# Carbohydrate Composition of Alfalfa Cell Walls Isolated from Stem Sections Differing in Maturity

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As plants mature, changes occur in the allocation of biomass between cell solubles and cell walls. Generally a larger proportion of the biomass accumulates in the cell-wall fraction. This study was undertaken to determine the cell-wall carbohydrate composition of alfalfa (*Medicago sativa* L.) stem sections of differing maturity. Stems were harvested from greenhouse-grown plants at bud stage and subdivided into apical, upper, and lower nodes and internodes. Cell walls isolated from each stem region were fractionated into pectic polysaccharides, alkaline-soluble polysaccharides, and cellulose. As expected, the proportion of pectic material in the cell wall decreased from apical to lower node regions. The extent of this decrease, approximately 50% in the lower regions, reflects the change in patterns of polysaccharide synthesis. Sugar composition of the pectic fractions did not change except in the lower regions, which contained higher proportions of xylose. For each stem region, 33% of the total pectic material was recalcitrant to calcium ion chelator extraction. This material is held in the cell-wall matrix by covalent linkages, some of which were alkaline labile. However, the majority of the recalcitrant pectic material was not solubilized until delignification of the wall. The ratio of alkaline-soluble polysaccharides to cellulose did not change with maturity of the stem section. The composition of the alkaline-soluble fractions changed with respect to stem regions. Apical nodes and internodes contained higher proportions of arabinose, galactose, and rhamnose residues. There was also a trend toward increased uronosyl residues in the lower stem regions within the alkaline-soluble fractions.

## INTRODUCTION

The stem of alfalfa plants represents a significant proportion of the total accumulated biomass even at early vegetative stages. As plants mature, the proportion of biomass in the stem fraction increases to 60–80%, depending upon the physiological stage of development and upon environmental conditions (Hirst et al., 1959). A large portion of the biomass results from accumulation of structural polysaccharides in the stem cell wall. These structural polysaccharides are a potential energy source for ruminants; nevertheless, their utilization is incomplete. Moreover, as maturity advances, utilization generally declines (Harkin, 1973; Morrison, 1979; Buxton et al., 1985). It is important to identify the chemical changes in cell walls (CW) that might be the underlying determinants for this change in digestibility.

The CW matrix of dicots such as alfalfa is composed of pectic polysaccharides (rhamnogalacturonans, arabinogalactans, arabinans), other noncellulosic polysaccharides (heteroxylans, mannans, heteroglucans), cellulose, lignin, and small amounts of protein (Bacic et al., 1988; Hatfield, 1989; Selvendran, 1983). As walls develop, synthesis of pectic polysaccharides decreases, while synthesis of cellulose and some noncellulosic polysaccharides increases (Northcote, 1969). There also may be changes in the substitution pattern of specific classes of polysaccharides as the functions of walls change. These metabolic changes affect characteristics of the total matrix. For example, walls generally advance from a more plastic, highly hydrated matrix to one that is more rigid and less hydrated (Selvendran, 1983). Alterations in the CW matrix, whether in composition, substitution pattern of the polysaccharides, or interactions among the components, may influence the efficient utilization of cell walls by ruminants.

Because of the large accumulation of biomass in alfalfa stems and the decline in digestibility, the present study focuses on alfalfa stem tissue. Alfalfa stem growth occurs from apical meristems, resulting in stem internodes that vary in maturity. The objective of this study was to determine the variation in stem carbohydrate composition from apical regions to stem bases. Stem sections were fractionated into cell-wall polysaccharide groups, and their composition was determined.

## MATERIALS AND METHODS

Alfalfa (*Medicago sativa*) plants were grown in a greenhouse under high-pressure sodium lamps using a 14/10 h day/night light regime. Plants were harvested 7.5 cm above the soil line at bud stage, freeze-dried, and separated into leaves with petioles attached and stems. To determine the compositional changes in cell-wall carbohydrate, pooled stems were subdivided into regions reflecting maturity. These included the lower seven or eight nodes and internodes (LN), the most mature regions, apical 2–3 cm of main stems and branches (AN), which was still undergoing some elongation and being the most immature region, and seven or eight nodes and internodes (UN) below the apical region which had recently stopped elongation, which should have an intermediate maturity. All extractions were from three sets of materials harvested on different dates except for the AN regions, which were from only two harvest dates.

**Cell-Wall Isolation.** Stem material from each maturity group was cut into pieces 5–10 mm long, and subsamples were removed for cell-wall isolation. The stem material was homogenized in cold (5 °C) phosphate buffer (10 mM $NaH_2PO_4$ plus 50 mM NaCl, pH 7.0, 50 mL/g of dry tissue) with a Waring blender. Two drops of octanol was added to reduce foaming. Each sample was blended for 2 min using 10-s bursts interspersed with 10-s rests. The homogenate was allowed to stand for 5 min and then blended for another 2 min as before. Cell-wall material was collected on two Teflon mesh filters (52 μm, Spectrum) as the homogenate was poured through a Fleaker filter apparatus (Spectrum). Insoluble residue was washed with cold 50 mM NaCl (2000 mL, 5 °C), to remove cytoplasmic contaminates, followed by acetone (−20 °C) until all of the residual pigment was removed. Chloroform/methanol (2:1, 500 mL) was added and allowed to drain slowly from the funnel. After the final wash, air was pulled through the CW until dry.

