chromatogram representing a 40-μL injection of a standard monosaccharide solution containing 25 ppm each of inositol (4.84 min), arabinose (13.99 min), rhamnose (15.29 min), galactose (16.63 min), glucose (19.53 min), xylose (22.88 min), and mannose (25.13 min). Retention times include 2.3 min for the autosampler cycle.

## RESULTS

Figure 1 presents a chromatogram of a 40-μL injection of a standard monosaccharide solution containing 25 ppm each of inositol, arabinose, rhamnose, galactose, glucose, xylose, and mannose. Figure 2 represents the neutral monosaccharide profile of an alfalfa sample after chemical hydrolysis procedure 2 (Theander and Westerland, 1986). The neutral monosaccharides detected are inositol, arabinose, rhamnose, galactose, glucose, xylose, and mannose. Peak separation was excellent, allowing all neutral sugars of interest to be identified and quantified. Detectability of monosaccharides was also excellent. Linear detectability ranges for the PAD II detector is dependent upon a variety of factors such as column type and sensitivity setting, as well as the component being detected. For the conditions and sensitivity settings outlined in Materials and Methods, all monosaccharides, excluding inositol, were in the linear response range with a sample concentration between 0.4 and 125 ppm. The internal standard, inositol, was within the linear range with a concentration between 0.4 and 50 ppm. The precision of the method of analysis was determined for 10 repeated injections into the HPLC of the same 50 ppm standard preparation. Standard deviations ranged from 0.07 ppm for galactose to 0.32 ppm for mannose. A recent review on electrochemical detection is available (Weber and Long, 1988). Monosaccharide recovery from the preparatory columns was 100% for the monosaccharides arabinose, xylose, glucose, galactose, mannose, rhamnose, and inositol. Percent recoveries of

**Table II. Neutral Monosaccharide Content (mg/g DM)[a] of Substrates Extracted Using Various Chemical Hydrolysis Procedures**

| procedure[b] | Ara | Xyl | Glc | Gal | Man | Rha | % rec[d] |
|---|---|---|---|---|---|---|---|
| | | | Alfalfa | | | | |
| 1 | 17.4[c] | 17.2[c] | 21.5[c] | 11.4[c] | 2.4[c] | 5.6[c] | 6.7 |
| 2 | 22.5[d] | 34.2[d] | 113.4[d] | 15.3[d] | 9.6[d] | 3.9[d] | 17.6 |
| 3 | 20.7[cd] | 27.2[cd] | 84.1[e] | 13.3[c] | 10.1[d] | 3.6[d] | 14.2 |
| SEM[c] | 1.0 | 3.3 | 8.6 | 0.6 | 1.2 | 0.2 | |
| | | | Apples | | | | |
| 1 | 12.1 | 6.4[c] | 172.3[c] | 7.9 | 0.0[c] | 0.6 | 17.9 |
| 2 | 13.6 | 10.0[d] | 231.8[d] | 7.9 | 2.9[c] | 3.1 | 24.2 |
| 3 | 13.8 | 9.3[cd] | 240.4[d] | 8.9 | 11.9[d] | 4.6 | 26.0 |
| SEM[c] | 0.8 | 1.0 | 15.9 | 1.6 | 1.2 | 1.4 | |
| | | | Microcrystalline Cellulose | | | | |
| 1 | 0.0 | 0.0 | 12.0[c] | 0.0 | 0.0 | 0.0 | 1.1 |
| 2 | 0.0 | 3.0 | 491.8[d] | 0.0 | 10.8 | 0.0 | 45.5 |
| 3 | 0.0 | 4.6 | 428.9[d] | 0.0 | 4.2 | 0.0 | 39.4 |
| SEM[c] | 0.0 | 3.2 | 33.6 | 0.0 | 4.4 | 0.0 | |
| | | | Wheat Bran | | | | |
| 1 | 74.5 | 110.5 | 158.4[c] | 8.9 | 5.5 | 0.0 | 31.8 |
| 2 | 91.9 | 148.9 | 250.5[d] | 10.5 | 0.0 | 0.0 | 44.7 |
| 3 | 89.6 | 131.8 | 201.7[cd] | 9.6 | 1.5 | 0.0 | 38.6 |
| SEM[c] | 6.6 | 15.5 | 26.9 | 0.9 | 3.3 | 0.0 | |
| | | | Wheat Straw | | | | |
| 1 | 23.8 | 109.5 | 25.9[c] | 6.1 | 0.0[c] | 0.0 | 14.6 |
| 2 | 23.3 | 137.7 | 244.3[d] | 6.8 | 7.2[d] | 0.0 | 37.4 |
| 3 | 23.0 | 128.7 | 165.0[e] | 5.5 | 9.4[d] | 0.0 | 29.6 |
| SEM[c] | 1.7 | 18.7 | 6.1 | 1.0 | 1.4 | 0.0 | |
| | | | Xylan | | | | |
| 1 | 0.0 | 283.2[c] | 98.6 | 0.0 | 300.5[c] | 0.0 | 60.8 |
| 2 | 0.0 | 316.8[cd] | 146.0 | 0.0 | 421.8[d] | 0.0 | 79.0 |
| 3 | 0.0 | 325.7[d] | 130.4 | 0.0 | 425.2[d] | 0.0 | 78.7 |
| SEM[c] | 0.0 | 8.6 | 11.3 | 0.0 | 25.4 | 0.0 | |

