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Note

Liquid chromatography in the methylation analysis of carbohydrates, and the use of combined refractometric–polarimetric detection

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We have previously described the high-pressure liquid-chromatographic separation of oligosaccharides with a C_{18} μBondapak column and water as eluent[1], and a review on various results obtained with carbohydrates has recently been published[2]. The present extension of our previous work includes adaptation of a polarimetric detector that permits the monitoring, by specific rotations $[\alpha]_\lambda$, of carbohydrates eluted. The technique is applied in methylation analysis of oligo- and poly-saccharides with direct, quantitative separation of the partially methylated components. The use of high-pressure liquid chromatography (l.c.) for this purpose has been reported by others[3,4].

EXPERIMENTAL

Compounds. — The following compounds were analyzed: scleroglucan from CECA (France), whose structure was recently confirmed by n.m.r. spectroscopy[5]; dextran DT 40 from Pharmacia (Sweden); amylose (Sigma type III) from potato; amylopectin from waxy-corn starch (Société des produits du Maïs, France), and cycloamyloses from Corn Products (U.S.A.). The methylated derivatives of D-galactose were kindly donated by Dr. G. Chambat. The derivatives of D-glucose were collected in l.c. experiments and identified by gas chromatography.

Methylation. — Methylation of the polymers was performed by the Hakomori method[6] with slight modifications[7,8]. The sample was dissolved in dimethyl sulfoxide and then treated with methyl iodide; permethylation was achieved by repeated treatments. Preliminary depolymerization was performed with formic acid for 1 h at 100°, and then the polysaccharide was completely hydrolyzed[8] during 4 h by 2M trifluoroacetic acid at 100°.

Chromatography. — Radial-Pak C_{18} or Dextropak cartridges, manufactured by Waters, were used at room temperature with a Waters chromatograph (model 201 U/6000) equipped with a differential-refractometer detector (type R 401). The flow

rate was 1 or 2 mL/min with water or 4:1 (v/v) water–methanol as eluent according to the degree of methylation. Peaks were integrated with an L.T.T. 2100 integrator (Lignes Télégraphiques et Téléphoniques, Paris, France). A microcell (10 μL) for recording $[\alpha]_{350}$ was constructed in our laboratory, and adapted to a Fica spectropolarimeter, model Spectropol 1b. Both detectors were operated simultaneously.

RESULTS AND DISCUSSION

Structural analysis. — This work was intended to simplify the methylation analysis of polysaccharides. The methylated saccharides are generally derivatized for gas-chromatographic analysis and identification, as, for example, as the alditol acetates[9,10]. A reverse-phase column has been found useful for analysis of methylated monosaccharides, but each compound gives two peaks, because of anomeric equilibration. In order to simplify the chromatograms, the sugars may be reduced to their alditols with aqueous sodium borohydride.

A systematic study of the elution of di-, tri-, and tetra-methyl ethers of glucose and galactose was first performed, and the results are given in Table I. It was impossible to elute di-, tri-, and tetra-methyl ethers with the same eluent; the di- and tri-methyl ethers were eluted with water or 49:1 (v/v) water–methanol, and tri- and tetra-methyl ethers with 4:1 (v/v) water–methanol. Reduction increased the solubility and the elution volume decreased. With two separate elution-modes, the molecular

TABLE I

ELUTION VOLUMES[a] OF METHYLATED DERIVATIVES OF GLUCOSE AND GALACTOSE ON A "DEXTROPAK" CARTRIDGE

Ether derivatives	*Eluent 2% MeOH*			*Eluent 20% MeOH*		
	Aldose		*Alditol*	*Aldose*		*Alditol*
	α	β		α	β	
Glucose						
2,3-Me_2	2.6	2.4	1.8			
2,4-Me_2	1.9	1.7	2.1			
2,3,4-Me_3	13.9	21.8	4.2	2.4	3.2	1.5
2,3,6-Me_3	6.0	5.2	4.5	1.6	1.6	1.5
2,4,6-Me_3	10.8	7.0	8.3	2.0	1.7	1.8
2,3,4,6-Me_4				6.9	7.4	2.7
Galactose						
2,3-Me_2	1.3	1.8	1.5			
2,3,4-Me_3	4.2	5.3	4.3	1.4	1.6	1.4
2,3,6-Me_3	3.9	6.1	4.8	1.4	1.5	1.5
2,4,6-Me_3	5.5	3.9	2.2	1.7	1.5	1.6
2,3,4,6-Me_4				3.2	3.2	3.2

[a]Relative elution-volumes are expressed by reference to the elution of solvent ($V_R = V_{solute}/V_{solvent}$).

