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A MULTIPLE-COLUMN APPROACH TO THE METHYLATION ANALYSIS OF PLANT CELL-WALLS*

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ABSTRACT

A procedure has been developed for the improved analysis of the neutral-sugar glycosidic linkages in plant cell-walls, utilising capillary g.l.c. and columns of three different phases for the separation of the products of methylation analysis. Retention coefficients are reported for a wide range of partially methylated alditol acetates on columns of CP-Sil88 and a bonded phase (BP-1) equivalent to OV-1. Using these phases and SP-1000, all cases of co-chromatography can be resolved. Computers were used to process the large amounts of data produced, to identify peaks and to assist in merging the results obtained using the three phases.

INTRODUCTION

Polysaccharides from whole plant cell-walls contain a wide range of sugars and linkages[1] which, on methylation, produce a complex mixture of partially methylated derivatives. The fractionation of large numbers of partially methylated sugars, as their alditol acetates, now can be achieved using capillary columns[2], especially if use is made of inert fused-silica columns and chemically bonded stationary phases[3]. However, the theoretical study[4] of the fractionation of complex mixtures has shown that the real peak-capacity of a column is far lower than its potential, and the probability of obtaining a peak as a singlet is quite low unless the chromatogram is mostly empty. As a result, it is not feasible to separate all the possible methylated sugars using a single column, given the large numbers and structural similarities of the compounds involved. Several commonly encountered methylated sugars remain difficult to separate by g.l.c. In particular, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol and -mannitol, and 2- and 3-*O*-methylxylitol are poorly resolved on most phases. The selectivity of various stationary phases must therefore be used to obtain particular resolutions[5].

Inevitably, the manual interpretation of results produced by the analysis of complex mixtures on more than one phase is both time-consuming and prone to

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error. The large amount of data generated can be processed more efficiently by computers[6]. With suitable programmes, peaks can be provisionally identified and information from different runs compared[7,8].

We have found that all instances of co-chromatography and doubtful assignment of partially methylated sugars derived from plant cell-walls can be resolved using three phases of different characteristics. Merging of the data obtained by using the three capillary columns was achieved with the assistance of computers. The methods described, when used in conjunction with the quantitative methylation procedure reported previously[1], allowed the complete analysis of neutral-sugar glycosidic linkages in plant cell-wall polysaccharides to be undertaken on a routine basis. Methylation analysis of cell walls prepared from Timothy grass and white clover are reported to show the applicability of the methods to both mono- and di-cotyledons.

EXPERIMENTAL

Plant material. — White clover (*Trifolium repens* cv. Aberystwyth S100) and Timothy grass (*Phleum pratense* cv. Percora) (~20 g) were collected from pure swards grown on trial plots at the North of Scotland College of Agriculture. The samples were extracted sequentially with chloroform–methanol (1:1, 700 mL), water (700 mL), and chloroform–methanol (1:1, 700 mL), and the remaining cell-wall material was dried at 60°. The cell walls were then freezer(cryo)-milled for 3 min at −196°, using a Spex 6700 Freezer-mill.

Methylation. — The milled plant material (~10 mg) was subjected to Hakomori methylation, using methyl β-D-allopyranoside as internal standard, as described previously[1].

G.l.c. — Carlo-Erba 4160 and 5160 gas chromatographs equipped with on-column injectors and flame-ionisation detectors were used throughout. Separations were performed, using helium as carrier gas, on wall-coated fused-silica columns (50 m × 0.33 mm, 0.22-μm film) of SP-1000 (130 kPa, 210°) and CP-Sil88 (130 kPa, 210°) (Chrompack), and on a fused-silica bonded-phase column (50 m × 0.33 mm, 0.5-μm film) of BP-1 (S.G.E.), equivalent to OV-1 (130 kPa, 195°). The thicker film of BP-1 was used to improve peak shape and the reproducibility of retention data.

Manipulation of g.l.c. data. — Initial integration of the chromatograms was performed using Spectra-Physics SP4270 and Trivector Trilab III integrators. Data from the integrators were fed into a Prime 550 computer through RS232 interfaces. Retention coefficients were calculated from the retention times of the peaks for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylallitol, derived from the added standard and quebrachitol using algorithms developed from those described previously[7]. Peaks were identified using a database of retention coefficients obtained for each phase by chromatography of a wide range of partially methylated sugars[7]. Provision was made in the programme for the manual forcing of particular identifications where,

perhaps in a crowded area of the chromatogram, other evidence (*e.g.*, mass spectra) suggested that the first identification by the computer was faulty.