[a] Key: Ara = arabinose, Xyl = xylose, Glc = glucose, Gal = galactose, Man = mannose, Rha = rhamnose, Ino = inositol. (c–e) Means in the same column within a substrate not sharing a common superscript are different ($P < 0.05$). [b] Procedures: 1, de Ruiter and Burns (1986); 2, Theander and Westerland (1986); 3, Neilson and Marlett (1983). [c] Standard error of the mean. [d] Prior to determining percent recovery, the monosaccharides were converted to anhydro monosaccharides with the conversion factors of 0.88 and 0.90 for the pentoses and hexoses, respectively.

monosaccharides from a standard solution following hydrolysis using procedures 1–3 are presented in Table I. For procedure 1, recovery values ranged from 93.6% for galactose to 102% for inositol. Recovery values ranged from 82.3% to 101% for xylose and inositol, respectively, when procedure 2 was used to hydrolyze the standard solution. Recoveries using procedure 3 were also satisfactory, with values ranging from 91.2% for galactose to 103% for inositol.

The monosaccharide values in Table II are not corrected for the recoveries reported in Table I. It was our purpose to report differences in the assays prior to alteration of values with correction factors. Total recovery, expressed as a percentage, was calculated as milligrams of total anhydro monosaccharides detected divided by 1 g of dry matter and multiplied by 100. The conversion factors 0.88 and 0.90 for pentoses and hexoses, respectively, were used to convert the neutral sugars to polysaccharides (i.e., anhydro monosaccharides). Our results are expressed on a dry matter basis of original material; therefore, recovery values are lower than those reported by other researchers who used neutral detergent fiber (NDF) as substrate. Chemical hydrolysis of original material rather than NDF was necessary to provide a complete carbohydrate profile of the substrates.

The neutral monosaccharide composition of alfalfa is presented in Table II. Arabinose and xylose concentrations in alfalfa were higher ($P < 0.05$) when procedure 2 was used compared to procedure 1. Glucose was higher ($P < 0.05$) for procedure 2 compared to procedures 1 and 3. Glucose was also higher ($P < 0.05$) for procedure 3 compared to procedure 1. Galactose recovery was higher ($P < 0.05$) with procedure 2 compared to procedures 1 and 3. Mannose recovery was higher ($P < 0.05$) with procedures 2 and 3 compared to procedure 1. Rhamnose was higher ($P < 0.05$) for procedure 1 compared to procedures 2 and 3. Percent total recovery of monosaccharides ranged from 6.7% for procedure 1 to 17.6% for procedure 2.

