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METHYLATION OF UNFRACTIONATED, PRIMARY AND SECONDARY CELL-WALLS OF PLANTS, AND THE LOCATION OF ALKALI-LABILE SUBSTITUENTS

JAMES A. LOMAX, ALEX H. GORDON, AND ANDREW CHESSON
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB (Great Britain)
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ABSTRACT

A procedure has been developed for the methylation of intact (mature) plant cell-walls in good yield. The method depended on the reduction of particle size by dry milling in liquid nitrogen to a level that allowed plant fragments to swell and disperse in the methylation solvent. Quantification of the results was achieved by using methyl β-D-allopyranoside as internal standard. Perennial ryegrass cell-walls, whole barley straw, and beechwood, with degrees of lignification of 1.93, 12.66, and 19.33%, respectively, were used to determine the efficiency of methylation after milling. The recoveries of parent sugars, calculated by summing values for the appropriate partially methylated sugars, were 116, 102, and 109%, respectively, of those determined on the basis of alditol acetates. Acetalation of the free hydroxyl groups of milled, whole cell-walls, using methyl vinyl ether, enabled selective methylation to be performed at sites of alkali-labile substitution. The resulting recovery of pentoses was nearly quantitative, but that of (cellulosic) glucose was very low. These procedures allow linkage analysis of the structural polysaccharides and determination of the location of sites of alkali-labile substitution in the matrix polysaccharides of the intact cell-wall.

INTRODUCTION

Improvements in the techniques of methylation analysis[1,2] and in the methods of separation and identification of partially methylated sugars[3] now enable structural analysis of polysaccharides to be undertaken on a routine basis. Although methylation analysis has been applied to fractionated polysaccharides from plant cell-walls[4,5] and to tissue-cultured, primary cell-walls[6,7], there have been no reports of the methylation of whole cell-walls from mature plant tissue. We now report a method which allows the methylation of unfractionated cell-walls of plants in good yield even when applied to highly lignified, hardwood cell-walls. The method depends on the reduction of the particle size of the material to such a level that swelling and dispersal in the methylation solvent occurs.

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Internal standards are not usually employed in methylation analysis, quantification being based on the relative proportions of the products[7]. This procedure assumes that incomplete methylation will be apparent from the ratio of terminal to branching points, or from the number of unmethylated sugars or sugars having more than two free hydroxyl groups. When the original material contains considerable proportions of unreactive or insoluble carbohydrate, or non-carbohydrate components, as with cell walls from mature plants, the methylation results must always be open to doubt unless the recovery of methylated products can be determined. Large-scale methylation, which can enable recovery to be calculated by weight[6], is not generally practical.

The inclusion of an internal standard enables the recovery of partially methylated sugars to be monitored, and the recovery of the parent sugars can then be computed and compared with the results of standard sugar analysis. To our knowledge, this is the first report of the use of such an internal standard in the methylation analysis of polysaccharides.

The investigation of unfractionated walls makes possible the study of labile structures that are lost or rearranged during fractionation and isolation procedures. However, the powerful Hakomori-methylation technique[8] destroys alkali-labile linkages that may be important in the overall architecture of the cell wall, particularly the secondary wall[9,10]. Modification of the methylation procedure, based on the acetalation of hydroxyl groups, when applied to intact cell-walls, enables determination of the location and quantification of alkali-labile linkages to the matrix polysaccharides of the cell wall.

EXPERIMENTAL

General. — Methyl sulphoxide was dried over molecular sieves, distilled under reduced pressure, and stored in a desiccator over P_2O_5. Acetone was dried over molecular sieves and distilled. Other solvents and chemicals were of analytical grade. All evaporations were performed at reduced pressure at less than 40°.