The CW material was transferred to a jar mill with 80% ethanol and brought to a final volume of 100 mL. Each sample was milled until the particle size was approximately 500 μm (4–5 h) and collected on a glass fiber filter with vacuum filtration. Milled CW was washed with 80% ethanol followed by acetone, air-dried, and transferred to a 250-mL centrifuge bottle. Each milled-CW sample was suspended in 50 mL of phosphate buffer containing sodium azide (10 mM $KH_2PO_4$ plus 0.02% $NaN_3$, pH 7.0) and heated in a boiling water bath for 1 h. Flasks were cooled to 60–65 °C, 10 IU of α-amylase (Sigma A-3403) was added, and then the flasks were placed in a waterbath at 55 °C for 1.0 h. After the initial incubation, pH was adjusted to 4.75, 10 units of amyloglucosidase (Sigma A-3514) was added, and the flasks were returned to the waterbath for an additional 1.5 h. CW material was pelleted at 2500*g* for 10 min following amyloglucosidase treatment. The supernatant was decanted into a filtration flask fitted with a glass fiber filter (GF 4, Fisher). Thirty milliliters of deionized water ($H_2O$) was added to each bottle and mixed thoroughly and the CW pelletized as before. After four wash cycles, the CW was transferred to a freeze-drying flask and lyophilized. The amylase extract and washes were combined, dialyzed against $H_2O$ (8–10 L) for 48 h, frozen, and lyophilized. This extract, primarily pectin, was treated as a hot water extract (HW-1) of the CW.

**Cell-Wall Fractionation.** Starch-free cell walls were subjected to sequential extraction to remove $Ca^{2+}$ bound pectic polysaccharides, lignin, and hemicellulosic material. The extraction sequence consisted of eight steps: (1) first pectin extract (PE-1), 0.5% ammonium oxalate, $(NH_4)_2C_2O_4$; (2) second pectin extract (PE-2), a repeat of step one; (3) lignin extract (LE-1), delignification; (4) third pectin extract (PE-3), 0.5% $(NH_4)_2C_2O_4$; (5) first potassium hydroxide extract (HE-1), 0.25 M KOH; (6) second KOH extract (HE-2), 0.50 M KOH; (7) third KOH extract (HE-3), 1.0 M KOH; (8) fourth KOH extract (HE-4), 4.0 M KOH. The remaining residue was primarily cellulose.

*$(NH_4)_2C_2O_4$ Extractions PE-1 and PE-2.* One gram of CW suspended in 150 mL of 0.5% $(NH_4)_2C_2O_4$, pH 3.5, was continuously stirred in a water bath (70–80 °C) for 1 h. After heating, the CW residue was pelleted by centrifugation and the supernatant (PE-1) decanted through a glass fiber filter (GF 4, Fisher). The CW residue was washed once with 0.5% $(NH_4)_2C_2O_4$ (75 mL) before being resuspended in 150 mL of 0.5% $(NH_4)_2C_2O_4$ and heated for an additional 1 h (70–80 °C). The original extract (PE-1) and the wash were combined and dialyzed against $H_2O$ for 48 h. The second extract (PE-2) was treated in the same manner except that the CW residue was washed three times with cold $H_2O$. The combined extract (PE-2) and washes (400 mL) were reduced by ultrafiltration (Amicon, PM-10 membrane) to a volume of 75 mL and dialyzed against $H_2O$. The filtrate was tested for total sugars and total uronics before being discarded.

*Delignification.* Partially depectinated CW residues were transferred to 125-mL flasks with 40 mL of $H_2O$ and delignifed using the procedure of Wise et al. (1946). Briefly, sodium chlorite (1.25 g of $NaClO_2$) and acetic acid (150 μL of 18 M $CH_3COOH$) were added to each flask and stirred until dissolved. A 25-mL Erlenmeyer flask was inverted into the top of each flask, and flasks were heated in a hot water bath (70 °C) in a fume hood for 1 h. Additional $NaClO_2$ (0.4 g) and $CH_3COOH$ (150 μL) were added to each flask, mixed thoroughly, and incubated for another 1 h. Total samples were transferred to 250-mL centrifuge bottles, and the CW was pelleted by centrifugation (2500*g*, 15 min); supernatants were then decanted through a glass fiber filter. The CW residues were resuspended in 30 mL of $H_2O$ and recentrifuged. This wash was repeated four times, and the total was combined with the original extract, dialyzed (48 h, $H_2O$), and lyophilized.

*Extract PE-3.* Delignified CW material was extracted with $(NH_4)_2C_2O_4$ and washed as described for the second pectin extract. The washed CW residues were shell frozen and lyophilized. Initial experiments were conducted with $(NH_4)_2C_2O_4$ solutions; however, subsequent experiments with $H_2O$ gave similar results.