No differences ($P > 0.05$) were detected among procedures 1–3 in arabinose, galactose, or rhamnose concentrations in apples (Tables II). Xylose recoveries were higher ($P < 0.05$) for procedure 2 compared to procedure 1. Glucose recoveries were higher ($P < 0.05$) with procedures 2 and 3 compared to procedure 1. Mannose recoveries were higher ($P < 0.05$) for procedure 3 compared to both procedures 1 and 2. Again, $H_2SO_4$ hydrolysis was superior to TFA hydrolysis with regard to xylose and glucose recoveries. Differences between $H_2SO_4$ procedures with regard to recoveries of the major cell wall monosaccharides were not apparent. Percent total recovery of monosaccharides ranged from 17.9% for procedure 1 to 26.0% for procedure 3.

The microcrystalline cellulose used in this study was devoid of arabinose, galactose, and rhamnose (Table II). Xylose and mannose contamination was apparent when the cellulose was hydrolyzed with $H_2SO_4$ (procedures 2 and 3). Glucose recoveries from microcrystalline cellulose were higher ($P < 0.05$) with procedures 2 and 3 compared to procedure 1. Percent total recovery of monosaccharides ranged from 1.1% for procedure 1 to 45.5% for procedure 2.

Arabinose, xylose, galactose, mannose, and rhamnose

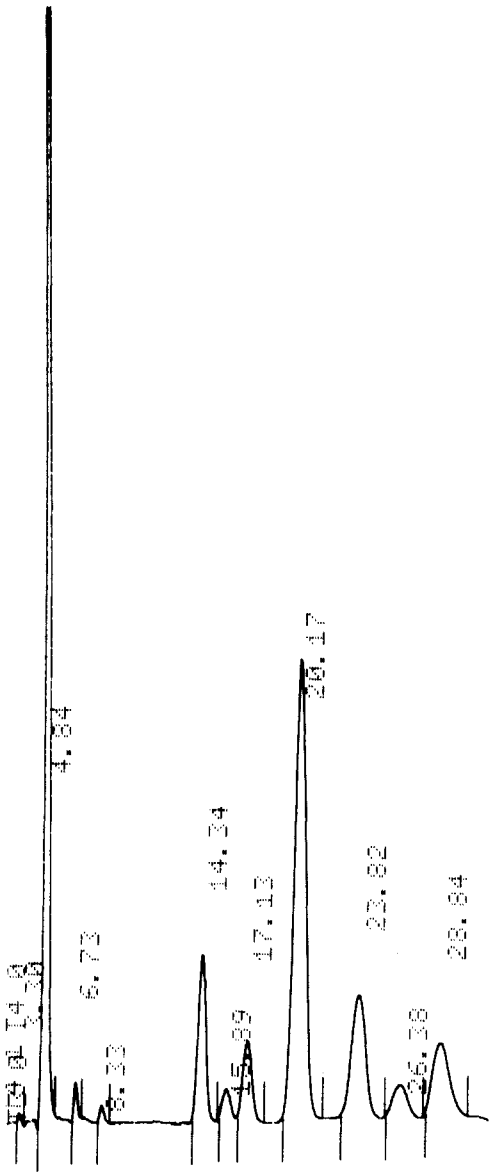


Figure 2. Neutral monosaccharide profile of source 2 alfalfa via procedure 2: inositol (4.84 min), arabinose (14.34 min), rhamnose (15.89 min), galactose (17.13 min), glucose (20.17 min), xylose (23.82 min), mannose (26.38 min).

concentrations in wheat bran were not different ($P > 0.05$) among the different procedures. Glucose concentrations were higher ($P < 0.05$) with procedure 2 compared to procedure 1. While the differences were not statistically significant, arabinose and xylose concentrations obtained by $H_2SO_4$ extraction (procedures 2 and 3) tended to be higher than concentrations obtained with TFA (procedure 1). Also, xylose and glucose values tended to be higher for procedure 2 compared to procedure 3. Percent total recovery of monosaccharides ranged from 31.8% for procedure 1 to 44.7% for procedure 2.