Plant material. — Perennial ryegrass (*Lolium perenne* L., cv. Talbot) (20 g), collected in May from an established field, was extracted sequentially with chloroform–methanol (1:1, 700 mL), water (700 mL), and chloroform–methanol. The remaining cell-wall material (~13 g) was air-dried at 60°. Barley straw (*Hordeum vulgare* L., cv. Golden Promise) was obtained from a commercial field which had been combine-harvested. The whole straw, including husks, was used. Beechwood (*Fagus sylvatica* L.) was obtained as shavings from a large piece. All plant material was ground to pass through a 1-mm mesh before use.

Milling. — Freezer(cryo)-milling was carried out dry in liquid nitrogen for 3 min, using a Spex 6700 Freezer-mill (Spex Industries Inc.).

Analyses. — Sugars were analysed as their alditol acetates. The method of Blakeney *et al.* [12] was used, except that the volumes used in the hydrolysis step were doubled, and glass rods were inserted into the hydrolysis tubes to assist mix-

ing. The duration of the initial hydrolysis with cold, aqueous 72% sulphuric acid was also increased to 1 h, and that of acetylation to 20 min.

Total phenolics were determined by the method of Morrison[13], using ferulic acid as standard. Lignin was measured by the Christian method[14].

Acetalation of plant material. — The sample (~10 mg) was dispersed in methyl sulphoxide (2 mL) with ultrasonication, and toluene-*p*-sulphonic acid (20 mg) was added followed by methyl vinyl ether (2 mL, condensed at −10°). The mixture was maintained at 15° for 3.5 h with intermittent mixing, and then centrifuged at 1600*g* for 10 min. The pellet was washed three times with acetone and then dried *in vacuo* over P_2O_5.

Methylation. — Sodium methylsulphinylmethanide was prepared by adding oil-free sodium hydride (300 mg) to methyl sulphoxide (12 mL) under nitrogen. The mixture was agitated under a stream of nitrogen in an ultrasonic bath at 60°, and reaction was complete in 1 h[15].

Samples for methylation (~10 mg) were dried *in vacuo* over P_2O_5, methyl sulphoxide (1 mL) containing methyl β-D-allopyranoside (0.145 mg) was added, and the mixture was ultrasonicated under nitrogen for 30 min. Freshly prepared sodium methylsulphinylmethanide in methyl sulphoxide (1 mL) was then added under nitrogen, and the tube was ultrasonicated for 30 min and stored at room temperature overnight. Methyl iodide (1 mL) was added in portions during 1 h with cooling, and, after a further 1 h, the mixture was poured into 5.5M NaCl (25 mL), the pH was adjusted to 1.5 with 2M HCl, and the mixture was extracted three times with chloroform. The combined extracts were washed with 0.05M HCl and water (4×), concentrated, and transferred to a tube containing quebrachitol (0.506 mg). The chloroform was evaporated and aqueous 90% formic acid (1 mL) was added. The tube was flushed with nitrogen and then heated at 100° for 5 h. Formic acid was then removed, 0.25M H_2SO_4 (1 mL) was added, and heating at 100° was continued for 16 h. The hydrolysate was neutralised with Amberlite IRA-400 (HCO_3^-) resin and then concentrated to dryness. Fresh, aqueous sodium borodeuteride (1 mL, ~10 mg) was added and, after 3 h at room temperature, excess of borodeuteride was destroyed by the addition of 50% acetic acid. Dry methanol acidified with acetic acid (100:1) was then added and evaporated, four times, followed by one addition of methanol. Acetic anhydride (1 mL) was added to the residue, the mixture was heated at 100° for 16 h, acetic anhydride was then removed by codistillation with toluene, and the residue was partitioned between chloroform and water. The chloroform layer was washed with water (4×) and then concentrated to dryness. The remaining partially methylated alditol acetates were dried *in vacuo* over P_2O_5.