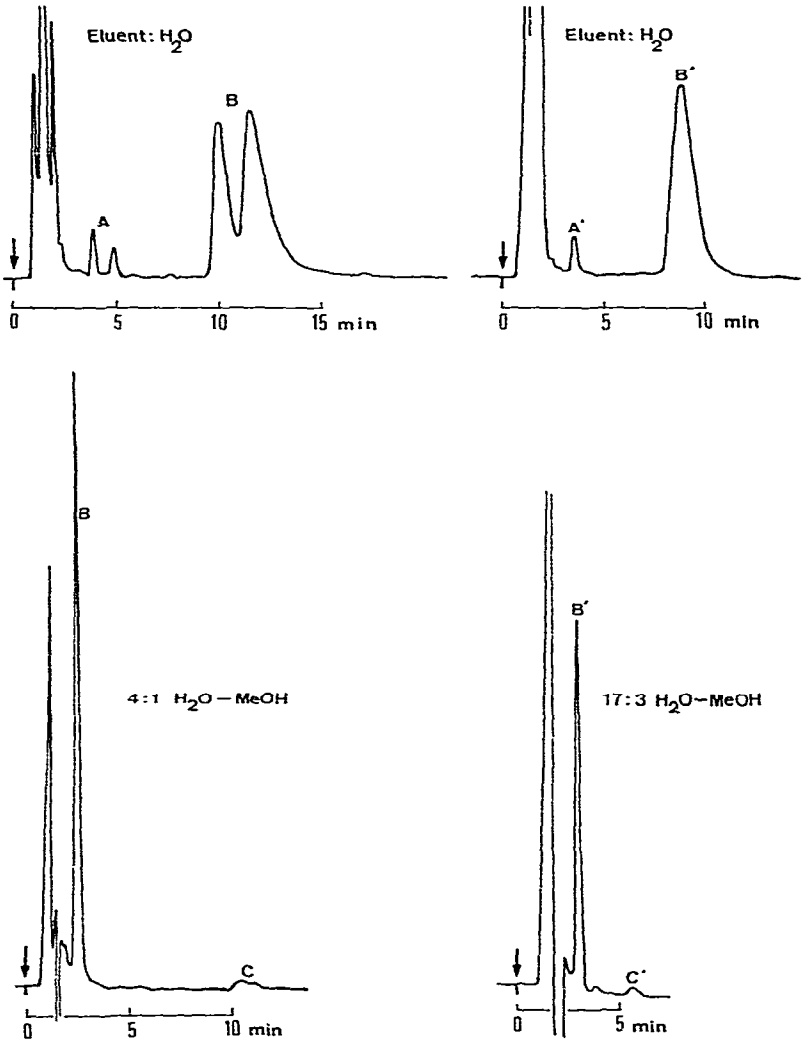

Fig. 1. Separation of the products of methylation analysis, and their alditols, for amylopectin on a C_{18} Radial-Pak cartridge. Flow rate = 2 mL/min; A = 2,3 di-*O*-methyl-D-glucose; B = 2,3,6-tri-*O*-methyl-D-glucose; C = 2,3,4,6-tetra-*O*-methyl-D-glucose. The primed letters denote the corresponding alditols.

structure of polysaccharides may be established by quantitative analysis of the proportions of di-, tri-, and tetra-methylated monosaccharides.

This work was restricted to homopolysaccharides of D-glucose. Amylose and cycloamyloses gave a chromatogram showing only 2,3,6-tri-*O*-methylglucose. Amylopectin, as shown in Fig. 1, gives equal amounts of 2,3-di-, and 2,3,4,6-tetra-*O*-methylglucose. The ratio 2,3,6-tri-*O*-methylglucose/2,3-di-*O*-methylglucose of 21 corresponds to an average distance between two branch points of 21 monomeric residues.