Rhamnose was not detected following extraction of wheat straw, regardless of procedure. Arabinose, xylose, and galactose concentrations were not different ($P > 0.05$) among procedures. However, there was a tendency for higher recoveries of xylose when $H_2SO_4$ vs TFA was used. Glucose concentrations were higher ($P < 0.05$) with procedure 2 compared to either procedures 1 or 3. Glucose concentrations were also higher ($P < 0.05$) with procedure 3 vs procedure 1. Mannose concentrations were higher ($P < 0.05$) with procedures 2 and 3 compared to procedure 1. Percent total recovery of monosaccharides ranged from 14.6% for procedure 1 to 37.4% for procedure 2.

Arabinose, galactose, and rhamnose were not detected in xylan, regardless of extraction procedure. More ($P < 0.05$) xylose was detected by procedure 3 than procedure 1. Also, more ($P < 0.05$) mannose was detected with procedures 2 and 3 compared to procedure 1. Although results were not significant, glucose concentrations tended to be higher when $H_2SO_4$ was used (procedures 2 and 3) compared to TFA (procedure 1). Percent total recovery ranged from 60.8% for procedure 1 to 79.0% for procedure 2.

Table III presents the neutral monosaccharide composition of different sources of substrate as determined by the three chemical hydrolysis procedures. Variation among substrate source is evident throughout the data set. For example, the glucose content of the various sources of alfalfa is different. Regardless of procedure used, source 2 alfalfa had the greatest amount of glucose. Arabinose and xylose content also is higher in source 2 alfalfa, independent of procedure.

Monosaccharide composition of apple sources also was variable. Arabinose and xylose contents were highest in source 1 when procedure 1 was used and highest in source 3 when procedures 2 and 3 were used. Glucose content was highest in source 1 when procedures 1 and 3 were used and highest in source 2 when procedure 2 was used.

Within procedure 1, glucose content of source 2 microcrystalline cellulose was greatest. Source 1 had the greatest amount of glucose when procedures 2 and 3 were used. Source 2 wheat bran consistently had the highest amount of arabinose and xylose compared to sources 1 and 3, regardless of procedure used to extract the neutral sugars. Glucose content of source 3 wheat bran was greatest, regardless of procedure used.

Within wheat straw sources, arabinose content was highest for source 3, regardless of procedure. Xylose content was greatest for source 3 when procedures 1 and 2 were used and greatest for source 2 when procedure 3 was used. Glucose content was greatest for source 3 when procedures 1 and 2 were used and greatest for source 1 when procedure 3 was used.

For xylan, xylose content was greatest in source 2 when procedures 1 and 2 were used and source 1 when procedure 3 was used. Glucose and mannose concentrations were highest in source 1 when procedures 1 and 3 were used and highest in source 2 when procedure 2 was used.

## DISCUSSION

A wide variety of substrates was chosen for this study. Wheat straw and alfalfa represent a monocot and dicot, respectively, and are used primarily for ruminant animal feeding. Apples and wheat bran, however, are substrates of interest in human nutrition as both are used as sources of dietary fiber. Microcrystalline cellulose and xylan are chemically isolated fiber fractions and represent the major types of structural carbohydrate (i.e., cellulose and hemicelluloses) present in most fibrous substrates. The study of these substrates provides information regarding the hydrolytic procedure most capable of providing an accurate estimation of the neutral monosaccharide profile of diverse fiber sources.

Recoveries from neutral monosaccharide standards using procedure 1 (Table I) are higher than those reported previously (de Ruiter and Burns, 1986). Values are similar to those of Barton et al. (1982) who used a single-stage hydrolysis with 2 N TFA. Similar standard recovery values were reported by Neilson and Marlett (1983) when using procedure 3. Sloneker (1971), using a procedure similar to our procedure 2, reported a xylose recovery of 80%. The presence of pyranosyl or furanosyl TFA esters formed in