RESULTS AND DISCUSSION

Chromatography of a range of methylated samples of known composition and of characterised mixtures of partially methylated products from individual sugars[7] enabled a database of retention coefficients to be produced for each phase which included all the commonly occurring, partially methylated sugars from plant material. Results for a column of SP-1000 have already been published[7]; Tables I and II give data for columns of BP-1 (OV-1) and CP-Sil88, respectively. From these results, it can be seen that all instances of co-chromatography should be capable of resolution on one or more phases using capillary columns with more than 100,000 theoretical plates.

The peak for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylallitol (= 2,3,4,6-All), derived from the internal standard methyl β-D-allopyranoside and used for quantification of results, had a retention coefficient (ρ) of 0.775 on SP-1000 which was well separated from other peaks and always occurred as a singlet. On CP-Sil88,

TABLE I

RETENTION COEFFICIENTS[a] OF PARTIALLY METHYLATED ALDITOL ACETATES ON CP-SIL88

Location of methyl groups	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Unmethylated	1.480	1.577	2.164	2.868	4.201	4.719	5.434
2	1.260	1.324	1.688	1.972	3.282	3.686	3.808
3	1.448	1.524	1.787	1.952	4.125	4.643	4.259
4	1.355			[b]	[c]	[c]	
5			1.335				
6	—	—	—	—	2.412	2.650	
2,3	1.000	1.114	1.195	1.319	2.471	2.972	2.850
2,4	0.968	1.066	1.213	1.198	2.703	3.132	2.648
2,5			1.067				
2,6	—	—	—	—	1.986	2.143	2.240
3,4	0.926	1.147	1.435	[d]	2.766		
3,5			0.981		[e]		
3,6	—	—	—	—	2.351	2.428	2.420
4,6	—	—	—	—	1.956	2.143	2.298
2,3,4	0.686	0.792	0.812	0.841	1.630	2.003	1.665
2,3,5			0.726				
2,3,6	—	—	—	—	1.540	1.688	1.759
2,4,6	—	—	—	—	1.449	1.541	1.428
3,4,6	—	—	—	—	1.439		1.480
2,3,4,6	—	—	—	—	0.977	1.102	1.000

[a]Retention coefficients were calculated using the 2,3,4,6-tetra-*O*-methylallitol derivative (ρ = 0.796), and the mono-*O*-methyl-L-inositol derivative (ρ = 4.000) as standards. [b]Identical to that from 2-*O*-methylxylose. [c]Identical to that from the corresponding 3-*O*-methylhexose. [d]Identical to that from 2,3-di-*O*-methylxylose. [e]Identical to that from 2,4-di-*O*-methylmannose.

TABLE II

RETENTION COEFFICIENTS[a] OF PARTIALLY METHYLATED ALDITOL ACETATES ON BP-1 (OV-1)

Location of methyl groups	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Unmethylated	1.554	1.530	1.615	1.739	4.481	4.780	4.618
2	1.137	1.153	1.161	1.222	3.461	3.655	3.538
3	1.300	1.319	1.259	1.241	3 942	4.167	3.800
4	1.237			[b]	[c]	[c]	3.996
5			0.910				
6	—	—	—	—	2.832	2.918	2.897
2,3	0.776	0.832	0.738	0.766	2.671	2.845	2.724
2,4	0.831	0.898	0.771	0.723	2.967		2.803
2,5			0.572				
2,6	—	—	—	—	2.163	2.203	2.219
3,4		0.898		[d]			2.814
3,5			0.570		[e]		
3,6	—	—	—	—	2.428	2.411	2.368
4,6	—	—	—	—	2.262		2.355
2,3,4	0.390	0.497	0.363	0.365	1.880	2.134	1.863
2,3,5			0.232				
2,3,6	—	—	—	—	1.621	1.618	1.674
2,4,6	—	—	—	—	1.683	1.744	1.595
3,4,6	—	—	—	—	1.593		
2,3,4,6	—	—	—	—	1.000	1.115	1.000

[a]Retention coefficients were calculated using the 2,3,4,6-tetra-*O*-methylallitol derivative (ρ = 0.827), and the mono-*O*-methyl-L-inositol derivative (ρ = 3.000) as standards. [b]Identical to that from 2-*O*-methylxylose. [c]Identical to that from the corresponding 3-*O*-methylhexose. [d]Identical to that from 2,3-di-*O*-methylxylose. [e]Identical to that from 2,4-di-*O*-methylmannose.