*KOH Extraction.* Cell wall residues were further fractionated using a modification of the gradient extraction procedure of Carpita (1984). The depectinated and delignified residues were extracted in four steps with increasing concentrations of KOH (0.25, 0.5, 1.0, 2.0 M). A sample of CW (500 mg) was placed in a 250-mL centrifuge bottle along with a magnetic stir bar and 100 mL of 0.25 M KOH containing 3 mg/mL $NaBH_4$. The bottle was flushed with argon gas, capped, and placed on a magnetic stirrer for 1 h at room temperature. At the end of the hour the stir bars were removed and rinsed with a small volume of $H_2O$ and the bottles recapped and centrifuged at 2500*g* for 15 min to pellet the CW residue. The supernatant was decanted through a glass fiber filter (GF 4). Small amounts of CW residue collected on the filter were rinsed back into the bottle with the next concentration of KOH. Stir bars were placed in the bottles, which were gassed with argon, capped, and stirred for an additional hour. The filtration and extraction steps were repeated for the remaining KOH solutions (0.5, 1, and 4 M containing $NaBH_4$).

After the 4 M KOH extraction, the CW residue was washed four times with 50 mL of $H_2O$ and the washes were combined with the 4 M KOH extract. The washed CW residue was subsequently shell frozen and lyophilized. Each extract was dialyzed against approximately 15 L of $H_2O$ for 48 h. At the completion of dialysis, the total volume was measured and samples were removed for total sugar, total uronics, and individual neutral sugar analysis.

*Additional Extractions.* Additional CW material was collected from lower internodes of stems and partially depectinated as described above (PE-1 and PE-2). Subsamples (250–500 mg) were subjected to additional extraction procedures before delignification in an attempt to better understand potential interactions between pectic polysaccharides and remaining cell-wall material. Treatments included extraction with sodium carbonate ($Na_2CO_3$) and sodium methoxide ($CH_3ONa$). For the $Na_2CO_3$ extract CW residues were stirred in a 0.1 M $Na_2CO_3$ solution (60 mL) at 5 °C for 16 h. Extracted residues were pelleted by centrifuging for 15 min (2500*g*), and the supernatant was decanted through a glass fiber filter. Sodium methoxide treatment consisted of suspending fresh samples of partially depectinated CW residues in 0.25 M $CH_3ONa$ in anhydrous methanol (25 mL) and stirring at room temperature for 20 h. Insoluble material was pelleted by centrifugation and methanol solution decanted. Treated walls were briefly washed with cold $H_2O$ to remove residual methanol/$CH_3ONa$ before resuspension in $H_2O$ (60 mL) and stirring in a waterbath (70–80 °C) for 1 h. Wall residues were pelleted by centrifuging, and the supernatant was collected. After these additional treatments, all CW residues were subjected to a $(NH_4)_2C_2O_4$ extraction, delignification with $NaClO_2$, and subsequent hot $H_2O$ extraction as described above.

**General Methods.** Isolated polysaccharides were hydrolyzed with 2 M TFA at 120 °C for 1.5 h. The TFA was removed by evaporation under a stream of dry filtered air. Monosaccharides were analyzed by GLC as their alditol acetates using the procedure of Blakeney et al. (1983). Total sugar was determined according to the phenol/sulfuric acid method (Dubois et al., 1956) using glucose, arabinose, and xylose as standards. Total uronics were determined using the 3-phenylphenol method (Blumenkrantz and Asboe-Hansen, 1973) with galacturonic and glucuronic acids as standards; corrections were made for interference from hexoses in the samples.

Neutral sugar composition of CW residues after extraction was determined using a modified Saeman hydrolysis (Saeman et al., 1963). Ten milligrams of CW residue was solubilized in 0.2 mL of 72% $H_2SO_4$ followed by dilution to 1.6 M $H_2SO_4$ and hydrolysis at 100 °C for 5 h. Two milligrams of inositol was added with mixing to each sample as an internal standard. Samples were cooled and filtered through glass fiber filters before neutralization with solid $BaCO_3$. The $BaSO_4$ precipitate was removed by centrifugation and filtration. Subsamples of hydrolysates (2.5 mL) were evaporated to dryness and analyzed according to the methods used for TFA hydrolysis.

Uronosyl residues were reduced to the corresponding neutral sugar using the method of Taylor and Conrad (1972) as modified by Anderson and Stone (1985). For these experiments $NaBH_4$ was used in place of the radioactive sodium borotritide. Neutral sugar composition of the CW extracts was determined before and after reduction using the method described above.