**Table III. Neutral Monosaccharide Composition (mg/g DM)[a] of Different Substrate Sources As Determined Using Various Chemical Hydrolysis Procedures**

| procedure[b] | source | Ara | Xyl | Glc | Gal | Man | Rha |
|---|---|---|---|---|---|---|---|
| | | | Alfalfa | | | | |
| 1 | 1 | 16.2 | 14.9 | 13.9 | 10.7 | 0.0 | 5.5 |
| | 2 | 19.2 | 22.2 | 26.2 | 13.0 | 3.1 | 6.0 |
| | 3 | 16.7 | 14.5 | 24.3 | 10.5 | 4.1 | 5.3 |
| 2 | 1 | 20.4 | 29.6 | 95.7 | 14.4 | 6.7 | 3.8 |
| | 2 | 24.1 | 41.0 | 130.8 | 16.1 | 11.6 | 3.7 |
| | 3 | 22.9 | 31.9 | 113.8 | 15.5 | 10.4 | 4.1 |
| 3 | 1 | 18.8 | 21.9 | 65.1 | 12.6 | 8.5 | 3.9 |
| | 2 | 21.9 | 34.2 | 99.7 | 13.5 | 11.6 | 3.4 |
| | 3 | 21.4 | 25.5 | 87.4 | 13.7 | 10.2 | 3.4 |
| | | | Apples | | | | |
| 1 | 1 | 14.6 | 7.0 | 205.2 | 5.3 | 0.0 | 0.0 |
| | 2 | 11.5 | 5.8 | 170.9 | 9.2 | 0.0 | 0.0 |
| | 3 | 10.2 | 6.3 | 140.8 | 9.2 | 0.0 | 1.7 |
| 2 | 1 | 13.9 | 8.3 | 241.5 | 4.8 | 2.4 | 4.2 |
| | 2 | 12.6 | 10.1 | 252.9 | 9.8 | 6.2 | 1.9 |
| | 3 | 14.4 | 11.6 | 201.0 | 9.2 | 0.0 | 3.1 |
| 3 | 1 | 14.0 | 7.3 | 265.4 | 5.2 | 13.9 | 1.9 |
| | 2 | 13.0 | 8.9 | 222.4 | 10.1 | 10.0 | 8.9 |
| | 3 | 14.3 | 11.8 | 233.4 | 11.5 | 11.8 | 2.9 |
| | | | Microcrystalline Cellulose | | | | |
| 1 | 1 | 0.0 | 0.0 | 5.5 | 0.0 | 0.0 | 0.0 |
| | 2 | 0.0 | 0.0 | 17.8 | 0.0 | 0.0 | 0.0 |
| | 3 | 0.0 | 0.0 | 12.8 | 0.0 | 0.0 | 0.0 |
| 2 | 1 | 0.0 | 9.0 | 555.6 | 0.0 | 21.7 | 0.0 |
| | 2 | 0.0 | 0.0 | 403.1 | 0.0 | 0.0 | 0.0 |
| | 3 | 0.0 | 0.0 | 516.8 | 0.0 | 10.8 | 0.0 |
| 3 | 1 | 0.0 | 13.9 | 468.0 | 0.0 | 12.7 | 0.0 |
| | 2 | 0.0 | 0.0 | 461.5 | 0.0 | 0.0 | 0.0 |
| | 3 | 0.0 | 0.0 | 357.3 | 0.0 | 0.0 | 0.0 |
| | | | Wheat Bran | | | | |
| 1 | 1 | 62.2 | 65.0 | 89.6 | 6.0 | 16.4 | 0.0 |
| | 2 | 89.4 | 143.6 | 161.2 | 10.3 | 0.0 | 0.0 |
| | 3 | 71.8 | 122.9 | 224.4 | 10.5 | 0.0 | 0.0 |
| 2 | 1 | 93.1 | 151.4 | 241.2 | 10.7 | 0.0 | 0.0 |
| | 2 | 100.6 | 156.8 | 233.6 | 10.2 | 0.0 | 0.0 |
| | 3 | 81.9 | 138.6 | 276.7 | 10.6 | 0.0 | 0.0 |
| 3 | 1 | 87.1 | 114.6 | 161.4 | 8.6 | 4.6 | 0.0 |
| | 2 | 101.4 | 154.7 | 205.6 | 10.3 | 0.0 | 0.0 |
| | 3 | 80.2 | 126.1 | 238.1 | 9.8 | 0.0 | 0.0 |
| | | | Wheat Straw | | | | |
| 1 | 1 | 19.8 | 61.5 | 23.1 | 5.0 | 0.0 | 0.0 |
| | 2 | 23.0 | 98.1 | 17.9 | 4.3 | 0.0 | 0.0 |
| | 3 | 28.5 | 168.9 | 36.8 | 9.0 | 0.0 | 0.0 |
| 2 | 1 | 23.2 | 127.4 | 249.5 | 7.3 | 8.7 | 0.0 |
| | 2 | 21.5 | 137.3 | 231.1 | 5.6 | 8.1 | 0.0 |
| | 3 | 25.2 | 148.4 | 252.4 | 7.4 | 4.7 | 0.0 |
| 3 | 1 | 22.6 | 119.8 | 171.7 | 6.8 | 7.6 | 0.0 |
| | 2 | 21.0 | 136.0 | 170.3 | 4.5 | 7.3 | 0.0 |
| | 3 | 25.5 | 130.3 | 152.9 | 5.2 | 13.4 | 0.0 |
| | | | Xylan | | | | |
| 1 | 1 | 0.0 | 269.1 | 116.0 | 0.0 | 343.6 | 0.0 |
| | 2 | 0.0 | 297.3 | 81.2 | 0.0 | 257.3 | 0.0 |
| 2 | 1 | 0.0 | 312.4 | 138.0 | 0.0 | 414.6 | 0.0 |
| | 2 | 0.0 | 321.2 | 154.0 | 0.0 | 428.9 | 0.0 |
| 3 | 1 | 0.0 | 327.3 | 134.2 | 0.0 | 429.4 | 0.0 |
| | 2 | 0.0 | 324.0 | 126.5 | 0.0 | 421.0 | 0.0 |

