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AN IMPROVED PROCEDURE FOR THE METHYLATION ANALYSIS OF OLIGOSACCHARIDES AND POLYSACCHARIDES

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ABSTRACT

An improved procedure for the methylation analysis of oligosaccharides and polysaccharides is described. Steps in the procedure were examined and optimised for quantitative recovery and speed. Methylation was shown to be complete by using [^{14}C]methyl iodide. All operations were performed in the same tube and the need to concentrate solutions containing acetylated alditols of methylated sugars was eliminated, thus minimising losses due to volatilization. The method is convenient, gives high recoveries of acetylated alditols of methylated sugars, and allows analysis of the glycosyl linkages of oligo- or poly-saccharides to be completed within a working day. A wide range of oligo- and poly-saccharides were methylated by this procedure.

INTRODUCTION

Methylation analysis is widely used to determine the position of linkages between component monosaccharide residues in oligo- and poly-saccharides. This is usually achieved by treating the carbohydrates with methylsulphinyl carbanion to form polyalkoxide ions, followed by methylation with methyl iodide[1,2]. The methylated carbohydrates are then hydrolysed and the partially methylated monosaccharides reduced and acetylated. The resulting alditol acetates of methylated sugars are separated by gas chromatography and identified by their retention times and mass spectra[3,4]. Quantitative aspects of methylation procedures as currently used are poorly understood. Factors that influence the recovery of alditol acetates of methylated sugars include undermethylation, incomplete hydrolysis, degradation and demethylation during hydrolysis, incomplete reduction and acetylation, selective losses of volatile components during evaporation of solvents, and losses on glassware. We now report the development of an analytical procedure for methylation analysis where attention has been given to optimizing the critical steps.

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We have recently used a similar approach for the analysis of monosaccharide mixtures in which the monosaccharides are quantitatively reduced and acetylated in a single tube without concentration[5].

MATERIALS AND METHODS

Reagents. — 2,2-Dimethoxypropane was obtained from Aldrich Chemical Inc., Milwaukee, Wis. U.S.A.; [^{14}C]methyl iodide from Amersham, Australia Pty. Ltd.; methyl iodide (Puriss, inhibited with silver), 1-methylimidazole (stored over molecular sieve type 4A) and 4-methylmorpholine from Fluka A.G., Buch, Switzerland; "Insta-gel" from Packard Instruments, Melbourne, Australia; and Blue Dextran 2000 from Pharmacia, Uppsala, Sweden. 2,3,4,6-Tetra-*O*-methyl-D-glucose was the kind gift of the late Dr. D. J. Bell. All other reagents were the purest grade commercially available. 4-Methylmorpholine *N*-oxide monohydrate (MMNO) was prepared from 4-methylmorpholine[6] and recrystallized from acetone, m.p. 75–76°. *myo*-Inositol hexa-acetate and D-glucitol hexa-acetate were prepared by the method of Blakeney *et al.*[5].

Samples for methylation. — Samples were obtained from the following commercial sources: cellulose (Avicel, a microcrystalline preparation) from Macherey Nagel and Co., Düren, Federal Republic of Germany; sucrose and potato amylose from BDH Ltd., Poole, U.K.; arabinose, inulin, melibiose, raffinose, stachyose, cellobiose, glycogen, pullulan, methyl β-L-arabinopyranoside, and methyl α-D-xylopyranoside from Sigma Chemical Co., St. Louis, Mo., U.S.A.; and 3-*O*-β-D-galactopyranosyl-D-arabinose from United States Biochemical Co., Cleveland, Ohio, U.S.A. Laminaratetraose was prepared by partial acid hydrolysis of laminaran and isolated by charcoal-column chromatography[7]. (1→3),(1→4)-β-D-Glucan was isolated from barley endosperm[8]. Arabinoxylan was isolated from wheat endosperm[9]. Cell walls of Italian ryegrass (*Lolium multiflorum* Lam.) were isolated as described previously[5].

Preparation of methylsulphinyl carbanion. — Sodium methylsulphinyl carbanion was prepared from sodium hydride[10]. Potassium methylsulphinyl carbanion was prepared from potassium hydride[11] and from potassium *tert*-butoxide[12].