## RESULTS AND DISCUSSION

The cell-wall content of alfalfa stems varied considerably with the developmental age of the stem region. Cell-wall

**Table I. Pectin and Pectic Fractions from Alfalfa Stems**

| | stem segment | | | | | |
|---|---|---|---|---|---|---|
| | AN | | UN | | LN | |
| cell-wall fraction[a] | g/g CW[b] | % uronosyl residues[c] | g/g CW | % uronosyl residues | g/g CW | % uronosyl residues |
| HW-1 | 0.0665 (0.0111)[d] | 51.51 (1.70) | 0.0524 (0.0007) | 50.63 (5.07) | 0.0391 (0.0030) | 52.24 (1.06) |
| PE-1 | 0.0623 (0.0064) | 58.63 (6.02) | 0.0489 (0.0027) | 56.36 (6.92) | 0.0326 (0.0013) | 54.35 (8.59) |
| PE-2 | 0.0267 (0.0052) | 44.40 (4.12) | 0.0092 (0.0057) | 46.13 (7.85) | 0.0057 (0.0020) | 42.41 (5.18) |
| LE-1 | 0.0444 (0.0007) | 41.18 (0.53) | 0.0406 (0.0052) | 42.40 (3.80) | 0.0312 (0.0030) | 40.70 (2.58) |
| PE-3 | 0.0266 (0.0031) | 48.73 (1.59) | 0.0151 (0.0054) | 48.10 (2.91) | 0.0085 (0.0023) | 51.10 (4.50) |
| total | 0.2265 | | 0.1662 | | 0.1171 | |

[a] Cell-wall fractions include HW-1 (hot water extract), PE-1 (first pectic extract), PE-2 (second pectic extract), LE-1 (delignification extract), and PE-3 (third pectic extract). [b] Total carbohydrate (neutral sugars plus uronosyl residues) solubilized by hot water, $(NH_4)_2C_2O_4$, and sodium chlorite treatment of alfalfa stem cell walls (CW). [c] Uronosyl residues are expressed as a percent of the total carbohydrate in the extract. [d] Standard error.

recoveries from stem regions were 478.0 (±10.0), 607.0 (±37.8), and 680.0 (±18.5) g $kg^{-1}$ from AN, UN, and LN, respectively. Isolated cell walls were fractionated into general groups of polysaccharides based on solubility in extraction solutions. Cell-wall fractionation based on chemical solubilization schemes does not result in separation of polysaccharides into homogeneous groups. It is clear from this study that polysaccharide groups overlap particularly among components of the pectic and hemicellulosic fractions. The use of the term hemicellulose has recently come under criticism because it does not reflect the polydispersity of polysaccharides that make up a given fraction (Bacic et al., 1988). The term hemicellulose is used here to simply reflect the extraction strategy utilized and does not represent a single type of polysaccharide. Chemical fractionation schemes reflect the nature of the wall, in terms of components and their interactions, rather than isolating a distinct polysaccharide. However, such schemes do provide a means of comparing compositional changes of CW carbohydrates differing in maturity and provide information on the physical and chemical relationships among wall components. For example, the delignification process solubilized appreciable CW material (Table I). This process, as proposed by Wise et al. (1946), is thought to cause only minor modifications of a depectinated CW. Upon analysis the solubilized material resembled pectic polysaccharides in terms of total uronic content and composition of associated neutral sugars. Therefore, the fractions that were considered extractable pectic materials (pectin and pectic polysaccharides) were HW-1, PE-1, PE-2, LE-1, and PE-3.

**Pectic Extracts.** The pectic fraction is associated with the middle lamella and primary cell wall of plants and is extracted with $Ca^{2+}$ chelators. Sequential extraction with hot water followed by $(NH_4)_2C_2O_4$ before and after delignification resulted in the solubilization of 22.6%, 16.6%, and 11.7% of the total matrix from apical (AN), upper (UN), and lower (LN) nodes and internodes, respectively (Table I). The decline of approximately 48% of extractabe pectic polysaccharides from AN to LN is one of the major changes in CW composition. This decline as a proportion of the total wall is most likely the result of a