[a] Key: Ara = arabinose, Xyl = xylose, Glc = glucose, Gal = galactose, Man = mannose, Rha = rhamnose, Ino = inositol. [b] Procedures: 1, de Ruiter and Burns (1986); 2, Theander and Westerland (1986); 3, Neilson and Marlett (1983).

the primary hydrolysis step with 2 N TFA (de Ruiter and Burns, 1986) or the sulfate esters formed in the primary hydrolysis step with 72% $H_2SO_4$ (Theander and Westerland, 1986) may account for some of the loss of monosaccharides. The secondary hydrolysis step in both procedures cleaves the esters formed in the primary step and regenerates the free monosaccharide; however, hydrolysis may be incomplete. Dehydration to furfurals and (hydroxymethyl)furfural products when $H_2SO_4$ was employed could also explain recoveries less than 100%. Talmadge et al. (1973) noted that the glycosidic linkage of cellulose was not hydrolyzed using 2 N TFA for 1 h at 121 °C. This could account for the lower recovery of glucose with procedure 1 compared to procedures 2 and 3 noted with alfalfa, apples, microcrystalline cellulose, wheat bran, and wheat straw. The significantly greater recoveries of the hemicellulosic monosaccharides, xylose and arabinose, for substrates such as alfalfa, apples, and xylan further suggest that $H_2SO_4$ is the superior hydrolytic agent. The recovery of arabinose or xylose following chemical hydrolysis never

was significantly greater using procedure 1 compared to procedures 2 or 3 for any substrate. Overall, $H_2SO_4$ hydrolysis resulted in greater recovery of the major monosaccharides, arabinose, xylose and glucose, found in plant material.

In certain instances, differences between procedures 2 and 3 were noted. With regard to alfalfa and wheat straw, more glucose was recovered following hydrolysis with procedure 2 compared to procedure 3. According to Table I, glucose recovery is not different between these procedures. Therefore, differences in glucose recovery from these substrates are due to the ability of the chemical to free glucose from the cellulose. The procedures differ significantly with regard to the second-stage hydrolysis step. As noted previously, the secondary hydrolysis step cleaves the esters formed in the primary step and regenerates the free monosaccharides. Apparently temperature, rather than duration of hydrolysis, plays a greater role in monosaccharide recovery in the case of wheat straw and alfalfa.