G.l.c. — Carlo–Erba Fractovap 4160 gas chromatographs, with on-column injection facilities, were employed throughout. Integrations were performed with either a Spectra-Physics SP4100 or a Trivector Trilab III computing integrator, and the data were fed directly into a Prime 550 computer for storage and further manipulation. Retention coefficients were calculated, and a provisional identification of the peaks was made using algorithms developed from those described previ-

ously[3]. The peak for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-allitol was used as the first retention-coefficient standard and as the internal standard. Quebrachitol was used as the second retention-coefficient standard. Separations were performed on glass columns (50 m × 0.3 mm) wall-coated with SP 1000 (Phase Separations). Ratios of 2- and 3-Xyl were obtained by chromatography on a wall-coated fused-silica column (50 m × 0.3 mm) of OV 1 (Hewlett-Packard)[16].

Alditol acetates of sugars were analysed by using a Pye GCD chromatograph equipped with columns (3 m × 3 mm) of 5% of SP 2340 on Chromosorb W (100–120 mesh)[17]. Peaks were integrated with a Trivector Trilab III computing integrator. Sugar compositions were calculated as "anhydro" residues.

Mass spectrometry. — A VG Micromass 16 mass spectrometer coupled to a VG 2025 data system was used. For g.l.c.–m.s., a wall-coated fused-silica column (30 m × 0.2 mm) of Silar-5CP was used: ionisation potential, 70 eV; source temperature, 200°.

Methyl β-D-allopyranoside. — Methyl glycosidation of D-allose was performed in the presence of strontium chloride[18]. The mixture of methyl glycosides was eluted from a column (60 × 1.8 cm) of Bio Rad AG1-X2 (HO) resin (200–400 mesh) with water, to give the α-D-pyranoside (16.9%), β-D-pyranoside (9.7%), α-D-furanoside (27.7%), and β-D-furanoside (7.6%). Contrary to the experience of Evans and Angyal[18], the α-pyranoside failed to crystallise. The β-pyranoside (81 mg, 7.5%), after recrystallisation from ethanol, had m.p. 150–151°; lit.[19] m.p. 151–152°. G.l.c. of the derived 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-allitol showed it to be chromatographically pure.

RESULTS

Internal standard. — Methyl β-D-allopyranoside was chosen as an internal standard which, after methylation, gave a single peak in g.l.c., with a recovery of 109% relative to methyl β-D-glucopyranoside. The retention coefficient of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-allitol on SP 1000 was 0.779 (relative to 2,3,4,6-tetra-*O*-methyl-D-glucose and quebrachitol) and was well separated from other partially methylated sugars likely to be derived from plant cell-walls (Fig. 1).

Methylation of plant cell-walls. — The average particle size of plant samples was reduced to 10–40-μm diameter on freezer-milling. Such particles swelled and dispersed in methyl sulphoxide, and could be methylated by the Hakomori technique with little apparent residue of unreacted material. To ascertain the extent of methylation, three plant samples, perennial ryegrass, barley straw, and beechwood, showing different degrees of lignification, were methylated. Methylation was performed directly on the freezer-milled hardwood and straw samples. However, in order to avoid the inclusion of non-structural sugar residues in any subsequent analysis, perennial ryegrass was first extracted with water and chloroform–methanol to provide a crude cell-wall preparation (66.8% recovery of original dry-matter).