the allose standard peak was found at ρ = 0.796 and occurred as a doublet with the peak for 2,3,4-Fuc (ρ = 0.792). Computations based on the resolving power of the column and the retention times and separation of the peaks showed that separation would be achieved using the SP4270 integrator down to trace amounts of 2,3,4-Fuc. On BP-1, the retention coefficient of 2,3,4,6-All was 0.827 which was very close to that (ρ = 0.831) for 2,4-Rha. No separation of these peaks was achieved under the chromatographic conditions employed. A correction would therefore be necessary if chromatography on the other phases showed the presence of 2,4-Rha.

The proposed identities of peaks in complex samples were made by computer, using the appropriate database file for retention coefficients. A variability in retention time of ±1% was allowed to ensure that compounds were not eliminated from the analysis simply because the retention coefficient of a peak was slightly altered due to overloading or slight non-ideal behaviour, or because of unresolved multiple-peaks. When multiple matches were found, the identifications were ranked in the report according to the closeness of fit to the target peak. Aggregated values for each parent sugar (based on the closest identification) were also reported in order to enable comparisons of sugar composition to be made with independent assays.

TABLE III

METHYLATION RESULTS (A) AND RESOLVED VALUES (B) FOR CELL WALLS OF TIMOTHY GRASS ON THREE PHASES AS A PERCENTAGE OF TOTAL SAMPLE

Sugar	*SP-1000*		*CP-Sil88*		*BP-1 (OV-1)*		*Average of B values*
	A	B	A	B	A	B	
2,3,4-Ara	0.102	0.102	0.149	0.149	k	k	0.126
2,3,5-Ara	2.849	2.849	2.743	2.743	2.635	2.635	2.742
2,3-Ara	0.468 C[a]	0.468 C	0.630 D	0.564 D	0.459	0.459	0.497
2,5-Ara	0.298	0.298	c	0.298 c	a	0.298 a	0.298
3,4-Ara			f	f			
3,5-Ara	0.339 A	0.339 A	a	0.356 a	0.671 A	0.373 A	0.356
2-Ara	h	h	0.277 H	H			
3-Ara	0.833 J	0.405 J	i	i			n.c.[f]
5-Ara	0.022 F	0.022 F					n.c.
Ara			0.063 N	0.063 N			n.c.
2,3,4-Xyl	0.287	0.287	0.319	0.319	0.327 K	0.327 K	0.311
2,3-Xyl	9.858 E	9.858 E	9.784 E	9.784 E	9.790 B	9.790 B	9.811
2,4-Xyl	d	0.066 d	d	0.066 d	0.066	0.066	0.066
3,4-Xyl	e	e					
2-Xyl	k	3.344 k	3.049 K	3.049 K	2.885	2.885	3.093
3-Xyl	4.088 K	0.744 K	0.545 Jk	0.545 Jk	0.774 D	0.774 D	0.688
Xyl	0.194	0.194		[d]	f	0.194 f	0.194
2,3,4-Rha	0.381	0.381	0.324	0.324	0.396	0.396	0.367
2,3-Rha	a	a	b	b	b	b	
2,4-Rha	b	0.074 b	0.074	0.074			0.074
3,4-Rha	0.091	0.091	0.067	0.067		[e]	0.079
2-Rha	f	f					
3-Rha	0.154	0.154		[c]	0.110	0.110	0.132
4-Rha					d	d	
Rha	i	i					
2,3,4-Fuc	0.046	0.046			0.038	0.038	0.042
2,3-Fuc			0.084	0.084			n.c.
2,4-Fuc	0.064	0.064	0.331 C	0.033 C	e		0.049
3,4-Fuc		[e]	0.178	0.178	0.181 L	0.181	0.180
2-Fuc			e	e			
3-Fuc			g	g			
Fuc							
2,3,4,6-Glc	0.853 B	0.752 B	0.840 B	0.840 B	0.774 C	0.747 C	0.780
2,3,4-Glc	j	0.378 j	0.386 h	0.386 h	j	0.370 j	0.378
2,3,6-Glc	42.369 I	42.142 I	40.400 I	40.400	41.362	41.362	41.303
2,4,6-Glc	1.426	1.426	1.546 F	1.546 F	1.896 E	1.508 E	1.493
3,4,6-Glc							
2,3-Glc	1.145 M	1.145 M	1.532	1.532	1.228	1.228	1.302
2,4-Glc							
2,6-Glc	0.716	0.716	0.741 nm	0.741 nm	0.710 G	0.710 G	0.722
3,4-Glc							
3,6-Glc	0.408 L	0.408 L	0.490 O	0.490 O	0.376 H	0.376 H	0.425
4,6-Glc	l	l			h	h	
2-Glc		[c]	0.120	0.120		[c]	0.120
3-Glc			0.042 Q	0.042 Q			n.c.
6-Glc					0.057 I	0.057 I	n.c.
Glc			0.064	0.064	0.064	0.064	0.064
2,3,4,6-Gal	0.828 Dc	0.762 Dc	0.751	0.751	0.754	0.754	0.756