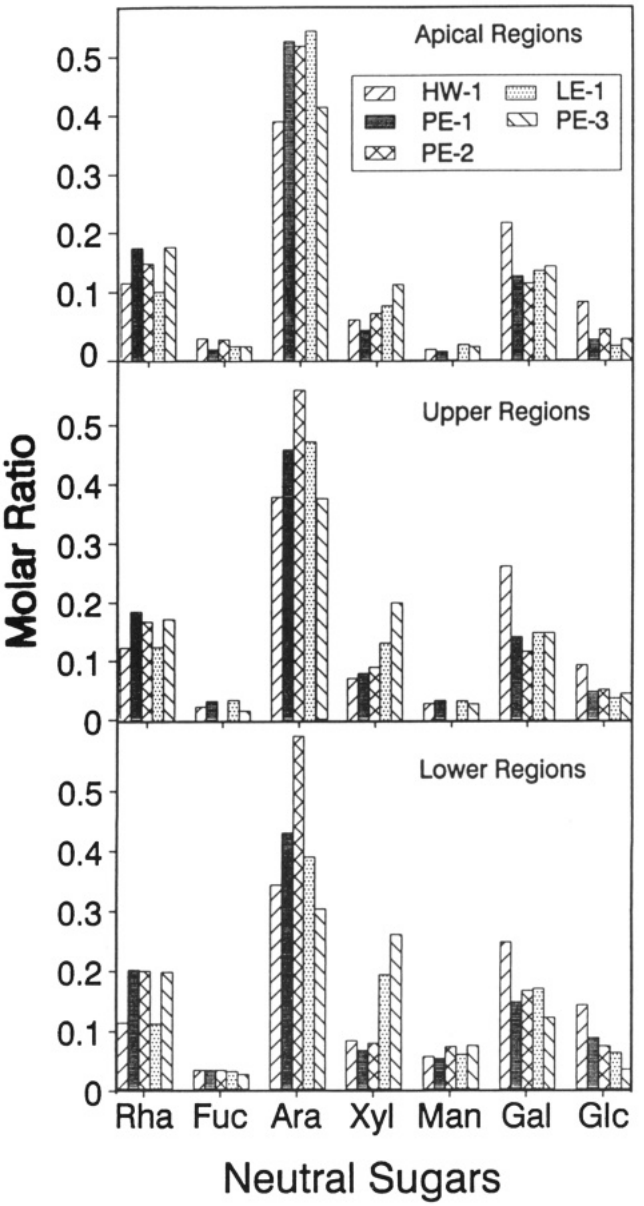


**Figure 1.** Neutral sugar composition of pectin and pectic polysaccharides solubilized during the sequential extraction of alfalfa stem cell walls. HW-1, hot water extract; PE-1, first $(NH_4)_2C_2O_4$ extract; PE-2, second $(NH_4)_2C_2O_4$ extract; LE-1, delignification extract; PE-3, third $(NH_4)_2C_2O_4$ extract.

shift from the synthesis of pectic polysaccharides to increased cellulose and xylan synthesis along with lignification. As additional CW is laid down, the major components become cellulose, hemicelluloses, and lignin. Total uronic acids (Table I) in each CW extraction did not change with maturity. However, HW-1 and PE-1 contained the highest proportions of total uronics (51–59%) in the isolated polysaccharides and LE-1 the lowest (41–42%). Galacturonic acid was the major unonosyl residue in these fractions, although glucuronic acid also was present in trace amounts (data not shown). This was based on the observed increase in galactose and glucose with respect to arabinose when uronosyl residues were reduced (Taylor and Conrad 1972) to their corresponding neutral sugars.

Neutral sugar composition of each fraction within the same stem section (AN, UN, or LN, Figure 1) indicates similarity of the isolated polysaccharides. Major neutral sugars associated with the extracts were arabinose, rhamnose, galactose, and xylose. Extracts LE-1 and PE-3 contained more xylose, especially in the LN sections. The

neutral sugar compositions of extracts compared across stem sections (compare AN, UN, and LN in Figure 1) were similar, with arabinose being the major sugar. This was particularly true for PE-1 and PE-2. Similarities are expected as this class of polysaccharides is among the first to be synthesized and laid down as part of the cell plate during cell division (Northcote, 1969), with synthesis ceasing by the time CW expansion stops (Northcote, 1969; Jarvis et al., 1981). Increased xylose concentration in LN extracts LE-1 and PE-3 may result from coextraction or might indicate specific polysaccharides that are covalently linked to the pectic materials. Recent work with corn seedling cell walls indicated fractions that contained rhamnogalacturonans covalently linked to arabinoxylans (Nishitani and Nevins, 1989; Kim and Carpita, 1991).

A portion of the pectic polysaccharides in each stem section was resistant to extraction with $(NH_4)_2C_2O_4$. This was not due to a limitation in the capacity of the $(NH_4)_2C_2O_4$ solutions to extract all of the pectic polysaccharides. When the concentration of $(NH_4)_2C_2O_4$ was increased (1%) or the incubation time increased (18 h at room temperature), the total amount of material extracted from LN was unchanged (data not shown). The observation that a proportion of the total pectic material is recalcitrant to normal $Ca^{2+}$ chelator extraction suggests that these polysaccharides were held in the CW matrix through covalent linkages. Delignification solubilized a portion of the pectic material and rendered another portion amenable to extraction (Table I). Chesson and Monro (1982) earlier noted that a portion of the pectic fraction (25%) in total alfalfa herbage was resistant to repeated chemical extraction with $Ca^{2+}$ chelators, although 80–90% of the total uronics was removed during in vitro degradation by rumen microorganisms. Fry (1983) identified ferulic acid bound to arabinose and galactose residues of pectic polysaccharides from spinach tissue culture cells. He also isolated small amounts of diferulic acid and proposed that cross-linkages through diferulic acid may bind the pectic polysaccharides together, resulting in a macromolecular structure that accounts for the resistance to chemical extraction yet is disrupted by $NaClO_2$. The proportion of total pectic polysaccharides solubilized by the delignification process did not change with maturity (Table I), suggesting that the interactions binding these fractions in the wall matrix occur early in cell-wall development.

Jarvis et al. (1981) have suggested that pectic polysaccharides form ester linkages to other CW components through the uronosyl residues, preventing complete extraction with $Ca^{2+}$ chelators. They have recommended a mild alkali treatment such as with 0.1 M $Na_2CO_3$ to cleave ester linkages and completely solubilize the pectic polysaccharides. Nonetheless, an overnight $Na_2CO_3$ extraction on alfalfa stem cell walls (LN), after the second $(NH_4)_2C_2O_4$ extraction (PE-2), removed only 33% of the remaining pectic material (Table II). Treatment with $NaClO_2$ solubilized additional polysaccharides with neutral sugar compositions that resembled the previous pectic fractions (Figure 2). This observation indicates that ester linkages play a role in the total matrix organization involving pectic polysaccharides but that other covalent linkages are also involved.