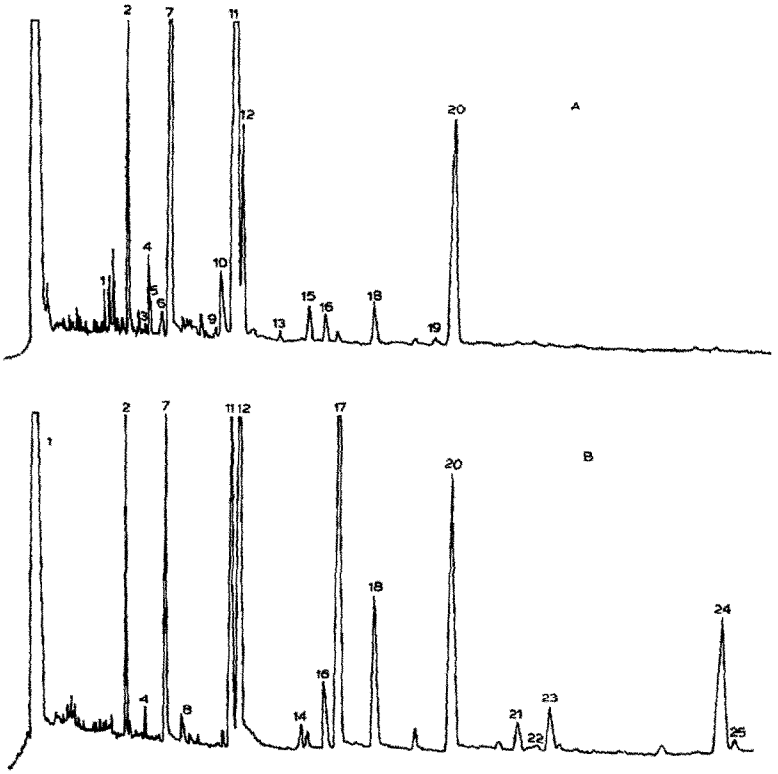

Fig. 1. Gas chromatograms of partially methylated alditol acetates obtained by methylation (A) and acetalation–methylation (B) of freezer-milled beechwood, run on a column (50 m × 0.3 mm) of SP 1000 WCOT at 220°, with helium carrier-gas (175 kPa): 1, 2,3,5-Ara; 2, 2,3,4,6-All (internal standard); 3, 2,5-Ara; 4, 2,3,4,6-Glc; 5, 2,3-Fuc; 6, 2,3-Ara; 7, 2,3-Xyl; 8, 2-Fuc; 9, 2,4,6-Glc; 10, 2,3,6-Man; 11, 2,3,6-Glc; 12, 2/3-Xyl; 13, 2,3,4-Gal; 14, Ara; 15, 2,6-Glc; 16, 4,6-Gal; 17, Xyl; 18, 2,3-Glc; 19, 3,4-Gal; 20, quebrachitol (standard); 21, 2-Glc; 22, 2-Gal; 23, 3-Glc; 24, Glc; 25, Gal.

The total contents of phenolics and Christian lignin for the three samples are shown in Table I. The discrepancy found between the two assay methods, when applied to barley straw and ryegrass, suggested that much of the phenolic material present in these samples was not in a polymeric form resistant to concentrated acid. No such discrepancy was found for beechwood.

TABLE I

ANALYSIS OF CELL WALLS FOR TOTAL PHENOLICS AND LIGNIN

Sample	*Dry matter (%)*	
	Total phenolics	*Lignin*
Perennial ryegrass, Talbot	7.63	1.93
Barley straw, Golden Promise	17.58	12.66
Beechwood	18.89	19.33

Table II shows the results of methylation analysis of the samples, with and without freezer-milling. There was a considerable increase in recovery after freezer-milling, notably for the most highly lignified samples, where the recoveries increased six-fold. The identities of the components (>0.5%) listed, and indicated by their chromatographic properties, were confirmed by mass spectroscopy.

The parent sugar composition of the samples was calculated from the methylation data by summing the values obtained for the appropriate partially methylated sugars. A comparison of these results with those obtained directly from acid hydrolysates by alditol acetate analysis, and expressed as percentages of the "anhydro" sugar residue, is shown in Table III. Good agreement between the "freezer-milled" methylation results and those from alditol acetate analysis was found for all three samples. No trend towards poorer recoveries with increasing lignification was discernible. Although the recovery of methylated products from samples without prior freezer-milling was low, especially for the more highly lignified samples, the relative proportions of the major products remained reasonably representative of the cell wall as a whole (Table IV). This similarity suggested that the freezer-milling process did not significantly modify the cell wall.

Acetalation of plant cell-walls. — The protection of free hydroxyl groups by acetalation[11] allows selective methylation to be performed at sites of alkali-labile substitution[1,2]. This reaction is carried out in methyl sulphoxide; since freezer-milled plant cell-walls could be dispersed in this solvent, acetalation of intact walls was attempted. The separation of acetalated plant-material and the products of reagent polymerisation after treatment with methyl vinyl ether proved difficult by chromatography[11] on Sephadex LH-20. The acetalated samples, however, could be recovered by centrifugation, and washing with acetone removed by-products that interfered with subsequent g.l.c. Since methyl β-D-allopyranoside would be lost in this process, it was added after acetalation, but before methylation.