Potassium methylsulphinyl carbanion was prepared routinely from potassium hydride as follows: a suspension of potassium hydride in oil (5 g), containing ~1 g of potassium hydride, was washed 4 times with hexane (20 mL) by centrifugation (500*g*, 2 min) in a 50-mL borosilicate-glass centrifuge tube. The potassium hydride was then resuspended in hexane (20 mL), transferred to a 250-mL conical flask, allowed to sediment, and the excess of hexane removed by aspiration. Residual hexane was removed with a stream of argon and the potassium hydride cooled on ice. (*Caution: After washing with hexane, the potassium hydride is very reactive and should be handled only in small quantities and with extreme care.*) Dimethyl sulphoxide (Me_2SO, 13 mL) was added and the solution mixed under argon as it warmed to room temperature. When the reaction ceased (usually after 30 min) the

solution was transferred to a 20-mL borosilicate-glass tube fitted with a Teflon-lined screw cap, and centrifuged (1500*g*, 3 min) to produce a clear, grey-green solution. The methylsulphinyl carbanion was stored at −20° under argon because it reacts with oxygen, carbon dioxide, and water[13]. The high density of argon relative to air makes it the preferred inert atmosphere. The concentration of methylsulphinyl carbanion was determined by titration under argon with anhydrous methanol, using triphenylmethane as indicator[14]. The usual concentration was 1.6M, which is lower than the theoretical value of 1.9M based on the reaction of 1 g of potassium hydride in 13 mL of Me_2SO.

Use of MMNO as a solvent for methylation. — MMNO was tested as a solvent for the methylation of samples insoluble in Me_2SO. Samples (1–2 mg) were intimately mixed with solid MMNO (100 mg) and heated for 10 min at 100° under argon with constant magnetic stirring. The solution was diluted with Me_2SO (50 μL) before cooling to room temperature. In these experiments, diethyleneglycol dimethyl ether was used as the solvent for reduction by sodium borohydride[15,16], and the alditols were acetylated by using 1-methylimidazole as the catalyst[5].

Radiochemical methylation experiments.. — In experiments to determine the extent of methylation, unlabelled methyl iodide (50 mL) was added to [^{14}C]methyl iodide (18.5 MBq) before use. The specific radioactivity of this solution was determined by counting an aliquot [after further dilution (1:100 v/v) with water] in Packard "Insta-gel".

The extent of methylation of pullulan was determined by counting fractions separated by gel-permeation chromatography. Fractions were transferred to scintillation vials, dried with a stream of hot air, and counted by using a toluene-based scintillation cocktail.

The extent of methylation of amylose and barley (1→3),(1→4)-β-D-glucan was determined as follows. Aliquots of the mixture, following the addition of methyl iodide, were dried onto glass-fibre discs (Whatman GF/A, 2.1 cm in diameter) by using an infra-red lamp. [^{14}C]Methyl iodide was removed during drying. Control methylation experiments without carbohydrate showed that non-volatile radioactive by-products, for example methyl ethyl sulphoxide, are also formed during methylation. These were removed by washing the glass-fibre discs in water before counting in Packard "Insta-gel".

All results are expressed on the basis of a calculation of the number of hydroxyl groups available for methylation in the polysaccharide sample.

Gel-permeation chromatography of methylation reaction mixtures. — Methylated pullulan was separated from low-molecular-weight components in the mixture following the addition of methyl iodide by using a column (30 × 2 cm) of Sephadex LH-20 with 2:1 (v/v) chloroform–methanol as the solvent[17]. The void volume of the column was determined by using an acetylated Blue Dextran 2000 prepared by the procedure of Connors and Pandit[18]; the bed volume was estimated by using Sudan III.

Gas chromatography. — Acetylated alditols of methylated sugars were sepa-

rated on a SCOT glass-capillary column[5] (28 m × 0.5 mm, i.d., Silar 10C) or on a WCOT capillary column[19] (6 m × 0.2 mm i.d. BP75 vitreous-silica, S.G.E. Pty. Ltd., Melbourne, Australia) in a Hewlett–Packard 5710A chromatograph equipped with a flame-ionisation detector and a modified S.G.E. "Unijector" capillary-injection system, used in the split mode. High-purity hydrogen was used as the carrier gas at a flow rate of 77 cm/sec (determined by using dichloromethane). The oven temperature was maintained for 4 min at 150° following injection and then raised at 4°/min to 230°. The injection port and detector were heated at 250 and 300°, respectively. Peak areas were recorded with a Hewlett–Packard model 3386A reporting integrator. The identity of the peaks was confirmed by using a Jeol JGC-20K gas chromatograph linked to a Jeol JMS-D100 mass spectrometer used in the electron-impact mode. The gas chromatograph was fitted with a BP75 column, which was inserted directly into the ion source of the mass spectrometer.

