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A critical assessment of a one-tube procedure for the linkage analysis of polysaccharides as partially methylated alditol acetates

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ABSTRACT

Methylation analysis data for the exudate gum from *Actinidia deliciosa*, obtained by a one-tube procedure, were compared with published data. Several discrepancies were apparent, which were subsequently attributed to differences in the partially methylated alditol acetate (PMAA) derivatisations used. These deficiencies were also noted during methylation analyses of pectic polysaccharides from potato, xyloglucan from tamarind seed, and amylase-resistant starches from maize and pea. Underestimation of terminal galactosyl and glucosyl groups was traced to degradation of their partially methylated alditol derivatives in the perchloric acid-catalysed acetylation procedure used as the final stage of PMAA preparation. In addition, extended acid-catalysed reactions resulted in low values for terminal arabinosyl and fucosyl groups. Trifluoromethanesulfonic (triflic) acid, adopted in these reactions as a safer, non-oxidative alternative to perchloric acid, gave the same losses although it catalysed acetylation of non-terminal derivatives successfully. Underestimation of terminal arabinosyl groups was traced to inadequate extraction of the corresponding PMAAs into dichloromethane. Subsequently, other highly methylated sugar derivatives, such as those obtained from the alditol end groups of reduced oligosaccharides, proved highly susceptible to this loss; in addition, their tendency to evaporative loss, particularly during the early stages of their derivatisation (i.e., after hydrolysis and reduction rather than after acetylation), was demonstrated. A method which minimises these losses is described. Long-term instability of samples for gas chromatography was prevented by washing them with water prior to storage to remove residual acetic acid.

INTRODUCTION

In our recent papers[1,2], we described a modified procedure for the methylation of oligosaccharides and complex polysaccharides, and outlined its advantages over existing methods. Here we consider the relative merits of the subsequent conversion of the permethylated polysaccharide into partially methylated alditol acetates (PMAAs), with particular reference to the simplified one-tube procedure described by Harris et al.[3] which is in increasing use.

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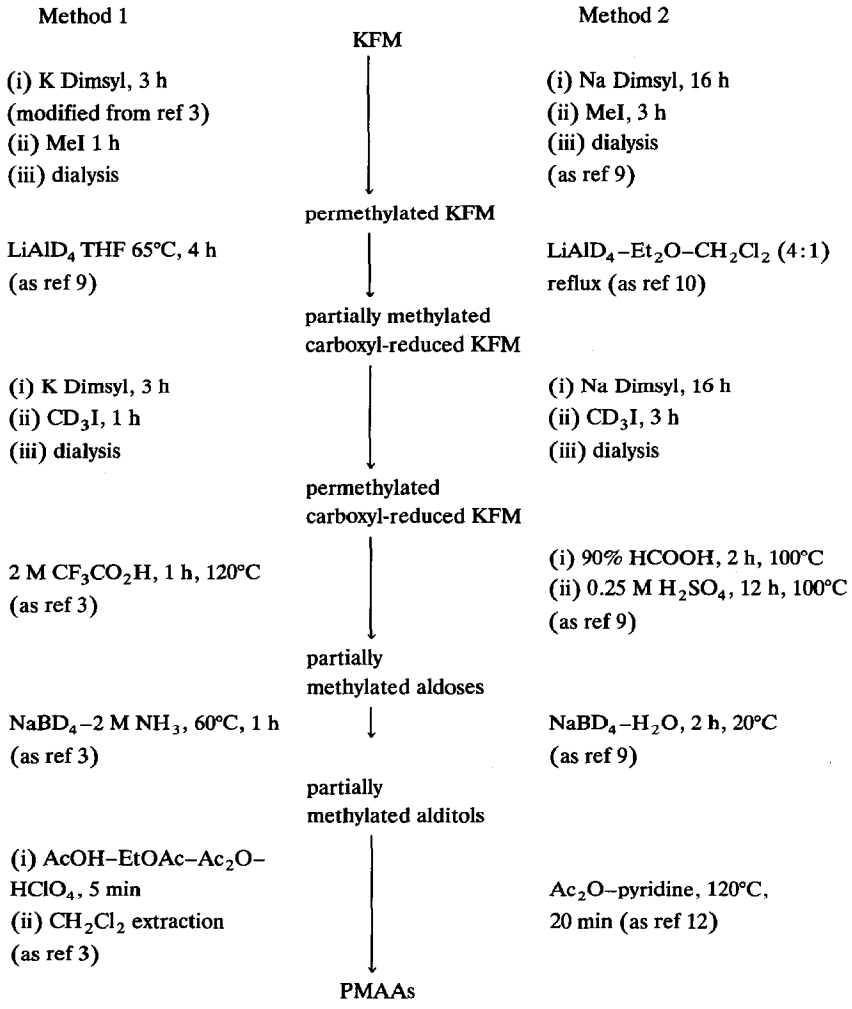
Linkage analysis of permethylated polysaccharides via conversion into PMAAs is a widely used and accepted technique[3]. Methylated material is hydrolysed to give a mixture of partially methylated glycoses which are reduced and acetylated to give PMAAs. The latter are then analysed by gas–liquid chromatography–mass spectrometry (GLC–MS). Conventional derivatisation methods are laborious and time-consuming[3]. Harris et al.[3] developed a rapid, convenient method of polysaccharide linkage analysis and tested it successfully on a wide range of linear polymers. The procedure allowed the one-tube derivatisation of material. In particular, it avoided the need for isolation of the methylated polymer by dialysis and for the removal of borate (after hydrolysis and reduction by borohydride) prior to acetylation (see below). In our hands, the acid-catalysed acetylation procedure of Harris et al.[3] led to underestimation of terminal residues, particularly when applied to branched polysaccharides. These losses, evident in a range of polysaccharides, and the factors which accentuate them have been studied. Model experiments with cellobiitol and cellotriitol, which helped to identify, quantify, and alleviate the losses in specific sugar derivatives from the oligosaccharides, are outlined.