TABLE III *(continued)*

Sugar	*SP-1000*		*CP-Sil88*		*BP-1 (OV-1)*		*Average of B values*
	A	B	A	B	A	B	
2,3,5,6-Gal	0.021	0.021					n.c.
2,3,4-Gal	0.238	0.238	0.293 L	0.293 L	0.291	0.291	0.274
2,3,6-Gal	0.213 H	0.213 H	h	0.277 h	e	0.245 e	0.245
2,4,6-Gal	i	0.227 i	g	0.227 g	0.421 F	0.227 F	0.227
2,5,6-Gal	g	0.178 g		[e]		[c]	n.c.
2,3-Gal			0.151	0.151		[c]	0.151
2,6-Gal			0.039 Mn	0 039 Mn	g	g	n c.
3,4-Gal	0.326	0.326					n.c.
3,6-Gal			o	o			
4,6-Gal	l	l	mn	mn			
3-Gal			0.033	0.033			n.c
6-Gal					i	i	
Gal			0.201	0.201			n.c
2,3,4,6-Man	b	0.027 b	0.383 A	0 027 A	c	0.027 c	0.027
2,3,4-Man	j	0.050 j	0.050	0.050	0.420 J	0.050 J	0.050
2,3,6-Man	0.326 G	0.148 G	0.375 G	0.148 G		0 148[b]	0.148
2,4,6-Man	h	h					
3,4,6-Man					e	e	
2,3-Man			0.139	0.139			n.c.
2,6-Man			l	l			
3,4-Man	m	m					
4,6-Man			jk	jk			
2-Man			0 047	0.047			n.c.
6-Man			o	o			
Man			q	q			
Total	68.943	68.943	67.343	67.343	66.685	66.685	67.567

[a]Upper-case letters after a value indicate that additional identifications were made. The additional identifications are denoted by the corresponding lower-case letter, in the same column [b]Rear shoulder on 2,4,6-Glc visible on chromatogram. [c]Small peak or trace visible on chromatogram, but not integrated. [d]Peak lost under very large 2,3,6-Glc peak. [e]Retention coefficient not known on this column [f]n.c. = Not confirmed on the other phases.

A separate computer programme (TAB) was used to abstract information on peak areas from the individual chromatogram reports according to the identifications made. Data were abstracted according to the closest identification, but cross-referencing within the tabulation was used to show other identifications that had been made (see Tables III and IV). Data from any number of chromatograms could be tabulated, so that runs on the same or different phases could be readily compared. A complete list of partially methylated sugars was usually used as the basis for the tabulation, and values for individual partially methylated sugars were expressed either as a percentage of total identified sugars or as a percentage of sample by weight. Averages for selected runs could also be generated.

Tabulation of results from the three columns for a sample allowed direct comparisons to be made of the results from different phases. Peaks that co-chromato-

TABLE IV

METHYLATION RESULTS (A) AND RESOLVED VALUES (B) FOR CELL WALLS OF WHITE CLOVER ON THREE PHASES AS A PERCENTAGE OF TOTAL SAMPLE