PROCEDURE FOR METHYLATION ANALYSIS

(*a*) *Preparation and dissolution of the sample.* — Dry the sample under vacuum over phosphorus pentaoxide for 16 h. Dissolve 1–5 mg in Me_2SO (50–200 μL) under argon in a borosilicate-glass tube (29 × 100 mm) fitted with a Teflon-lined screw cap and perform all subsequent steps in the same tube. Dissolution of samples is assisted by heating in a water bath (100°) and mixing by using a vortex mixer or by sonication.

If the sample is insoluble in Me_2SO (as with cellulose or plant cell-walls), ball-mill for 45 sec in an Ultramatt Amalgamator (Southern Dental Industries, Bayswater, Victoria, Australia) fitted with a stainless-steel capsule (28 mm × 8 mm i.d.) containing a single, stainless steel ball-bearing (diameter 6 mm) and sealed with a rubber stopper. Then dissolve the sample, using two preliminary methylations, as described in Section (*c*).

(*b*) *Methylation of samples soluble in Me_2SO.* — Add potassium methylsulphinyl carbanion (200 μL), generated from potassium hydride as already described, to the sample dissolved in Me_2SO under argon and mix. (Use a vortex mixer for all mixing steps). After 10 min, cool the solution on ice and add ice-cold methyl iodide (150 μL). Allow the mixture to warm to room temperature and mix during thawing. Ten min after adding the methyl iodide, add 2:1 (v/v) chloroform–methanol (3 mL) followed by water (2 mL), mixing after each addition. Centrifuge (200*g*, 30 s) to aid phase separation. Remove the upper phase by aspiration, taking care not to disturb the interface. Repeat this washing procedure 4 times using water (2 mL). After the final aspiration, add 2,2-dimethoxypropane (2 mL), 18M acetic acid (20 μL), and 2 or 3 anti-bumping granules. Mix, and place the tube in a water bath (90°) until all but ~200 μL of solvent has evaporated. Evaporate the remaining solvent under a stream of argon. The residue is frequently oily.

(*c*) *Methylation of samples insoluble in Me_2SO.* — Add Me_2SO (50–200 μL) under argon to the dry, finely ground sample (1–2 mg). Dissolve by two rapid, pre-

liminary methylations as follows: add potassium methylsulphinyl carbanion (20 μL), mix, cool on ice, add ice-cold methyl iodide (5 μL), mix, add potassium methylsulphinyl carbanion (60 μL), mix, cool on ice, add ice-cold methyl iodide (15 μL), and mix. The sample should now be in solution. Finally, methylate as for samples soluble in Me_2SO as described in section (*b*).

(*d*) *Hydrolysis.* — Add 2M trifluoroacetic acid (0.3 mL) to the methylated sample and autoclave[20] for 1 h at 121°. Cool, place the sample tube in a water bath (40°), and evaporate to dryness with a stream of nitrogen.

(*e*) *Reduction and acetylation.* — Add a freshly prepared solution of 0.5M sodium borohydride (1 mL) in 2M ammonia to the dry hydrolysate and reduce for 60 min at 60°. Add acetone (0.5 mL) to stop the reaction and then place the tube in a water bath (40°) and evaporate to dryness in a stream of nitrogen. Dissolve the residue in 18M acetic acid (0.2 mL) and then acetylate by adding ethyl acetate (1 mL) and acetic anhydride (3 mL), mix, add perchloric acid (70%, 100 μL) and mix. After 5 min, cool on ice and then add water (10 mL) followed by 1-methylimidazole (200 μL), mix and allow to stand for 5 min. Add dichloromethane (1 mL), containing *myo*-inositol hexa-acetate (1 mg) as the internal standard, and mix. After phase separation, remove the lower phase with a Pasteur pipette and store at −20° in a screw-capped glass vial prior to gas chromatography.

RESULTS AND DISCUSSION

Generation of methylsulphinyl carbanion. — Methylsulphinyl carbanion has usually been prepared[1,2] by the reaction of sodium hydride with Me_2SO. However, the subsitution of potassium hydride for sodium hydride has the advantages that the anion can be prepared rapidly and without heating, by a slight modification of the method of Phillips and Fraser[11]. In this procedure, the suspension of potassium hydride is washed three times with hexane and then treated with Me_2SO at room temperature. However, we found that the reaction is vigorous at the hydride–Me_2SO interface, causing frothing and preventing rapid mixing. Potassium methylsulphinyl carbanion is more conveniently prepared by mixing potassium hydride, which had been thoroughly washed with hexane, with Me_2SO at 0°. However, if the potassium hydride is not thoroughly washed, its reaction with Me_2SO is slow at room temperature, and residues of oil may interfere with subsequent analysis.

Although potassium methylsulphinyl carbanion may also be prepared simply from potassium *tert*-butoxide[12], this method results in the formation of an equilibrium mixture of methylsulphinyl carbanion and butoxide that we have shown to be unsuitable for methylation.