The result obtained with scleroglucan (Fig. 2) shows as much 2,4-di-*O*-methyl-

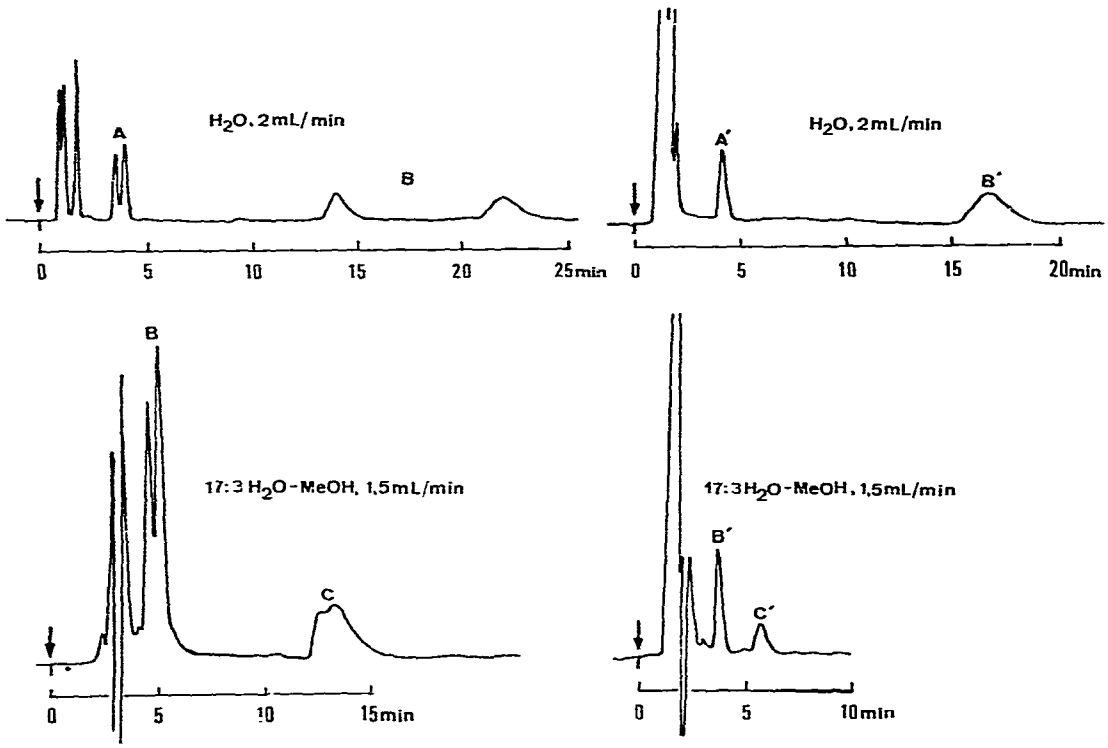

Fig. 2. Separation of the products of methylation analysis, and their alditols, for scleroglucan on a C_{18} Radial Pak cartridge. A = 2,4-di-*O*-methyl-D-glucose; B = 2,4,6 tri-*O*-methyl-D-glucose; C = 2,3,4,6-tetra-*O*-methyl-D-glucose. The primed letters denote the corresponding alditols.

glucose as 2,3,4,6-tetra-*O*-methylglucose, and the ratio of 2,4,6-tri-*O*-methyl glucose to 2,4-di-*O*-methylglucose (~2) agrees with the proposed structure[4].

We conclude that, with proper choice of experimental conditions for methylation:

(*a*) There is no accumulation of 2,3,4,6-tetra-*O*-methylglucose when amylose or cycloamyloses are used, and so there is no partial hydrolysis of the polymer chain during methylation.

(*b*) Methylation is complete and there is no partial hydrolysis of methyl groups, as no di-*O*-methylglucoses are found in the hydrolyzates of methylated amylose.

(*c*) Structural information on the polysaccharide may be deduced directly, without further derivatization, by a simple separation in water of the partially methylated monosaccharides.

This method is thus very effective with homopolysaccharides, but is much more difficult to apply with heteropolysaccharides, as with other analytical methods, because of the necessity for suitable standards. Moreover, the separation and quanti-