Sugar	*SP-1000*		*CP-Sil88*		*BP-1 (OV-1)*		*Average of B values*
	A	B	A	B	A	B	
2,3,4-Ara	0.140	0.140	0.189	0.189	n	0.145 n	0.158
2,3,5-Ara	1.017	1.017	0.900	0.900	1.055	1.055	0.991
2,3-Ara	1.229 C[a]	1.229 C	1.285 D	1.285 D	1.225	1.225	1.246
2,4-Ara	e	e			b	b	
2,5-Ara	0.136	0.136	c	0.128 c	a	0.136 a	0.131
3,4-Ara			f	f			
3,5-Ara	0.132 A	0.132 A	a	0.202 a	0.367 A	0.231 A	0.188
2-Ara	Jk	0.143 jk	1.185 I	0.215 I	0.286	0.286	0.215
3-Ara			j	0.220 j	0.220 E	0.220 E	0.220
5-Ara	0.109 F	F					n.c.[b]
Ara	o	o			g	g	
2,3,4-Xyl	0.438	0.438	0.417	0.417	0.573 N	0.428 N	0.428
2,3-Xyl	2.463 E	2.463 E	2.070 E	2.070 E	2.051 B	2.051 B	2.196
2,4-Xyl	d	d	d	d			
3,4-Xyl	e	e					
2-Xyl	n	n	0.113 L	0.113 L	0.092	0.092	0.103
3-Xyl	2.886 N	[c] N	0.110 Kl	0.110 Kl	0.207 D	0.110 D	0.110
Xyl	0.037 Q	Q	p	p	i	i	
2,3,4-Rha	0.261	0.261	0.225	0.225	0.321	0.321	0.269
2,3-Rha	a	a	0.627 B	B	b	b	
2,4-Rha	b	0.207 b	0.246 a	0.246 a			0.227
3,4-Rha	0.259	0.259	0.111	0.111		[d]	0.185
2-Rha	f	0.109 f	0.104	0.104			0.107
3-Rha	0.361	0.361			0.191	0.191	0.276
4-Rha	0.165 G	0.165 G			d	0.097 d	0.131
Rha	l	l	0.108 G	G			
2,3,4-Fuc	0.197	0.197			0.156	0.156	0.177
2,3-Fuc			0.106	0.106			n.c.
2,4-Fuc	0.138	0.138	0.128 C	C			n.c.
3,4-Fuc			0.485	0.485			n.c.
2-Fuc	g	g	e	e			
3-Fuc	h	h					
4-Fuc	0.032	0.032					n.c.
Fuc	0.088	0.088					n.c.
2,3,4,6-Glc	0.810 B	0.603 B	b	0.627 b	0.578 C	0.578 C	0.603
2,3,4-Glc	n	n					
2,3,6-Glc	20.606 L	20.606 L	21.342 J	21.122 J	20.239 H	20.239 H	20.656
2,4,6-Glc	0.117	0.117	0.361 F	0.117 F	0.313 F	0.117 F	0.117
3,4,6-Glc	0.129	0.129	g	0 108 g		[d]	0.119
2,3-Glc	1.122 R	1.122 R	1.257 P	1.257 P	1.159	1.159	1.179
2,4-Glc					0.094 Lm	0.094 Lm	n.c.
2,6-Glc	0.321	0.321	0.395	0.395	0 382 J	0.382 J	0.366
3,4-Glc					0.112 Ml	0.112 Ml	n.c.
3,6-Glc	0.249 P	0.249 P	0.339 O	0.302 O	0.149 K	0.149 K	0.233
4,6-Glc	p	p			k	k	
2-Glc			0.091	0.091			n.c.
Glc			0.138	0.138			n.c.
2,3,4,6-Gal	1.082 Dc	1.082 Dc	0.876	0.876	0.949	0.949	0.969

TABLE IV *(continued)*

Sugar	*SP-1000*		*CP-Sil88*		*BP-1 (OV-1)*		*Average of B values*
	A	B	A	B	A	B	
2,3,5,6-Gal	0.026	0.026					n.c.
2,3,4-Gal	0.323	0.323	0.390 M	0.390 M	0.331	0.331	0.348
2,3,6-Gal	0.983 K	0.983 K	i	0.971 i	2.133 G	0.941 G	0.965
2,4,6-Gal	0.393 Ml	0.393 Ml	h	0.485 h	0.577 I	0.577 I	0.485
2,5,6-Gal	ı	i					
3,4,6-Gal	m	m					
2,3-Gal			0.051	0.051			n.c.
2,5-Gal	0.038	0.038					n.c.
2,6-Gal			0.141 N	0.141 N	j	j	n.c.
3,4-Gal	0.324	0.324					
3,6-Gal	q	0.037 q	o	0.037 o			0.037
4,6-Gal	p	p	n	n			
Gal			0.088	0.088			n.c.
2,3,4,6-Man	b	b	0.202 A	A	c	c	
2,3,4-Man	m	m	0.257	0.257			n.c.
2,3,6-Man	1.326 I	1.326 I	1.257 H	0.772 H	g	1.192 g	1.097
2,4,6-Man	0.143 Jk	Jk			h	h	
3,4,6-Man	0.232 H	0.232 H	f	0.244 f	f	0.196 f	0.224
2,3-Man			0.257	0.257	0.049	0.049	0.153
2,6-Man	0.111 O	0.111 O	m	m			n.c.
3,4-Man	r	r					
4,6-Man	o	o	kl	kl			
2-Man							
6-Man			o	o	m	m	
Man	0.186	0.186					n.c.
Total	38.694	35 537	35.851	35.851	33.809	33.809	35.910