Dissolution of polysaccharides for methylation analysis. — If the polysaccharide is soluble in Me_2SO, its complete methylation can be achieved in a single step. However, additional treatments are necessary for polysaccharides not readily soluble in Me_2SO. MMNO is a good solvent for most polysaccharides[21] and has been proposed[22] as a solvent for the methylation analysis of polysaccharides insolu-

ble in Me_2SO. We found that to dissolve a polysaccharide in MMNO it was necessary to grind it to a fine powder and then intimately mix it with the solid MMNO. However, when MMNO was used as a solvent, the yields of partially methylated alditol acetates from cellulose and walls of ryegrass cells were low as compared with samples dissolved by multiple methylation. For example, in the presence of MMNO, the yield of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol from cellulose was only 45% of that obtained by multiple methylation. When cellobiose was methylated in Me_2SO in the presence of MMNO, the yields of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol were 2 and 3% respectively, of the yields obtained in Me_2SO without MMNO. These low yields may be a result of the oxidising capacity[23] of MMNO. Repeated methylation is often used to dissolve polysaccharides for structural analysis[24]. We found that two rapid preliminary methylations were sufficient to dissolve cellulose and the ryegrass walls in Me_2SO. After three methylations, cellulose yielded 42% more 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol than after a single methylation. This procedure was as easy to perform as dissolution in MMNO. However it would degrade glycosyluronic acid-containing polysaccharides by β-elimination[25,26].

Quantitative methylation. — (*a*) *Determination of the extent of methylation.* Estimation of the extent of methylation is difficult[26]. Under-methylation is often detected by i.r. spectroscopy, but traces of water interfere, and the method is rela-

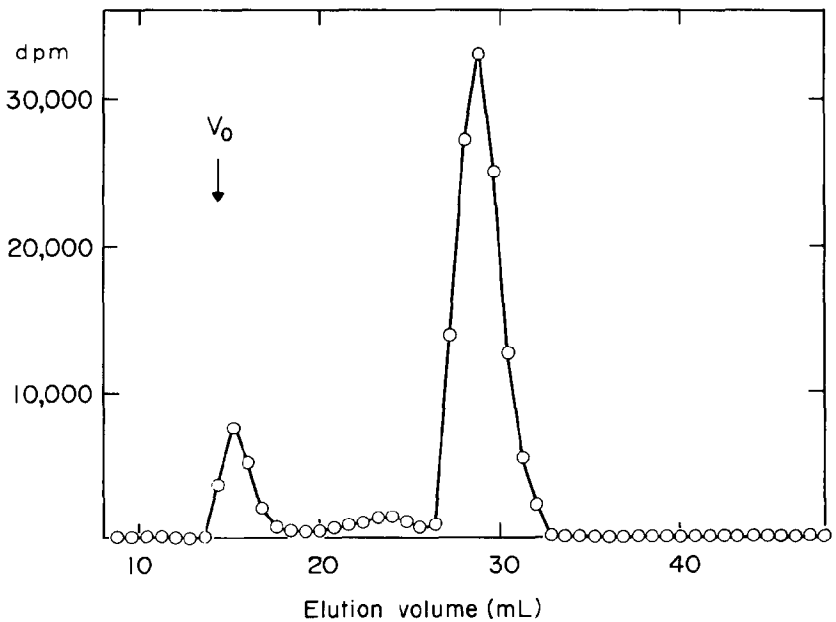

Fig. 1. Gel filtration of ^{14}C-labelled products of methylation of pullulan on a column of Sephadex LH-20 with 2:1 (v/v) chloroform–methanol as solvent. The apparent V_0 is indicated by the arrow and was determined by using acetylated Blue Dextran 2000.

tively insensitive. Measurement of the incorporation of ^{14}C from [^{14}C]methyl iodide into the polysaccharide is a more-direct method. By this method, the extent of methylation was determined with the polysaccharide, pullulan, which is readily soluble in Me_2SO. The labelled pullulan was separated from methyl iodide and other low-molecular-weight components by gel filtration on a column of Sephadex LH-20 (Fig. 1). Labelled polysaccharide was eluted near the void volume of the column (at 15–20 mL) and was well separated from the major peak of radioactivity (Fig. 1) close to the bed volume (at ~30 mL). In calculating the extent of methylation, the radioactivity in the peak near the void volume was corrected for the small amount of radioactivity found eluting in this region when the products of control methylations with glucose or with no carbohydrate were chromatographed.