In examining the data, it might be concluded that total recovery of the neutral monosaccharides from any substrate was not achieved, regardless of the chemical hydrolysis procedure used. The greatest percent total recovery was obtained with xylan. Also, recovery values were highest for the procedures utilizing $H_2SO_4$ as the hydrolytic agent. Considering the data obtained for microcrystalline cellulose, the lack of complete recovery for all assays may be due primarily to the inability to completely hydrolyze cellulose. Sloneker (1971), using a procedure similar to our procedure 2, reported glucose recoveries from the microcrystalline cellulose Avicel of approximately 98%. Although Avicel was not used in our experiment, we believe that none of the procedures studied possess the ability to completely hydrolyze microcrystalline cellulose to glucose and that the values reported based on three cellulose sources substantiate this.

Examples of differences among sources within a substrate and procedure are noted in Table III. Several sources of a given substrate (sample population) must be analyzed if valid statistical comparisons among procedures are to be made. Analysis of only one sample from a population, a practice followed by many researchers, may lead to incorrect conclusions regarding a particular comparison (i.e., superiority of a given hydrolytic procedure over another). Wheat bran provides an excellent example of a substrate whose composition differs depending on the source. This variation may arise from wheat processors using different extraction procedures to isolate wheat bran. The inability to detect significant differences among procedures (Table II) is due to the large variation in composition of the three wheat brans (Table III). Barton et al. (1982) also noted significant differences among three sources of coastal Bermuda grass. Differences among sources within substrate would be a more serious problem for certain fibers than for others.

The HPLC procedure outlined is superior to other HPLC techniques previously reported. Anion-exchange chromatography allows the separation of all neutral monosaccharides of interest. Other researchers utilizing HPLC have been unable to attain this separation. For example, Barton et al. (1982) were unable to completely dissociate the glucose and galactose peaks. Neilson and Marlett (1983) encountered similar difficulties with regard to arabinose and mannose. Also, the sensitivity of HPLC with refractive index detection is much poorer than that of PAD II detection reported here. Samples and standards containing the neutral monosaccharide in concentrations of 1000–10 000 $\mu$g/mL are detected by refractive index (Slavin and Marlett, 1983). Windham et al. (1983) used neutral sugar working standards in the range of 10 000–20 000 $\mu$g/mL, and a concentration of 10 000 $\mu$g/mL was required for optimal resolution of sugar standards and was used as an external standard to determine the concentration of sugars in the hydrolysate. Samples and standards at concentrations less than 125 $\mu$g/mL are within the linear range of the PAD II detector using our procedure.

Our HPLC technique is also superior to GLC techniques reported in the literature. The ability of each to separate and detect neutral sugars is comparable. However, sample preparation is much less tedious for HPLC compared to GLC. The GLC methodology requires that the neutral monosaccharides be converted to alditol acetates prior to separation and detection (Albersheim et al., 1967; de Ruiter and Burns, 1987). Also, incomplete reduction or acetylation of all or certain neutral sugars may occur, which would result in poor recoveries.

In conclusion, anion-exchange HPLC with pulsed amperometric detection is a viable alternative to GLC for quantification of neutral monosaccharides. This method provides superior separation and detection of neutral monosaccharides compared to previous HPLC techniques (Slavin and Marlett, 1983; Windham et al., 1983). Destruction of monosaccharides was less apparent when TFA was used compared to $H_2SO_4$; however, recoveries within all assays were acceptable. Losses can be corrected mathematically provided monosaccharide recoveries are determined simultaneously to sample analysis. Within all substrates, differences ($P < 0.05$) in monosaccharide composition were detected when different assays were used. On the basis of the data presented, it appears that the procedures utilizing $H_2SO_4$ as the hydrolytic agent are superior to those utilizing TFA. Also, in certain instances, temperature (procedure 2) rather than duration of hydrolysis (procedure 3) resulted in greater recoveries of the monosaccharides when $H_2SO_4$ was used as the hydrolytic agent. It is also important to note that while TFA has been recommended as a hydrolytic agent for hemicelluloses (de Ruiter and Burns, 1986; Olson et al., 1988), in several instances, xylose and arabinose recoveries were less than those obtained with $H_2SO_4$ hydrolysis. Also, it should be emphasized that hydrolytic procedure, not HPLC technology, is the major limitation in neutral sugar analysis. Better hydrolytic methods are needed that might further advance the science of carbohydrate chemistry and nutrition.