RESULTS AND DISCUSSION

Many modified methods of methylation analysis are tested solely or mainly on non-acidic monomeric, oligomeric, or unbranched homoglycans. This may represent an insufficiently stringent test in certain applications. Before any new linkage analysis technique is introduced into a laboratory, it must be compared with existing methods using representative samples of interest, and any analytical differences critically assessed. Undermethylation must be suspected if a branch point to terminal sugar ratio of greater than one is observed and/or minor, "unusual" PMAAs are produced; if improvements in both areas are found on remethylation (if the polymer is neutral and hence β-elimination of esterified uronosyl residues is not a problem), the evidence is strong. On the other hand, variable yields of particular PMAAs between different derivatisation procedures are seldom reported; material may be scarce and comparison with other methods is not usually attempted. Often, comparing an independently derived sugar analysis[4,5] of the polymer with the sum total of the various linkage patterns of each sugar can provide a useful reference point, though account must be taken of known procedural deficiencies (for example, loss of neutral sugars as aldobiouronic[6] or pseudoaldobiouronic[7] acids during sugar analysis and β-eliminative degradation of esterified uronosyl residues during methylation analysis).

We first suspected deficiencies in the procedure of Harris et al.[3] during further studies of the exudate gum from *Actinidia deliciosa* (kiwi fruit mucilage, KFM). This structurally complex glucuronomannan[8] contains a wide variety of sugar and linkage types and has proved a useful testing ground for new techniques. A sample of KFM form the earlier work[8] was converted into PMAAs via methylation with



Scheme 1. A comparison of methylation analyses of KFM used by us (Method 1, after Harris et al.[3]) and Redgwell et al.[8] (Method 2).

potassium dimsyl (potassium methylsulfinylmethanide, "K Dimsyl") and methyl iodide, carboxyl-reduced with lithium aluminium deuteride in tetrahydrofuran[9], trideuteriomethylated to reveal any initially unmethylated sites and also to trideuteriomethylate the CD_2OH groups which resulted from carboxyl reduction, hydrolysed with trifluoroacetic acid, reduced with sodium borodeuteride in ammonia, acetylated in the acid-catalysed procedure of Harris et al.[3], and finally analysed by GLC (see Scheme 1, Method 1). The data obtained (Table I) showed significant differences when compared with those of Redgwell et al.[8] who had used Method 2, Scheme 1. Most noticeably, the method of Harris et al.[3] indicated (*i*) a consider-