(*b*) *Time required for the formation of polyalkoxide ions and their reaction with methyl iodide.* The times allowed for formation of polyalkoxide ions have varied from a few min[28] to overnight[24], and times of exposure of the polyalkoxide ions to methyl iodide have also varied widely. Methylation of pullulan by treatment with methylsulphinyl carbanion (generated from potassium hydride) for 10 min, followed by addition of [^{14}C]methyl iodide and reaction for 10 min, gave an apparent degree of methylation of 101%. We found that exposure of amylose to methylsulphinyl carbanion (generated from potassium hydride) for 5 min or 1 h, followed by reaction of the resulting polyalkoxide ions with methyl iodide for 5 min or 1 h, did not give significantly different results. Extended exposure of carbohydrates to the methylsulphinyl carbanion, as recommended by many workers, increases the possibility of anion breakdown because of the possible slow diffusion of oxygen and carbon diodide from the atmosphere into the tube.

(*c*) *Source of methylsulphinyl carbanion.* Methylsulphinyl carbanion generated from potassium hydride is said to give more-complete methylation than that generated from sodium hydride[29]. On the other hand, it has also been reported that, at low carbanion concentrations, sodium methylsulphinyl carbanion effects a more complete reaction with hydroxyl compounds than does potassium methylsulphinyl carbanion[30,31]. We have routinely used potassium methylsulphinyl carbanion because of its ease of preparation, however sodium methylsulphinyl carbanion also gave complete methylation when used in the procedure described. A further advantage of the potassium carbanion is that the methylated carbohydrate may be extracted from the mixture free from contaminating potassium iodide, as potassium iodide is less soluble in chloroform than is sodium iodide[11].

Potassium methylsulphinyl carbanion prepared from potassium *tert*-butoxide and from potassium hydride were compared for the methylation of pullulan by using [^{14}C]methyl iodide. Reaction for 10 min with methylsulphinyl carbanion followed by exposure to methyl iodide for 10 min gave apparently complete methylation when the methylsulphinyl carbanion was prepared from potassium hydride, but only 49% methylation when it was prepared from potassium *tert*-butoxide; longer reaction-times did not substantially increase the extent of methylation. Thus it appears that the critical step in the methylation of carbohydrates is in the formation of polyalkoxide ions.

(*d*) *Concentration of methylsulphinyl carbanion.* The incomplete methylation of pullulan observed when using methylsulphinyl carbanion produced by treating potassium *tert*-butoxide with Me_2SO may have been due to the lower concentration of methylsulphinyl carbanion produced by this reaction[32,33] as compared to that produced using the hydride. A high initial concentration of methylsulphinyl carbanion may be necessary for the quantitative formation of polyalkoxide ions.

Rauvala[34,35] stated that no undermethylation results if, after treatment of the carbohydrate with the methylsulphinyl carbanion, the mixture gives a red colour with triphenylmethane as a result of formation of the triphenylmethane carbanion. However, when potassium *tert*-butoxide was used to generate the methylsulphinyl carbanion, we found a positive reaction with triphenylmethane under conditions that resulted in the incomplete methylation of pullulan. Thus a positive triphenylmethane reaction does not necessarily indicate that complete methylation will result. Triphenylmethane is a very sensitive indicator of the presence of methylsulphinyl carbanion[14], but does not indicate if a sufficient concentration is present to convert all the hydroxyl groups in the carbohydrate into alkoxide ions.

Isolation of methylated oligosaccharides and polysaccharides from the mixture. — In addition to the methylated oligo- or poly-saccharide, the mixture after methylation contains: methyl iodide, Me_2SO, potassium iodide, methyl ethyl sulphoxide formed by reaction of methyl iodide with potassium methylsulphinyl carbanion (reaction *1*)[13], and trimethyloxosulphonium iodide formed by reaction of methyl iodide with Me_2SO (reaction *2*)[36].

$$CH_3I + K^+ C^-H_2 SOCH_3 \rightleftharpoons CH_3CH_2SOCH_3 + K^+I^- \qquad (1)$$

$$(CH_3)_2 SO + CH_3I \rightleftharpoons (CH_3)_3 SO^+I^- \qquad (2)$$

The excess of reactants and by-products interfere in subsequent steps in the analysis. Some of these may be removed by dissolving the methylated carbohydrate in chloroform–methanol and extracting with water. After partitioning, the methylated oligosaccharide or polysaccharide and most of the unreacted methyl iodide remains in the organic solvent phase, whereas Me_2SO and methyl ethyl sulphoxide are extracted into the water phase. Using pullulan methylated with [^{14}C]methyl iodide, we tested how much radioactivity was removed from the organic solvent phase by water washing. Three water washes removed a total of 10.6% of the total label added, of this 7.7% was removed in the first wash, 1.9% in the second, and 1.0% in the third. Thus the five washes routinely used in the procedure should effectively remove all water-soluble components containing carbon atoms arising from methyl iodide. The main radioactive component removed by water-washing was probably methyl ethyl sulphoxide. The methyl iodide and chloroform are removed from the methylated oligosaccharide or polysaccharide by evaporation. Methyl iodide must be removed, as even traces were found to interfere with the subsequent reduction step.