Table V shows the methylation products of the major sugars obtained after acetalation, and the recoveries based on the sugar composition calculated from the alditol acetate analysis (Table III). The recovery of pentoses was nearly quantitative, but that of glucose was very low. Recoveries may have been reduced by manipulation losses before the addition of the internal standard, and some components of the cell walls may have been extracted by methyl sulphoxide and lost on

TABLE II

COMPARISON OF METHYLATION RESULTS FOR UNTREATED (*A*) AND FREEZER-MILLED (*B*) CELL-WALLS AS A PERCENTAGE OF THE TOTAL SAMPLE[a]

Sugar	*Retention coefficient (p)*[b]	*Ryegrass*		*Barley straw*		*Beechwood*	
		A	*B*	*A*	*B*	*A*	*B*
2,3,4-Rha[c]	0.492	0.03	0.04	0.02	—	—	—
2,3,5-Ara	0.521	1.04	2.77	0.38	1.51	0.08	0.09
2,3,5-Fuc	0.590	0.13	0.14	0.23	0.03	—	—
2,3,4-Fuc	0.611	0.28	0.17	0.24	0.18	—	0.35
2,3,4-Ara	0.645	0.03	0.04	0.02	—	—	—
2,3,4-Xyl	0.658	0.03	1.00	0.03	0.18	—	0.16
3,4-Rha	0.875	0.08	0.11	0.02	0.05	0.08	0.05
3,5-Ara	0.929	0.12	0.30	0.04	0.17	—	—
2,5-Ara	0.968	0.15	0.36	0.09	0.24	—	—
2,3,4,6-Glc	1.000	0.31	0.51	0.38	0.80	0.73	0.65
2,3,5,6-Gal	1.070	—	—	0.03	—	—	—
2,4-Fuc	1.080	—	—	—	0.04	0.40	—
2,3,4,6-Gal	1.149	0.71	1.21	0.21	0.80	0.08	0.23
2,3-Xyl	1.227	2.02	7.35	1.56	12.25	2.85	23.70
2-Rha	1.426	0.05	0.08	0.09	0.04	—	—
4-Rha	1.495	0.05	0.10	—	—	0.56	—
3-Rha	1.580	0.04	0.21	0.02	0.07	0.49	0.04
3,4,6-Man	1.658	—	0.04	0.02	—	—	—
2,4,6-Glc	1.725	1.84	1.24	—	0.38	0.07	1.02
2,3,6-Man	1.800	0.15	0.21	—	0.26	0.14	—
2,4,6-Man	1.855	—	—	0.04	—	0.10	0.14
2,3,6-Gal	1.873	0.09	0.30	—	—	—	—
2,3,6-Glc	1.931	21.35	40.37	4.22	35.72	4.15	30.70
2-Xyl[d]	2.021	0.28	1.60	0.35	3.07	0.22	2.32
3-Xyl[d]	2.021	0.52	2.97	0.24	2.05	0.07	0.99
2,3,5-Gal	2.380	0.22	—	0.04	—	—	—
2,3,4-Gal	2.427	—	0.24	—	0.03	—	0.07
2,6-Man	2.654	0.03	0.05	—	—	—	—
2,6-Glc	2.761	0.71	0.90	—	—	—	0.39
4,6-Gal	2.902	0.46	0.61	0.10	—	0.09	0.23
3,4-Man	3.430	1.09	1.43	0.23	0.03	0.13	—
2,3-Glc	3.496	—	—	—	0.88	—	0.51
6-Man	3.594	0.03	—	0.06	—	—	—
2,3-Gal	3.673	—	—	0.02	—	—	—
2,4-Man	3.774	0.03	—	0.87	—	—	—
3,4-Gal	4.115	—	0.28	—	0.11	—	—
2-Glc	5.051	0.03	—	0.04	—	—	—
Total	—	31.90	64.60	9.55	58.90	10.23	61.64

[a]Components were separated on SP 1000 (See Experimental). Methyl β-D-allopyranoside was used as the standard for quantification. [b]Retention coefficients are based on the standards 2,3,4,6-All (p = 0.779) and quebrachitol (p = 4.300). [c]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc.* [d]The ratio of 2- and 3-Xyl was obtained by g.l.c. on OV 1.