[a]Upper-case letters after a value indicate that additional identifications were made. The additional identifications are denoted by the corresponding lower-case letter, in the same column. [b]n.c. = Not confirmed on more than one phase. [c]2/3-Xyl peak on SP-1000 was broad and misshapen, suggesting the presence of other components. [d]Retention coefficient not known on this column.

graphed on one phase could be resolved using information from one or both of the other phases, and choices between multiple identifications could be made based on the results from other phases. Because of the number of possible partially methylated sugars and the complexity of the samples examined, this resolution and merging of the results was not a simple process. Many peaks were involved in multiplets or had multiple possible identifications on one or more columns. However, because of the differing selectivity of the phases, the alternative identifications were usually different. Unambiguous characterisation was therefore possible through sequential elimination of the contributions of particular identifications. The logical complexity of the situation made the writing of a computer programme to perform this task a considerable undertaking. An additional consideration was that an experienced chromatographer could be assisted in decision making by additional information not available to the computer. Clues below the thresholds

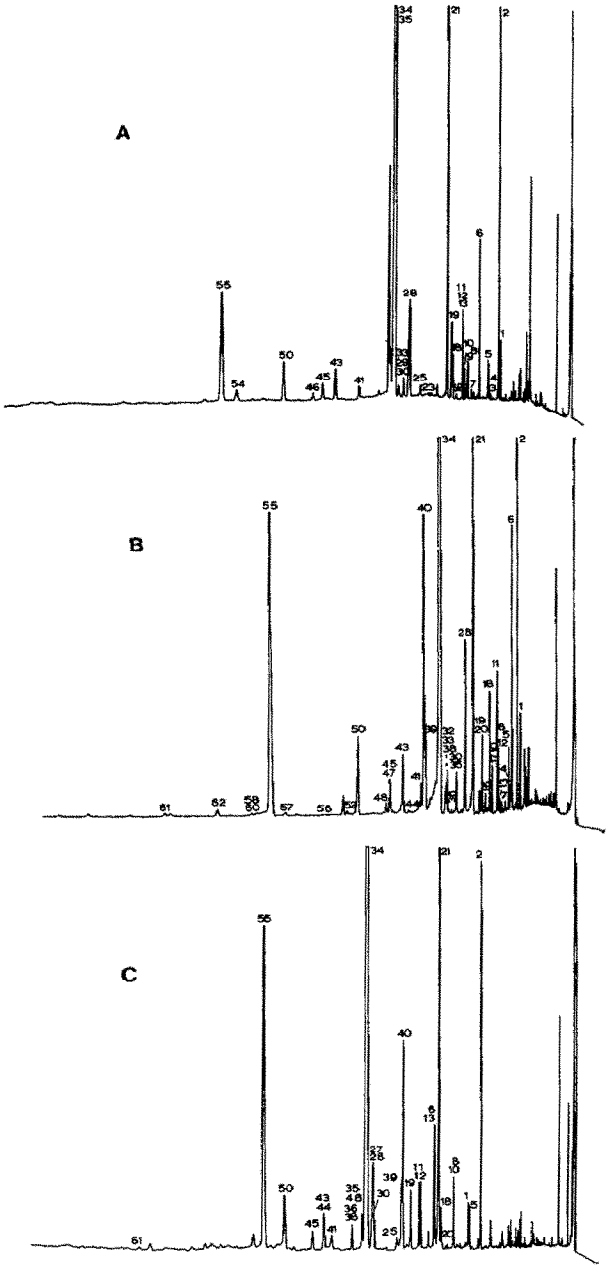

Fig. 1. Gas chromatograms of partially methylated alditol acetates from cell walls of Timothy grass on SP-1000 (A), CP-Sil88 (B), and BP-1 (OV-1) (C) (for conditions, see text): 1, 2,3,4-Rha; 2, 2,3,5-Ara; 3, 2,3,4-Fuc; 4, 2,3,4-Ara; 5, 2,3,4-Xyl; 6, 2,3,4,6-All (Std); 7, 3,4-Rha; 8, 3,5-Ara; 9, 2,3-Rha; 10, 2,5-Ara; 11, 2,3,4,6-Glc; 12, 2,3,4,6-Man; 13, 2,4-Rha; 14, 2,3-Fuc; 15, 3,4-Fuc; 16, 2,3,5,6-Gal; 17, 2,4-Fuc; 18, 2,3-Ara; 19, 2,3,4,6-Gal; 20, 2,4-Xyl; 21, 2,3-Xyl; 22, 2-Rha; 23, 5-Ara; 24, 4-Rha; 25, 3-Rha; 26, 3-Fuc; 27, 3,4,6-Man; 28, 2,4,6-Glc; 29, 2,5,6-Gal; 30, 2,3,6-Man; 31, 2,4,6-Man; 32, 2-Ara; 33, 2,3,6-Gal; 34, 2,3,6-Glc; 35, 2,4,6-Gal; 36, 2,3,4-Man; 37, 3-Ara; 38, 2,3,4-Glc; 39, 3-Xyl; 40, 2-Xyl; 41, 2,3,4-Gal; 42, Ara; 43, 2,6-Glc; 44, 2,6-Gal; 45, 3,6-Glc; 46, Xyl; 47, 3,6-Gal; 48, 2,3-Man; 49, 2,4-Glc; 50, 2,3-Glc; 51, 3,4-Glc; 52, 2,3-Gal; 53, 6-Glc; 54, 3,4-Gal; 55, quebrachitol (std); 56, 2-Man; 57, 2-Glc; 58, 3-Glc; 59, 3-Gal; 60, Man; 61, Glc; 62, Gal.