Care was taken not to disturb the interface and lower phase when removing the water washes, and this resulted in a layer of water remaining after the final wash. This residual water is difficult to evaporate, but may be removed by using 2,2-dimethoxypropane, which reacts quantitatively with water in the presence of acid to form methanol and acetone[37]. On heating with 2,2-dimethoxypropane, a single phase was formed and speeded evaporation. Where more water remains, the addition of extra 2,2-dimethoxypropane may be necessary; 1 mL of water reacts with 6.8 mL of 2,2-dimethoxypropane[38].

Analysis of methylated saccharides. — The methylated oligo- and poly-saccharides were hydrolysed in 2M trifluoracetic acid. The trifluoracetic acid was removed by evaporation at 40° to decrease the potential loss of low-boiling-point components.

The partially methylated monosaccharides were reduced rapidly by using sodium borohydride in ammonia. Unlike underivatized monosaccharides[5], we found that partially methylated monosaccharides were incompletely reduced when diethyleneglycol dimethyl ether or Me_2SO was used as solvents for sodium borohydride. Reduction of partially methylated pentoses and 2,3,4,6-tetra-*O*-methyl-D-glucose by sodium borohydride in 2M ammonia was apparently complete in 60 min at 60°, whereas the reduction of some partially methylated hexoses was not complete (>98% reduced). Further reduction was not achieved by increasing either the concentration of sodium borohydride or the time of reduction, or both.

Although the acetylation of most partially methylated alditols in the presence of borate by the method of Blakeney *et al.*[5] was found to be quantitative, there were exceptions. For example, when (1→3),(1→4)-β-D-glucan from barley endosperm was successively methylated, hydrolysed, and acetylated[5], unexpectedly little 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol was formed. We attribute this to the strong borate-complexing of 2,4,6-tri-*O*-methyl-D-glucitol. To overcome this problem we have used acid-catalysed rather than base-catalysed acetylation. Fritz and Schenk[39] recommended the use of perchloric acid in ethyl acetate for the quantitative acetylation of the hydroxyl groups of organic compounds, and Smith *et al.*[15] used it to prepare alditol acetates. We found that perchloric acid catalysed the quantitative acetylation of all partially methylated alditols tested.

It is necessary to remove the large excess of acetic anhydride to minimize "tailing" of the solvent front during chromatography. To aid in this removal, 1-methylimidazole was added to catalyze its conversion into acetic acid.

The improved procedure for methylation analysis was tested upon oligo- and poly-saccharides of known structure. Methylation analysis of cellobiose produced 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol in equal amounts (Fig. 2), as would be expected. The recovery of these derivatives was found to be >80%, as estimated by using *myo*-inositol hexa-acetate (in dichloromethane) as an internal standard and applying the relative, effective carbon-response factors described by Sweet *et al.*[40]. Peaks 2a and 2b

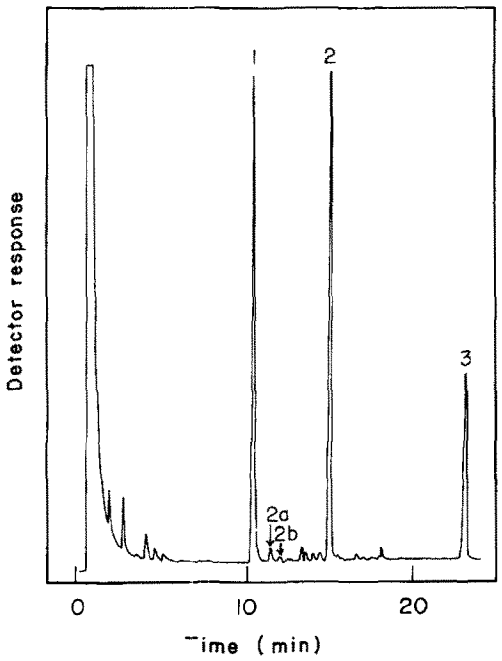

Fig. 2. Gas chromatography of alditol acetates of methylated sugars formed in methylation analysis of cellobiose: 1, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 2, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; 2a and b, 1,4-di-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucose; and 3, *myo*-inositol hexa-acetate (internal standard).