TABLE I

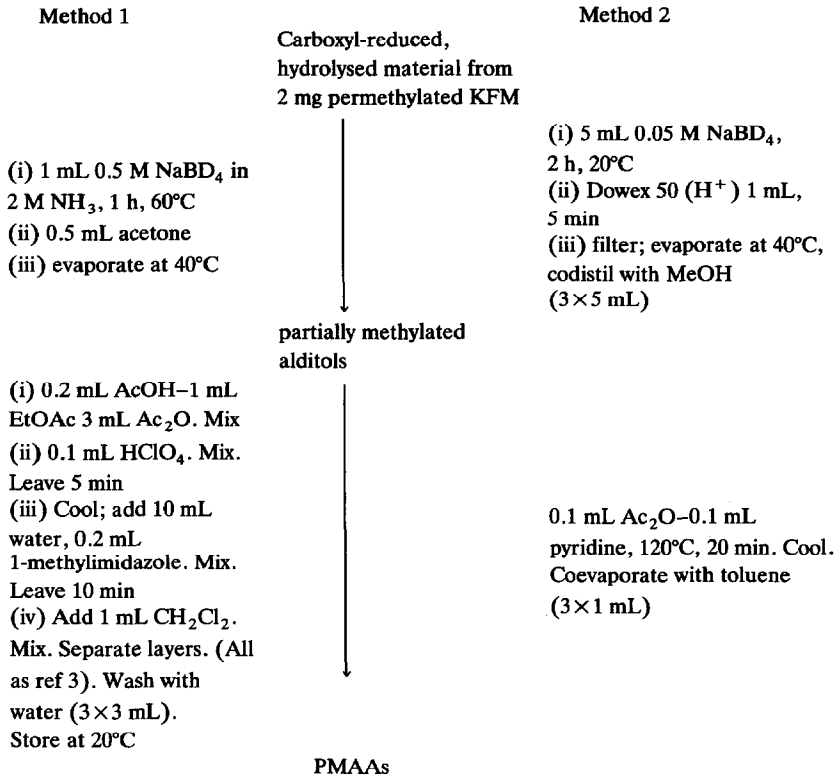
Methylation analysis data for the exudate gum from *A. deliciosa* (KFM)

Methylated alditol [b]	Molar composition (mol%) [a]		Linkage
	Method 1[3]	Method 2[8]	
2,3,5-Me_3-Ara	9.4	12.5	T-Ara*f*
2,3,4-Me_3-Ara	3.8	3.2	T-Ara*p*
2,3,4-Me_3-Fuc	13.6	14.5	T-Fuc
2,3-Me_3-Ara	5.9	4.4	1,5-Ara*f*
2,3,4,6-Me_4-Gal	2.2	12.0	T-Gal
3,4,6-Me_3-Man	–	0.3	1,2-Man
2,4,6-Me_3-Gal	14.0	11.8	1,3-Gal
2,3,6-Me_3-Glc [c]	7.1	4.4	1,4-GlcA
4,6-Me_2-Man	12.2	9.6	1,2,3-Man
4,6-Me_2-Gal	12.9	10.2	1,2,3-Gal
2,6-Me_2-Glc [c]	7.0	5.1	1,3,4-GlcA
2,4-Me_2-Gal	2.7	2.2	1,3,6-Gal
4-Me-Gal	9.2	9.8	1,2,3,6-Gal
Branch points: terminal sugars	1.83	1.11	

[a] Values corrected using the molar response factors given by Sweet et al.[11]. [b] 2,3,5-Me_3-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc. The PMAAs are listed in order of increasing GLC retention time. [c] 6,6-Dideuterio derivatives (see text).

ably lower proportion of terminal galactosyl groups in the polysaccharide, (*ii*) a slightly lowered proportion of terminal arabinofuranosyl groups, and (*iii*) a considerable discrepancy in the branch point to terminal sugar ratio. The acetylation conditions used differed fundamentally and so were examined in detail.