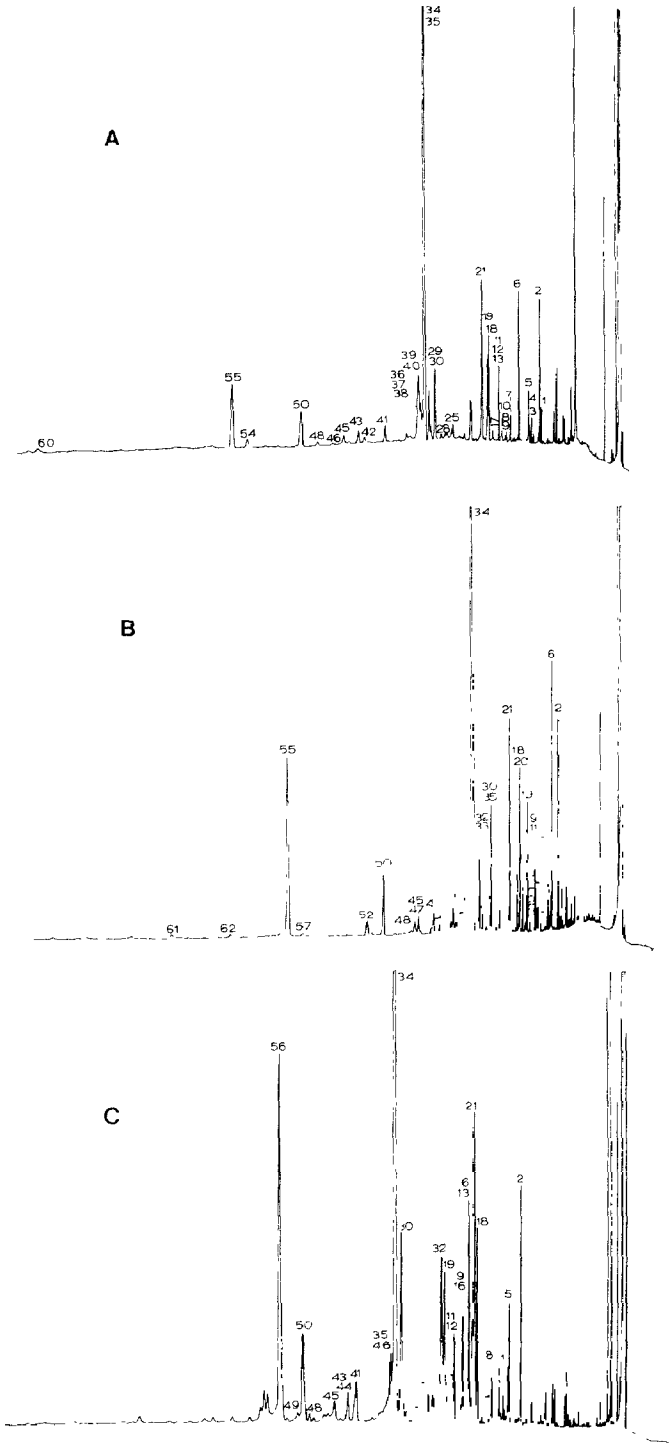

Fig. 2. Gas chromatograms of partially methylated alditol acetates from cell walls of white clover on SP-1000 (A), CP-Sil88 (B), and BP-1 (OV-1) (C) (for conditions, see text). Peak numbers are given in the legend to Fig. 1.

of the computing integrators could be obtained by direct appraisal of the chromatogram. Slight shoulders and other peak-shape irregularities could be easily assessed by eye. Account could be taken of small components which might be submerged in neighbouring very large peaks, and suspicion of non-ideal chromatographic behaviour could be considered. Also, the chromatographer could take account of mass-spectrometric results which were not available in a suitable machine-readable form.

An interactive "speadsheet" programme was therefore used, maintaining full operator-control and allowing "provisional" attempts at resolving problems to be altered. Using this programme, data from the three phases could be reconciled and merged into an overall analysis of the sample*.

Analysis of plant cell-wall samples. — The method is illustrated by the methylation analysis of two plant cell-wall samples, Timothy grass and white clover, a mono- and di-cotyledon, respectively. Figs. 1 and 2 show chromatograms of the partially methylated alditol acetates obtained. The different properties of the three phases can be seen from the different order of elution and retention of the various components. Peak shape was good on each phase although, for the OV-1 equivalent (BP-1), a thicker bonded phase was necessary to achieve this resolution.

Tables III and IV show the output of the TAB programme for the two samples. The results for each phase are shown, together with final analysis obtained by merging and reconciling these results. The need for operator involvement is demonstrated by the number of external decisions, shown in footnotes, that were required.

Almost all of the peaks in the chromatograms were identified; for Timothy grass, 93.5% of the total peak-area was identified on SP-1000, 96% on CP-Sil88, and 95.6% on BP-1. The corresponding figures for white clover were 92.3, 89.3, and 85.9%, respectively. Nearly all of the identifications were confirmed on one or both of the other phases; for both samples, <1% of the sample was unconfirmed.