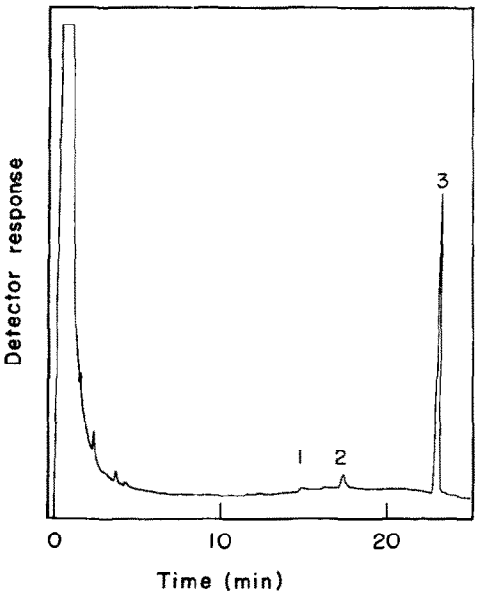

Fig. 3. Gas chromatography of products formed in the methylation procedure in the absence of carbohydrate (blank): 1 and 2, unknowns; 3, *myo*-inositol hexa-acetate (internal standard).

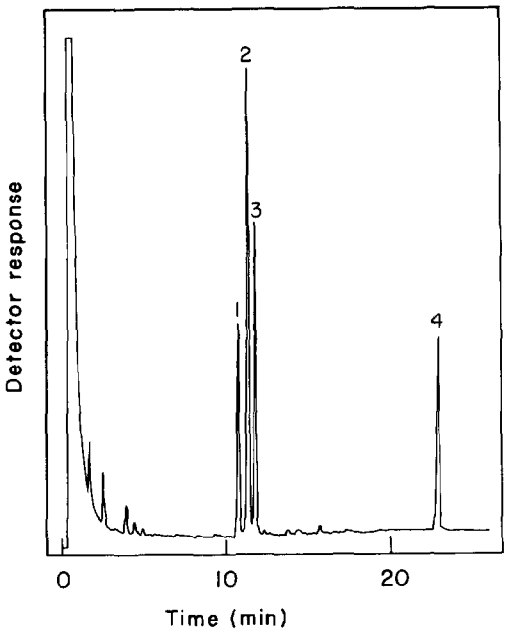

Fig. 4. Gas chromatography of alditol acetates of methylated sugars formed in methylation analysis of 3-*O*-β-D-galactopyranosyl-D-arabinose: 1, 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl-D-arabinitol; 2, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol; 3, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-arabinitol; and 4, *myo*-inositol hexa-acetate (internal standard).

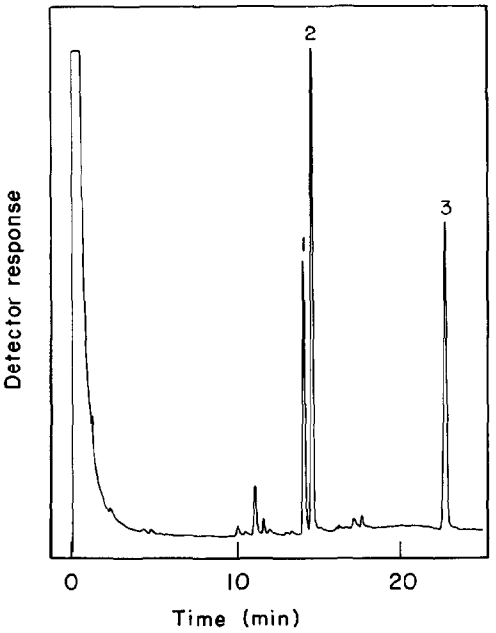

Fig. 5. Gas chromatography of alditol acetates of methylated sugars formed in methylation analysis of pullulan: 1, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; 2, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; and 3, *myo*-inositol hexa-acetate (internal standard).