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# Fractionation and Identification of Some Low Molecular Weight Grape Seed Phenolics

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Grape seeds were extracted in order to isolate low molecular weight phenolics. These were fractionated by analytical and semipreparative HPLC, Sephadex LH-20 chromatography, and analytical thin-layer chromatography. Ten compounds were separated and identified: gallic acid, (+)-catechin, (−)-epicatechin, dimer procyanidins B1, B2, B3, and B4, procyanidin C1 (trimer), procyanidin B2 gallate, (−)-epicatechin gallate.

The high phenolic content of grape seed is of critical interest as these compounds form a large proportion of wine tannins (Singleton and Draper, 1964; Glories, 1978; Singleton, 1980; Oszmianski et al., 1986). They are involved in quality aspects as they affect the color and the flavor of wines (Rossi and Singleton, 1966; Glories, 1978, 1982) and some may be involved in oxidative browning of grapes (Romeyer, 1984; Oszmianski et al., 1985). Although it is known that grape seed is rich in condensed tannins (Glories, 1978), the consistent role of lower molecular weight phenolics, which are also present in grape seeds, in some wine characteristics has been demonstrated recently (Glories, 1982, 1986; Dournel, 1985). As a consequence, it was of interest to improve knowledge of this latter part of grape seed tannins. This is the purpose of the present work.

## MATERIALS AND METHODS

**Plant Material.** Determinations were carried out in seeds from grapes cv. Carignane, picked during 1986 vintage in the INRA vineyards at the Chapitre Experimental Station near Montpellier (France), after veraison (ca. 15° Brix). This stage of the evolution of the fruit was chosen as the initial point of the study, which will be extended to the whole maturation period, as the phenolics level in grape seeds from unripe fruit is known to be higher than in fully ripe grapes (Su and Singleton, 1969; Czochanska et al., 1979; Singleton, 1980; Romeyer et al., 1986). Seeds were removed from the grapes, immediately frozen in liquid nitrogen, and crushed under liquid nitrogen in a ball grinder. A very fine powder was obtained and used immediately for the extraction of phenolic compounds.

**Preparation of the Phenolic Extract.** A 30-g portion of grape seed powder were extracted twice by stirring for 15 min at +4 °C with 100 mL of 70% acetone in water in the presence of 2000 ppm $SO_2$ to avoid oxidation. The mixture was filtered on sintered glass, and the filtrate was treated with chloroform (100 mL) in order to eliminate acetone and compounds other than phenolics. The aqueous phase containing phenolics was recovered; rinsing water (30 mL) of chloroformic phase was added (Jerumanis, 1985). Crystallized NaCl was then added to saturation point in order to precipitate the more polymerized tannins, thus enabling further fractionation of low molecular weight phenolics (Michaud et al., 1971). The preparation was filtered on Buchner filter and the filtrate extracted with ethyl acetate (3 × 90 mL). The EtOAc was concentrated under vacuum to approximately 20 mL, and 100 mL of chloroform was added to precipitate oligomer tannins. A filtration was made, the filtrate was discarded, and the precipitate was recovered to be redissolved in 96% EtOH. Referred to as "grape seed phenolic extract", it was subsequently fractionated by different chromatographic patterns.

**Fractionation of Phenolic Compounds.** *Fractionation of Acidic and Neutral Phenolic Compounds.* This was carried out according to Salagoity-Auguste and Bertrand (1984).

*Semipreparative Fractionation. Gel Chromatography.* Fractionation of compounds located in the grape seed phenolic

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