TABLE III

COMPARISON OF SUGAR COMPOSITION[a] OBTAINED BY METHYLATION ANALYSIS OF UNTREATED CELL-WALLS (*A*) AND FREEZER-MILLED CELL-WALLS (*B*), AND BY ANALYSIS OF ALDITOL ACETATES (*C*)

Sugar	*Composition*[a] *(%)*								
	Perennial ryegrass			*Barley straw*			*Beechwood*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Rhamnose	0.14	0.38	0.25	0.09	0.07	0.15	0.50	0.07	0.33
Fucose	0.40	0.37	—	0.42	0.12	—	0.18	0.32	—
Ribose	0.15	0.30	0.14	0.05	—	0.41	—	—	0.40
Arabinose	1.18	3.05	3.65	0.80	1.70	2.51	0.67	0.08	0.66
Xylose	3.26	10.58	11.79	1.65	15.71	14.69	2.76	23.91	15.68
Mannose	1.81	1.58	0.14	0.39	0.31	0.40	0.22	0.23	0.95
Galactose	1.23	2.16	1.33	0.18	1.11	1.01	0.11	0.97	0.55
Glucose	21.83	38.72	31.70	4.43	34.56	33.28	4.42	29.70	31.95
Total	30.00	57.14	49.00	8.01	53.58	52.45	8.86	55.28	50.52

[a]All results are given as percentages of "anhydro" residue

TABLE IV

COMPARISON OF METHYLATION RESULTS FOR UNTREATED (*A*) AND FREEZER-MILLED (*B*) CELL-WALLS AS A PERCENTAGE OF TOTAL AREA

Sugar	*Ryegrass*		*Barley straw*		*Beechwood*	
	A	*B*	*A*	*B*	*A*	*B*
2,3,5-Ara[a]	3.19	4.34	4.26	2.51	0.81	0.14
2,3,4-Fuc	0.84	0.27	2.64	0.30	—	0.57
2,3,4-Xyl	0.08	0.16	0.30	0.30	—	0.26
3,5-Ara	0.35	0.47	0.48	0.28	—	—
2,5-Ara	0.45	0.56	0.97	0.40	—	—
2,3,4,6-Glc	0.96	0.80	4.21	1.34	1.88	1.05
2,3,4,6-Gal	2.17	1.89	2.33	1.34	0.78	0.36
2,3-Xyl	6.18	11.50	17.41	20.43	28.77	38.25
2,4,6-Glc	5.62	1.94	0.86	0.63	0.60	0.44
2,3,6-Man	0.46	0.34	—	0.44	1.20	1.42
2,3,6-Gal	0.27	0.47	—	—	—	—
2,3,6-Glc	65.21	63.31	47.13	59.57	60.71	49.49
2-Xyl	1.76	2.50	3.95	5.12	2.23	3.74
3-Xyl	3.27	4.64	2.63	3.41	0.67	1.60
2,6-Glc	2.17	1.41	—	1.20	—	0.64
4,6-Gal	1.40	0.96	1.06	0.12	1.06	0.36
3,4-Man	3.32	2.24	2.57	0.05	1.66	—
2,3-Glc	—	—	—	1.47	—	0.82

[a]2,3,5-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, *etc*

centrifugation. Extraction of the samples with methyl sulphoxide, under conditions similar to those of the acetalation reaction, gave material which was methylated (Table VI) and represented 7.5, 3.4, and 1.9% of the dry matter of ryegrass, barley, and beechwood, respectively. In-chain glucosyl (2,3,6-Glc) and xylosyl (2,3-Xyl) residues accounted for 60–70% of the sugars in the soluble material, and were found in proportions similar to those obtained by methylation of the whole sample. A higher proportion of terminal glucosyl groups was found in methyl sulphoxide extracts of barley and beechwood, which may have reflected the presence of small proportions of free glucose in these samples.