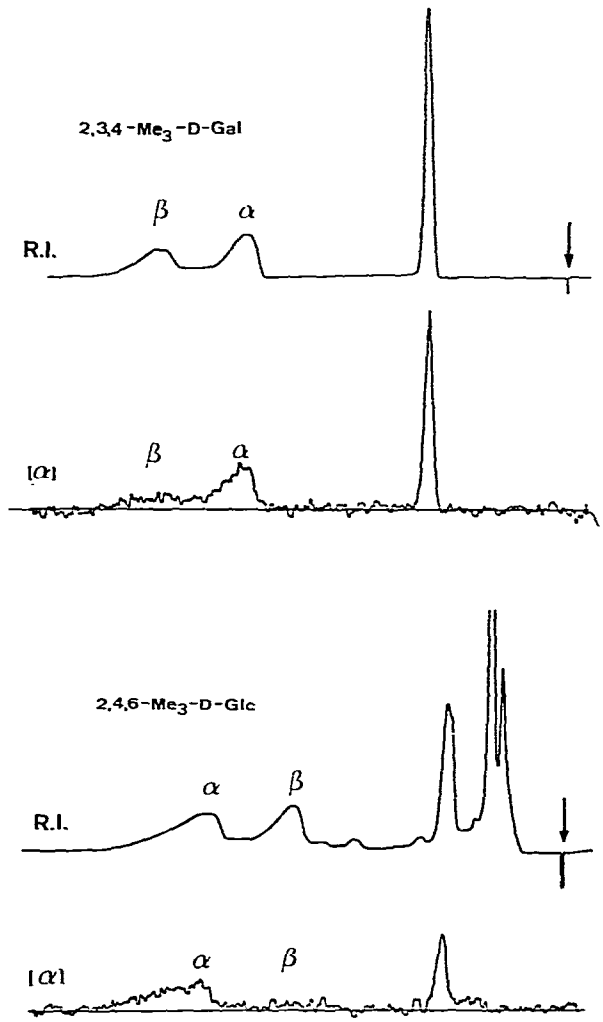

Fig. 3. Assignment of the anomeric form by a coupled polarimetric–refractometric detector. Top: 2,3,4-tri-*O*-methyl-α- and β-D-galactose (upper trace, refractometric detector; lower trace polarimeter). Bottom: 2,4,6-tri-*O*-methyl-α- and β-D-glucose (upper trace, refractometric detector, lower trace, polarimeter).

tation of hexoses containing equal numbers of methyl substituents may be critical in some instances.

Improvements in detection of sugars. — Refractometric detection is most widely used, but it does not differentiate between solvent, oligosaccharides, or other compounds. As it is important to identify each compound in sugar analysis, we undertook the linkage of a polarimeter to the refractometric detector. A sugar is detected by its rotation. If we assume that dn/dc is almost identical for most oligosaccharides, it is

possible with an internal standard to ascertain the identity of a sugar from its specific rotation (n, is the refractive index and c, the concentration of the compound).

Thus the following equation:

$$[\alpha]_D = \frac{S_p \times S_{RI}\ (\text{standard})}{S_{RI} \times S_p\ (\text{standard})} \times [\alpha]_D\ (\text{standard}), \qquad (1)$$

where S_p and S_{RI} are respectively the peak area of the polarimetric signal and the refractometric signal, permits calculation of the optical rotation of the unknown sugar. This system has good accuracy, but poor sensitivity, and we have used a spectropolarimeter and performed measurements at 350 nm to afford better sensitivity. Application of Eq. (*1*) remains a source of error when the specific rotation at 589 nm has to be estimated by using the Drude equation for comparison with literature values. In this context, it is evident that sensitivity of the method depends on the magnitude of the specific rotation. Good accuracy (±5%) has been obtained with 100 μg of sample injected if its $[\alpha]_D$ value is $>50°$.

We have used this double detection for attribution of the α and β anomers of 2,4,6-tri-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-galactose (Fig. 3). As β-D anomers have lower rotations than the α-D anomers[11], the peaks may be individually attributed. It may be noted that the elution order of the anomers is reversed for the two sugar derivatives. Another interesting and important feature is that the double detector provides a sub-mg method of determining chirality (D or L) of the sugars.

CONCLUSION

As methylation analysis is very important in the structural analysis of polysaccharides, a rapid and reliable analytical method is of considerable significance. It is shown that l.c. on a C_{18} support provides a convenient system for analysis; no further derivatization is necessary to provide volatile derivatives, and possible decomposition of samples is avoided. Although heteropolysaccharides pose some problems, the reverse-phase support may be useful as a complementary control tool. Furthermore, we have demonstrated from l.c. analysis that, with suitable experimental conditions for methylation and hydrolysis, the validity of the structural determination is not prejudiced by secondary reactions. On the other hand, the carbohydrate analysis may be improved by coupling a refractometric detector with a polarimeter.

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