The very wide range in concentration of the components made accurate analysis of the samples difficult. Many of the trace components were approaching background levels, and the 2,3,6-Glc peak was only just within the range of the integrators. Noise levels could be reduced by decreasing sensitivity and applying more sample, but a larger sample produced poorer peak shapes, particularly on BP-1, and this led to difficulties in identification.

The reproducibility of results from duplicate runs on the same phase was 0.45% relative standard deviation. Comparison of the results from the three phases gave an overall, relative standard deviation of 1.2% for Timothy grass and 1.5% for white clover. No real differences were found in the reproducibility of the estimates for those components that were fully separated on each phase and for those that were found in multiplets on one or more phases.

*Further details of all computer programmes will be published elsewhere; in the meanwhile, they are available from the authors.

TABLE V

COMPARISON OF SUGAR COMPOSITION AS PERCENTAGE DRY-MATTER FROM METHYLATION ANALYSIS (A) AND DIRECT ALDITOL ACETATE ASSAY (B)

Sugar	*Timothy grass*		*White clover*	
	A	B	A	B
Ara	4.02	4.18	3.15	2.73
Xyl	14.10	11.96	2.84	2.63
Rha	0.65	0.21	1.20	0.79
Fuc	0.23	—	0.14	—
Glc	46.59	32.25	23.27	19.09
Gal	1.65	1.71	2.80	2.66
Man	0.23	0.28	1.47	1.58
Total	67.47	50.59	34.87	29.48

Comparison of the aggregated sugar values from methylation analysis with the results of sugar assay as alditol acetates showed good agreement (Table V). As expected from previous work[9], overall recoveries, and particularly that for glucose, were somewhat higher from methylation analysis.

These results show that results obtained by methylation analysis can be improved by using three columns for the fractionation of the methylation products. The method does not increase the sensitivity of the analysis, but permits a much wider range of products to be accurately and individually determined on a routine basis.

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