Morrison (1977) proposed that treatment of plant cell walls with anhydrous sodium methoxide may hydrolyze ester bonds, particularly those involving phenolic acids. Subsequent treatment with hot water would solubilize a portion of the cell-wall matrix without using harsh alkali treatments. Treatment of partially depectinated alfalfa CW in this manner resulted in a significant solubilization of polysaccharides (Table II). However, the composition of this fraction was enriched in xylose, indicating that the major polysaccharides isolated were xylans. It is possible that this fraction also contained the polygalacturonans extracted by $Na_2CO_3$ treatment since it is feasible that ester linkages involving galacturonosyl residues would also be disrupted. It is important to note that the polysaccharides extracted during delignification after the sodium

**Table II. Polysaccharide Fractions Extracted from Partially Depectinated Cell Walls after Treatment with Sodium Carbonate or Sodium Methoxide**

| cell-wall treatment | polysaccharide fraction | % of total extracted[b] | % of depectinated CW residue[c] | % uronosyl residues[d] |
|---|---|---|---|---|
| sodium carbonate | SC-1 | 33.0 | 2.2 | 43.0 |
| | PE-3 | 3.0 | 0.2 | 36.0 |
| | LE-1 | 43.0 | 2.9 | 49.0 |
| | PE-4 | 21.0 | 1.4 | 51.0 |
| sodium methoxide | SM-1 | 51.0 | 4.9 | 33.0 |
| | PE-3 | 4.0 | 0.4 | 44.0 |
| | LE-1 | 35.0 | 3.3 | 36.0 |
| | PE-4 | 10.0 | 0.9 | 39.0 |

[a] Polysaccharide fractions included SC-1 (sodium carbonate treatment), PE-3 ($(NH_4)_2C_2O_4$), LE-1 (sodium chlorite treatment), PE-4 (hot $H_2O$ treatment), and SM-1 (sodium methoxide treatment followed by hot $H_2O$ treatment). [b] Percent of the total carbohydrate (neutral sugars plus uronosyl residues extracted by the fractionation scheme) solubilized by each treatment from partially depectinated cell walls. [c] Percent of the CW residue after partial pectin extraction solubilized by each treatment. [d] Total uronosyl residues expressed as a percent of the total carbohydrate.

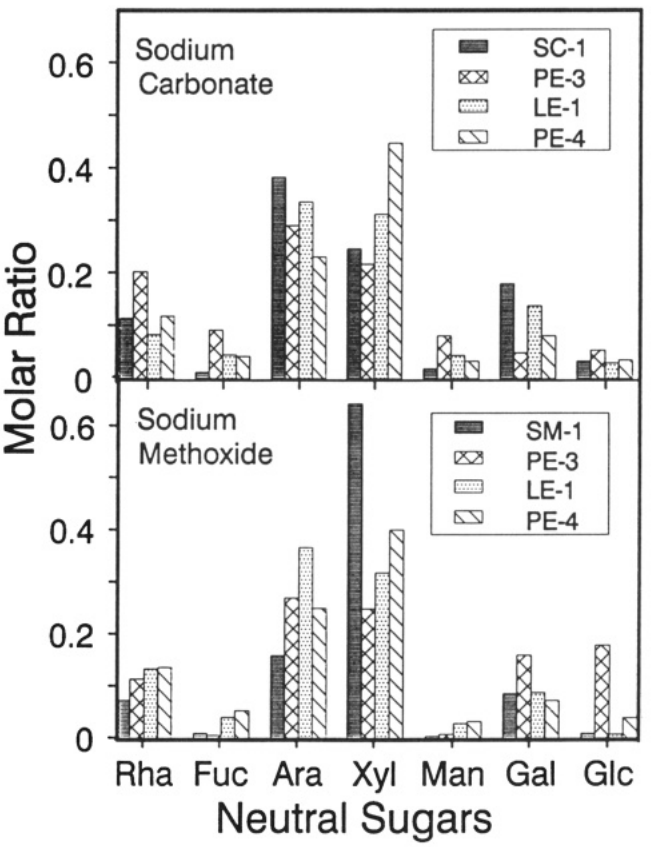


**Figure 2.** Neutral sugar composition of polysaccharides solubilized from partially depectinated lower alfalfa stem sections following treatment with sodium carbonate or anhydrous methanolic sodium methoxide. SC-1, $Na_2CO_3$ extract; SM-1, hot $H_2O$ extract after sodium methoxide treatment; PE-3, $(NH_4)_2C_2O_4$ extract; LE-1, delignification extract; PE-4, hot $H_2O$ extract.