DISCUSSION

The chemical fractionation of plant cell-walls often modifies polysaccharide structure and may lead to the isolation of non-representative products[20]. The method described here enabled linkage analysis of the unfractionated material and provided a base against which fractions obtained by chemical or enzymic treatments could be compared.

TABLE V

ACETALATION–METHYLATION OF FREEZER-MILLED CELL-WALLS

Sugar	*Location of methyl groups*	*Ryegrass*		*Barley straw*		*Beechwood*	
		Yield[a] *(%)*	*Recovery*[b] *(%)*	*Yield*[a] *(%)*	*Recovery*[b] *(%)*	*Yield*[a] *(%)*	*Recovery*[b] *(%)*
Xylose	2,3	0.67	5.0	1.18	7.1	2.45	13.8
	2	2.26	16.9	2.53	15.2	5.83	32 7
	3	1.22	9.1	2 97	17 8	3.14	17.6
	unmethylated	6.40	47.8	9.71	58 2	7.77	43.6
	Total	10.55	78.8	16.39	98.3	19 19	107.7
Arabinose	2,3	—	–	0.03	1.1	0.03	4.0
	2,5	0.13	3 1	0.33	11.6	0.04	5.3
	3,5	0.14	3.4	0.14	4.9	0 04	5.3
	2	0.06	1.5	—	—	—	—
	5	0.67	16.2	0.62	21.7	0.10	13.3
	unmethylated	2.05	49.4	1.54	54.0	0.31	41 3
	Total	3.05	73.6	2.66	93.3	0 52	69 3
Glucose	2,3,4,6	0.14	0.4	—	—	—	—
	2,3,6	2.53	7.2	5.33	14 4	3.94	11.1
	2,3	1.85	5.3	3.79	10.3	—	—
	2	—	—	0.07	0.2	0.73	2.1
	3	—	—	0.05	0.1	1.21	3.4
	unmethylated	5.52	15.7	3.47	9.4	5.16	14.5
	Total	10.04	28.6	12.71	34.4	11.04	31.1

[a]As % of the dry sample. [b]Based on the alditol acetate analysis.

TABLE VI

METHYLATION OF THE FRACTION OF FREEZER-MILLED CELL-WALLS[a] SOLUBLE IN METHYL SULPHOXIDE

Sugar	*Ryegrass*		*Barley straw*		*Beechwood*	
	Area (%)	*Yield*[b] (%)	*Area* (%)	*Yield*[b] (%)	*Area* (%)	*Yield*[b] (%)
2,3,5-Ara[c]	4.83	0.36	1.93	0.07	—	—
2,3,4-Fuc	0.60	0.05	3.02	0.10	—	—
2,3,4-Xyl	—	—	—	—	2.42	0.07
2,5-Ara	0.79	0.06	1.75	0.06	—	—
2,3,4,6-Glc	2.72	0.20	12.86	0.43	5.99	0.11
2,3,4,6-Gal	4.16	0.31	3.98	0.13	—	—
2,3-Xyl	12.73	0.95	12.67	0.43	36.30	0.67
2,4,6-Glc	9.38	0.70	8.02	0.27	—	—
2,3,6-Man	1.13	0.09	2.04	0.07	16.30	0.30
2,3,6-Glc	51.07	3.81	51.78	1.74	39.00	0.75
2-Xyl[d]	5.90	0.44	—	—	—	—
3-Xyl[d]	3.75	0.28	—	—	—	—
2,3-Glc	2.75	0.21	1.96	0.07	—	—
Total	100.00	7.46	100.00	3.37	100.00	1.90

[a]Components were separated on SP 1000 (see Experimental), using methyl β-D-allopyranoside as internal standard. [b]As % of the dry sample. [c]2,3,5-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, *etc.* [d]The ratio of 2- and 3-Xyl was obtained by g.l.c. on OV 1.