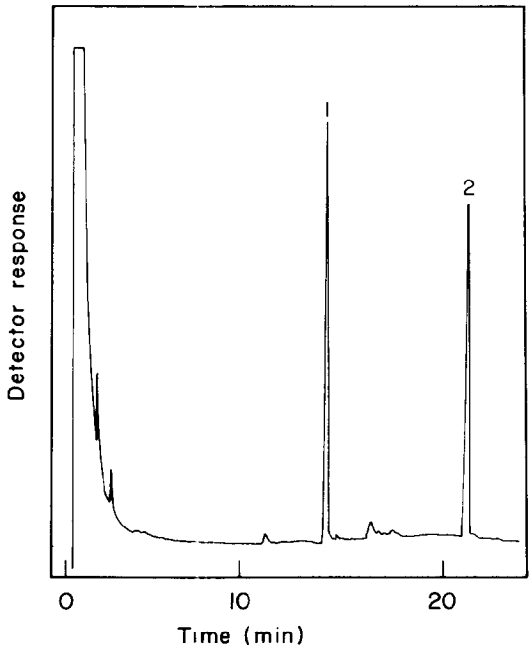

Fig. 6. Gas chromatography of alditol acetates of methylated sugars formed in the methylation analysis of Avicel (microcrystalline cellulose): 1, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; 2, *myo*-inositol hexa-acetate (internal standard).

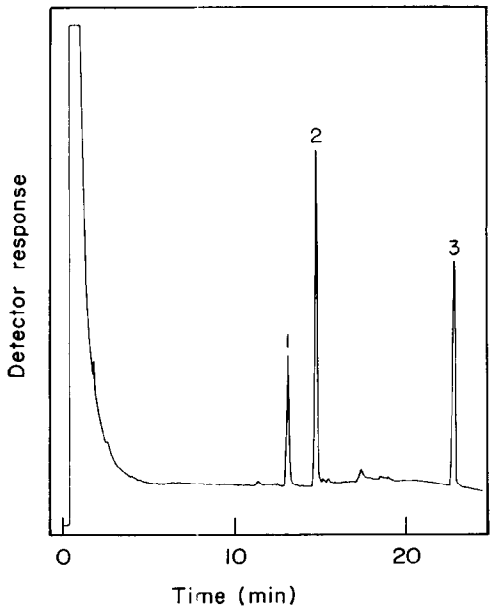

Fig. 7. Gas chromatography of alditol acetates of methylated sugars formed in the methylation analysis of (1→3),(1→4)-β-D-glucan from barley endosperm: 1, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; 2, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; and 3, *myo*-inositol hexa-acetate (internal standard).

arise from the α- and β-anomers of 1,4-di-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucose. These peaks were not observed when base-catalysed acetylation was used, possibly indicating incomplete acetylation by these methods. Other peaks in the chromatogram were also present in a methylation blank containing no carbohydrate (Fig. 3). These peaks may arise from impurities in the reagents, and emphasizes the need for high-purity reagents. Plasticizers are common contaminants of laboratory chemicals and some have similar retention-times to alditol acetates of methylated sugars[16].

Methylation analysis of 3-*O*-β-D-galactopyranosyl-D-arabinose produced three components: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol corresponding to the terminal, non-reducing galactosyl group, 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl-D-arabinitol corresponding to the 3-substituted arabinosyl residue in the furanose ring-form, and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-arabinitol, corresponding to the pyranose ring-form (Fig. 4). As expected, equal amounts of the hexose and pentose derivatives were found.

Methylation analysis of pullulan, a polysaccharide highly soluble in Me_2SO, produced only 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (Fig. 5) in the expected ratio of 2:1. Very little 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol was detected, as expected for a high-molecular-weight glucan. Methylation analysis of cellulose, a polysaccharide of low solubility in Me_2SO, gave 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol as the only major component (Fig. 6). Methylation analysis of (1→3),(1→4)-β-D-glucan from barley endosperm gave 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol in the ratio of 28:72 (Fig. 7), which is similar to the ratios previously reported for (1→3),(1→4)-β-D-glucan from barley endosperm[41].

We have also methylated many other carbohydrates by this method, including, arabinose, methyl β-L-arabinopyranoside, methyl α-D-xylopyranoside, melibiose, sophorose, sucrose, raffinose, stachyose, laminaratetraose, inulin, glycogen, arabinoxylan from wheat endosperm, and isolated whole cell-walls from suspension-cultured endosperm cells of Italian ryegrass.

CONCLUSION

We have developed an improved procedure for the methylation analysis of oligo- and poly-saccharides that is both rapid and convenient. The method permits routine analysis of large numbers of samples, which is impractical with previous methods. An attempt has also been made to improve the quantitative recovery of partially methylated monosaccharide residues. Under the conditions used, methylation was complete. All operations were performed in the same tube, thus eliminating loss during transfer. No evaporations under diminished pressure were necessary and the number of concentration steps was decreased to minimise evaporative losses. Hydrolysis of the methylated oligosaccharides and polysaccharides was

not quantitatively investigated and probably constitutes the remaining cause of incomplete recovery. The speed and simplicity of the procedure allows a range of hydrolysis conditions to be tested for any sample, thus permitting an assessment of the most appropriate hydrolysis procedure for each sample.

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