The two acetylation methods are compared in Scheme 2. The older, base-catalysed technique used by Redgwell et al.[8], Method 2, uses an equimolar mixture of pyridine and acetic anhydride at 100°C for 10 min[9] or, more often (as here) 120°C for 20 min[12], to acetylate the partially methylated alditols. It is necessary first to remove borate formed in the preceding reductive step. This avoids complexation of the alditols under the basic conditions which would prevent complete acetylation. It is achieved by treatment with a cation-exchange resin, in its acidic form, to give boric acid which is removed as the volatile trimethyl borate by repeated coevaporation with methanol[9]. This is time-consuming and risks loss of the more highly volatile components (see below). The avoidance of repeated evaporations with methanol was one of the main aims of Harris et al.[3]. Blakeney et al.[4] had reported a simple procedure for the acetylation of alditols, using acetic anhydride and 1-methylimidazole, which was subsequently modified by Englyst et al.[5]. Harris et al.[3] found that, although the method was suitable for the production of PMAAs in most cases, exceptions existed. As an example when a (1 → 3),(1 → 4)-β-D-glucan from barley endosperm was analysed, only a small amount of 1,3-linked glucose was indicated. This was ascribed to the strong complexing with borate of the 1,3,5-triol intermediate. Acid-catalysed acetylation, which avoided problems with borate complexation, was proposed as an alternative by Harris et al.[3], based on the



Scheme 2. A comparison of the partially methylated aldose reduction and acetylation methods used initially by us (Method 1, after Harris et al.[3]) and Redgwell et al.[8] (Method 2, after Lindberg[9]) to produce PMAAs from KFM.

earlier work of Abdel-Akher et al.[13]. Acetylation was achieved by treating samples dissolved in an acetic acid–ethyl acetate mixture with perchloric acid and acetic anhydride. The PMAAs produced were isolated, after hydrolysis of the excess of acetic anhydride into acetic acid with 1-methylimidazole and water, by a single extraction into dichloromethane. The method successfully derivatised a range of linear polysaccharides[3]. This was the procedure we had used (Method 1, Schemes 1 and 2) for the analysis of KFM.

Our investigation of the relative merits of the acetylation procedures was designed to meet another concern. Mixtures of acetic anhydride and 72% perchloric acid are known to be unstable, and many explosions have been reported, even at room temperature and during small-scale reactions[14]; the acid catalyses the reaction of residual water with the anhydride to give anhydrous perchloric acid and so we sought an alternative catalyst. Triflic acid (trifluoromethanesulfonic acid), which has been advocated as a substitute for perchloric acid[15], is an even stronger acid than perchloric and is non-oxidative, although it fumes readily in air and is

hygroscopic. We have found that it can be conveniently handled by enclosure in a glass vial fitted with a "Mininert" valve (Pierce Co.) from which the rubber septum has been removed, and transferred using a glass syringe fitted with a stainless steel needle.

The results of our investigation into the influence of the acetylation conditions used on the PMAA ratios obtained from KFM are shown in Table II. Minor, unassignable peaks, where present, were not taken into account when calculating molar percentages; see below and Table III. The following points may be made. (*i*) Triflic acid catalysis under the same conditions as perchloric acid catalysis (except that the perchloric acid was replaced with an equal volume of triflic acid which represented an approximately equimolar amount) gave very similar results; compare A with C, and B with E, see below. The terminal galactosyl group content was still depleted relative to the base-catalysed acetylation result, see columns H and I. (*ii*) Reduction of the amount of triflic acid by 90% led to poor recovery of PMAA and altered ratios (see D), suggesting incomplete acetylation under these conditions due to the lower acidity of the medium. The increase in the relative recovery of the PMAA (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol) derived from former terminal galactosyl groups suggested that acid-catalysed degradation of this PMAA might be responsible for its former loss and prompted us to try extended acid-catalysed acetylation. This progressively lowered the recovery of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (see E, F, and G). When acid-catalysed acetylation was replaced with pyridine catalysis (i.e., Method 2, Schemes 1 and 2), the losses were restored (see H and I). (*iii*) Smaller losses of the PMAAs derived from former terminal fucosyl and arabinosyl groups were evident during extended acid-catalysed acetylations compared with the base-catalysed values (compare G with H and I). (*iv*) Threefold extraction with dichloromethane gave increased recoveries of both terminal arabinosyl derivatives, when either perchloric or triflic acid was used (compare A with B, and C with E). Extraction three times with 1 mL of dichloromethane was sufficient; extraction with 3×10 mL did not improve the recovery (compare B and E). The recovery of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, derived from the terminal xylosyl groups of a xyloglucan, was similarly dependent on the extraction conditions[16].