Freezer-milling, which was the only treatment necessary before methylation, was selected as least likely to modify cell-wall structure. The use of liquid nitrogen avoided problems caused by localised heating (which occur with other comminution methods) and the loss of plant material with the milling solvent. The results indicated that the linkage patterns of cell walls were not altered detectably by freezer-milling and that structural polysaccharides may be recovered and converted, in good yield, into their partially methylated components. However, a small fraction of plant material released by milling was soluble in methyl sulphoxide. Although this was not important for the methylation procedure, loss of the soluble sugars may be unacceptably high for the acetalation–methylation method, where only the insoluble residues are retained for analysis. Optimisation of milling-time may help to reduce these losses. Alternatively, acetalated soluble-material could be examined in tandem with the insoluble fraction.

The relative proportions of partially methylated sugars obtained from plant material before and after freezer-milling were similar (Table III). This constancy in composition, even for beechwood, where the recovery from unmilled material was <10% of the dry matter, indicated that a low recovery was not associated with undermethylation. It appeared that a substantial fraction of cell-wall polysaccharide remained totally unavailable to the methylation reagents. Therefore, the increase in surface area produced by milling appears to be an important facet of the pre-treatment method.

Quantification of the results was dependent on the inclusion of methyl β-D-allopyranoside as internal standard, and its derivative, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-allitol, gave a single peak in an empty area of the chromatogram (Fig. 1). It was assumed that the equal weight response-factors for the various methylated sugars, relative to the allitol derivative, were close to unity; for studies of the changes in cell-wall structure of plant material undergoing degradation by micro-organisms[21], this assumption was valid. Ideally, all response factors should be determined, but, as Sweet *et al.*[22] pointed out, this is a long and arduous task. As an alternative, they suggested that response factors calculated on the basis of the Effective Carbon Response theory[23] could have some validity for partially methylated sugars, and they have published values, relative to terminal hexose, ranging from 0.86–1.20. Application of their data to the results shown in Table I suggests that the recovery of 2,3,6-Glc might be overestimated (~6%), whereas that of the methylated pentoses could be underestimated (~6%).

The recovery of parent sugars, calculated from the results of methylation analysis of whole cell-walls, was equal to or higher than that predicted by conventional methods of sugar analysis. There are considerable problems associated with the routine analysis of plant cell-wall polysaccharides. Methods involving acid hydrolysis always represent a compromise between rates of hydrolysis of the more resistant polysaccharide components and rates of destruction of released monosaccharides. Complete analysis of plant cell-walls rarely sums to 100%, and some of the shortfall is almost certainly caused by an underestimate of total sugars. Hydrolysis of methylated cell-walls appears to be more straightforward, with a lower range of labilities shown by the various glycosidic linkages. However, as a method of sugar analysis, methylation has two major disadvantages, namely, the long and complicated methodology, and the need for sophisticated instrumentation to separate and identify the large number of compounds produced.

Alkali-labile linkages, which are an important feature of plant cell-walls[9,10], are destroyed during methylation. In order to locate and quantify substituents attached by alkali-labile linkages, the technique developed by de Belder and Norrman[11] for the location of acetyl groups in partially acetylated dextrins was applied to milled plant material. Although the recovery of pentoses from acetalated walls was satisfactory, recovery of glucose was very low. The high proportion of partially methylated glucose residues detected suggested that protection by acetalation was incomplete. It seems likely that polymerisation products formed at the surface of cellulose microfibrils prevented complete acetalation and subsequent penetration by the methylation reagents. Although the method is clearly not applicable to cell-wall glucans (notably cellulose), in our experience, it produces valuable information when restricted to the matrix polysaccharides of the cell wall[10].

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