**Table III. Alkaline-Soluble Fractions from Alfalfa Stems**

| | stem segment | | | | | |
|---|---|---|---|---|---|---|
| | AN | | UN | | LN | |
| KOH, M | g/g CW[a] | % uronosyl residues[b] | g/g CW | % uronosyl residues | g/g CW | % uronosyl residues |
| 0.25 | 0.1102 (0.0028)[c] | 21.91 | 0.1182 (0.0028) | 26.92 | 0.1338 (0.0102) | 27.47 |
| 0.50 | 0.0604 (0.0082) | 9.82 | 0.0461 (0.0067) | 17.51 | 0.0573 (0.0081) | 15.83 |
| 1.00 | 0.0516 (0.0067) | 3.94 | 0.0494 (0.0153) | 9.99 | 0.0474 (0.0011) | 11.17 |
| 4.00 | 0.1292 (0.0018) | 3.43 | 0.0730 (0.0013) | 6.88 | 0.0843 (0.0190) | 9.82 |
| total | 0.3514 | | 0.02867 | | 0.3228 | |

[a] Total carbohydrate (neutral sugars plus uronosyl residues) solubilized from depectinated and delignified alfalfa stems by each molarity of the KOH step gradient. [b] Total uronosyl residues expressed as a percent of the total carbohydrate extracted. [c] Standard error.

methoxide treatment were similar in neutral sugar composition to those previously extracted (Figures 1 and 2, lower stem regions).

Whether this resistance to extraction was through direct bonding between polysaccharides or lignin or physical entrapment is unknown. In wood, pectic polysaccharides have been proposed to be covalently linked to lignin (Minor, 1982). Thus, pectic extraction would be incomplete until the lignin is removed or at least partially degraded. Another possible cell-wall interaction may occur in which some of the pectic polysaccharides become physically bound to cellulose through cross-linking of adjacent extensin molecules that surround the microfibrils (Lamport and Epstein, 1983). Because the cross-linking unit is isodityrosine, $NaClO_2$ would disrupt this covalent interaction, allowing the solubilization of entrapped polysaccharides. Intramolecular isodityrosine has been identified in native extensin molecules, but intermolecular isodityrosine, which would be necessary to cross-link adjacent molecules (Fry 1986), has not.

**KOH Extracts.** The total material solubilized from depectinated and delignified CW by KOH treatment varied from 29% to 35% (Table III). Earlier work by Hirst et al. (1959) indicated that as alfalfa plants mature there is a shift to increased cellulose synthesis in the cell wall. This work indicates that within alfalfa stems there is an increase in the proportion of cellulose in the mature walls. The proportion of KOH-soluble polysaccharides remains relatively constant, although the composition of these fractions may change. The trend of slightly higher amounts of KOH-soluble polysaccharides in the AN suggests their synthesis may be higher in immature stems; the increase in cellulose synthesis occurs relatively early in stem development as there was no significant difference in the ratio of KOH-soluble polysaccharides to cellulose between upper and lower regions.

The KOH step gradient effectively fractionated the solubilized polysaccharides into subgroups based on composition (Figure 3). These polysaccharides appear to be mainly glucuronoxylans with varying amounts of uronosyl substitution (Table III). Xylose accounted for 70–80% of the total neutral sugars present in most of the extracts. With increased maturity of stem sections there is a decrease in the diversity of polysaccharides extracted. Aspinall and McGrath (1966) indicated that all xylans in alfalfa were glucuronoxylans without arabinose substitution. However, Gaillard (1965) isolated a xylan from alfalfa that contained 8% arabinose but only 1.5% uronic acid. This suggests that the small amount of arabinose contained in the extracts may be part of a minor group of xylans. It has been shown in corn cell walls that the substitution of glucuronic acid and arabinose on xylans varies with maturity (Carpita, 1984).