Two additional deficiencies were noted in both the acid-catalysed acetylation procedures. Firstly, samples for GLC analysis were not stable for more than about 2 months, even when stored at -20°C. Washing the organic extract of PMAAs with water prevented decomposition (see Method 1, Scheme 2). The instability was presumably due to residual acetic acid. Secondly, the acid-catalysed procedure gave, on most occasions, an additional peak of variable, though frequently large, area at a retention time very close to that of the PMAA resulting from 1,2-linked rhamnosyl residues. Apart from the possibility of obscuring peaks of interest, the component was only produced when partially methylated alditols were present (although dependent on their origin — apart from kiwi fruit mucilage, a variety of pectins and xyloglucans gave it, although cellobiitol, cellotriitol, and resistant

TABLE II

Comparative methylation analysis data for KFM [a]

Methylated alditol [b]	Molar compositions (mol%) [c] obtained using either perchloric acid, triflic acid, or pyridine as acetylation catalyst									Linkage
	Perchloric acid		Triflic acid					Pyridine		
	A [d]	B	C	D	E	F	G	H	I	
2,3,5-Me_3-Ara	7.0	9.8	6.7	6.0	9.7	8.9	7.8	9.5	9.3	T-Ara*f*
2,3,4-Me_3-Ara	4.5	6.4	4.2	4.4	6.4	7.1	7.2	5.9	5.6	T-Ara*p*
2,3,4-Me_3-Fuc	12.8	14.0	12.9	15.5	14.5	14.4	12.8	12.7	12.2	T-Fuc
2,3-Me_2-Ara	7.9	7.9	7.2	7.3	7.7	7.9	9.4	7.1	7.2	1,5-Ara*f*
2,3,4,6-Me_4-Gal	4.5	4.7	6.5	15.6	6.2	1.8	0.4	10.0	10.0	T-Gal
3,4,6-Me_3-Man	16.1	15.3	13.3	16.5	15.4	17.2	18.0	13.6	14.3	1,2-Man
2,4,6-Me_3-Gal	12.1	10.4	11.4	6.7	9.3	9.0	11.0	8.9	8.8	1,3-Gal
4,6-Me_2-Man	14.1	10.6	13.9	13.2	10.9	11.6	13.9	10.7	10.7	1,2,3-Man
4,6-Me_2-Gal	5.1	5.6	6.5	7.5	5.3	6.1	6.7	6.2	6.5	1,2,3-Gal
2,3-Me_2-Glc	4.8	4.9	4.9	2.6	5.0	5.7	6.2	5.1	4.4	1,4-GlcA
2,4-Me_2-Gal	2.6	2.6	2.8	1.1	2.2	2.3	2.8	2.5	2.7	1,3,6-Gal
2-Me-Glc	8.5	7.9	9.7	3.4	7.3	8.2	10.0	8.0	8.3	1,3,4-GlcA
4-Me-Gal	1.76	1.27	1.73	0.73	1.14	1.40	1.91	1.13	1.16	1,2,3,6-Gal

[a] All methylated as one batch by Method 1 (see text) (except no remethylation was used and the acetylation conditions were varied as shown below) and then split into the individual samples; each represented 2 mg of KFM. [b] 2,3,5-Me_3-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc. The PMAAs are listed in order of increasing GLC retention time. [c] Values corrected using the molar response factors given by Sweet et al.[11]. [d] Acetylation conditions: A, 100 μL $HClO_4$, 5 min, 1×1 mL extraction; B, 100 μL $HClO_4$, 5 min, 3×10 mL extraction; C, 100 μL CF_3SO_3H, 5 min, 1×1 mL extraction; D, 10 μL CF_3SO_3H, 5 min, 1×1 mL extraction; E, 100 μL CF_3SO_3H, 5 min, 3×1 mL extraction; F, 100 μL CF_3SO_3H, 30 min, 3×1 mL extraction; G, 100 μL CF_3SO_3H, 2 h, 3×1 mL extraction; H, Ac_2O–pyridine, 120°C, 20 min (method 2, Fig. 2); I, as H.