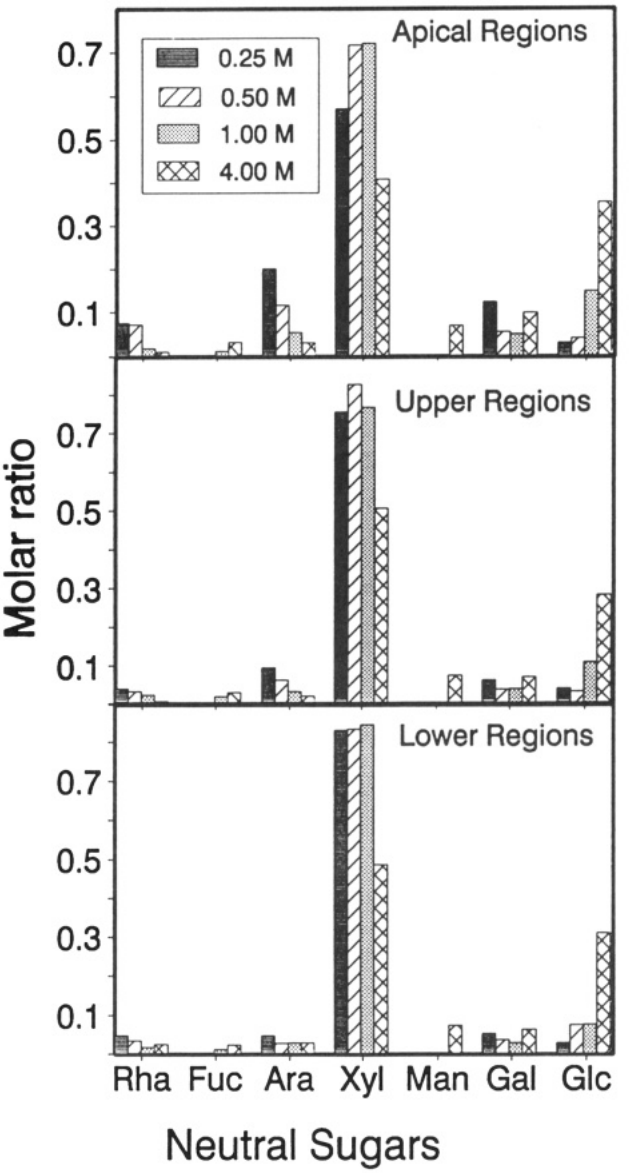


**Figure 3.** Neutral sugar composition of polysaccharides solubilized from alfalfa stem sections by KOH solutions ranging in concentration from 0.25 to 4.0 M.

The relatively higher amounts of arabinose along with rhamnose and uronosyl residues in 0.25 M KOH extracts resemble the pectic extracts. Sugar analysis of a reduced sample of the AN extract revealed galacturonic acid as the major uronosyl sugar. This would indicate that 0.25 M KOH solubilized an easily extracted xylan along with a resistant pectin fraction. Aspinall and McGrath (1966) have shown that alfalfa xylans contain a small amount of rhamnose; however, the high levels in the 0.25 M KOH extracts would seem to exceed the level of rhamnosyl residues in xylans, indicating some other polysaccharide being present. The trend toward higher total uronics in the KOH extracts from mature tissues (UN and LN sections) does not necessarily indicate increased amounts of resistant pectic material but rather a shift in the poly-

saccharide synthesis. In LN sections (Figure 3) extracted polysaccharides are much less diverse in terms of neutral sugar composition, indicating that these walls contain a higher proportion of glucuronoxylans.

In all stem sections, the 4 M KOH extract contained large amounts of glucose (Figure 3), which was most likely derived from xyloglucans synthesized during primary cell-wall development. The extracts also contained fucose and galactose. Previously, dicot xyloglucans have been shown to have a complex structure containing both of these sugars (Bacic et al., 1988; Hatfield, 1989; Selvendran, 1983). Release of these polysaccharides required strong alkali treatment to disrupt hydrogen bonding between the xyloglucans and cellulose microfibrils (Valent and Albersheim, 1974; Aspinall et al., 1969).

**Cellulose Residue.** The neutral sugar composition of the cellulose residue indicated that for all stem sections noncellulosic polysaccharide extraction was nearly complete. Glucose accounted for 91–92% with xylose, mannose, rhamnose, and fucose accounting for the remainder of recovered sugars after hydrolysis. The identification of non-glucose sugars in the CW residue indicates the presence of other polysaccharides that are tightly associated with the cellulose microfibrils. The nature of these associations may be extensive hydrogen bonding to the surface of microfibrils or the intermingling of non-glucan polymers with the amorphous regions of cellulose microfibrils (Selvendran, 1983).

**Conclusions.** During the development of alfalfa stems the CW matrix undergoes compositional changes in structural carbohydrates. The CW carbohydrate composition varies considerably from the apical regions to the lower internodes of stems. The most significant difference is the decrease in the proportion of pectic polysaccharides which is replaced by alkaline-soluble polysaccharides and cellulose from apical regions to lower stem regions. A portion of the pectic polysaccharides is held in the wall matrix by covalent linkages that require treatment with $NaClO_2$ to solubilize them completely. The unchanged proportion of these pectic polysaccharides with increased stem maturity would suggest that if lignification is involved, it occurs early in development. Entrapment by extensin molecules may be important since the timing of their synthesis would most likely correspond to the synthesis of the pectic polysaccharides. The alkaline-soluble fraction does not change significantly as a proportion of the total cell-wall matrix. The dilution of highly digestable polysaccharides such as those in the pectic fraction (Chesson and Monro, 1982; Dehority et al., 1962; Gradel and Dehority, 1972) with more slowly and less extensively digested polysaccharides decreases the utilization of potential energy in the total cell-wall matrix. Germplasm that contains higher proportions of pectic materials throughout the whole stem would have higher digestibilities and provide increased energy.

## ABBREVIATIONS USED

AN, apical nodes and internodes; UN, upper nodes and internodes; LN, lower nodes and internodes; CW, cell wall; GLC, gas–liquid chromatography; TFA, trifluoroacetic acid.

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**Registry No.** Cellulose, 9004-34-6; pectin, 9000-69-5; rhamnose, 3615-41-6; arabinose, 147-81-9; galactose, 59-23-4; xylose, 58-86-6; fucose, 2438-80-4; mannose, 3458-28-4; glucose, 50-99-7.