TABLE III

Retention time and peak area data for the early-running peaks observed in triflic acid-catalysed acetylations of polysaccharides

Peak number	Retention time [a,b]	Peak areas [b]			Ion *m/z* (in order of decreasing intensity)
		E [c]	F	G	
1	0.457	0.04	0.09	0.12	43, 100, 55, 54, 86, 83, 82, 71, 114, 69, 125, 143, 153, 168
2	0.503	0.32	0.40	0.62	110, 43, 81, 53, 54, 111, 152, 81
3	0.535	0.02	0.23	0.66	43, 84, 98, 126, 140, 99, 124, 121, 150
4 [d]	0.714	0.59	0.54	0.38	43, 123, 139, 138, 95, 127, 51, 53, 65, 68, 137, 110, 169, 180, 210, 240

[a] Using cold column injection onto a capillary column (30 m×0.32 mm i.d., Restek OV225) in a Carlo Erba 5160 model chromatograph equipped with a flame-ionisation detector. High-purity He was used as carrier at a head pressure of 0.96 kgm^{-2}. 10 s after injection, the cooling was switched off, and the oven temperature maintained at 55°C for 1 min, raised at 45°C min^{-1} to 140°C and immediately raised at 2.5°C min^{-1} to 218°C. This temperature was maintained for 37 min. The detector temperature was 220°C. Integration was performed using a Spectra-Physics SP4400 integrator. [b] 1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methylmannitol as an internal standard. [c] As Table II. [d] Later-running anomalous peak discussed in the text.

starches did not). A blank experiment did not give the peak, which suggested that it was formed from the alditols; this would inevitably lead to analytical error. Its identity was elusive; its electron ionisation (EI) mass spectrum and retention time data are given in Table III (compound **4**). These data were unchanged when sodium borohydride, rather than deuteride, was used for the reduction. Chemical Ionisation (CI) mass spectrometry suggested a molecular weight of 240. It was not further investigated. The amount of this component produced did not increase with increasing underestimation of terminal galactosyl groups. However, three minor early-running peaks did appear in increasing amounts on extended acid-catalysed acetylation. This suggested that they might result from degradation of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol derived from former terminal galactosyl groups. Their mass spectral data are summarised in Table III (compounds **1**, **2** and **3**); they were not further investigated.

Underestimation of terminal galactosyl groups by procedures involving acid-catalysed acetylation was not confined to the KFM system. Methylation analysis of a hot-water-extracted tamarind xyloglucan exhibited the same effect (see Table IV); the content of terminal xylosyl group was also underestimated, although to a lesser extent. (The residual imbalance of branch points and end groups is a feature of linkage analyses of xyloglucans[17]. Its cause is unknown, but see below.) Underestimation of terminal galactosyl groups was also noted in analyses of pectic polysaccharides from potato[18], from an imbalance of branch points and end groups. Why 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol should be most subject to degradation is unclear, particularly as the other galactosyl PMAAs were seemingly unaffected. In general, many side reactions of PMAAs in these strong acids are plausible: ether cleavage, unimolecular elimination or substitution, rearrangements, and formation of acetoxonium ions are all known[19] with resultant changes of configuration and/or structure. The reaction was not further investigated.

A further deficiency in the acid-catalysed procedure became apparent during analysis of amylodextrins obtained by alpha-amylase digestion of retrograded plant starches (so called "resistant" starches[2,20]). Table V gives typical analytical data for a resistant starch from maize. All the samples were derived from a single portion of methylated, hydrolysed, and reduced material. Methylation was achieved by our modification[1,2] of the method of Ciucanu and Kerek[21], and hydrolysis and reduction by the method of Harris et al.[3] (Method 1, Scheme 1). Sample A was acetylated by the perchloric acid-catalysed procedure of Harris et al.[3] (see Method 1, Scheme 2), sample B by a pyridine-catalysed procedure after removal of borate[12] (Method 2, Scheme 2), and sample C by an adaptation[2] of the 1-methylimidazole-catalysed alditol acetylation procedure of Englyst et al.[5] (no borate removal — see above). Sample D was acetylated under extended (2 h) perchloric acid catalysis (triflic acid gave comparable results). The following points may be made: (*i*) acid catalysis results in underestimation of terminal glucosyl groups compared with either base-catalysed procedure; (*ii*) the two base-catalysed procedures, in this case, give highly comparable data (although see below); (*iii*) extended acid-cata-

TABLE IV

Methylation analysis data for water-extracted tamarind xyloglucan

Methylated alditol [a]	Molar composition (mol%) [b]		Linkage
	A[c,d]	B	
2,3,4-Me_3-Xyl	11.9	15.4	T-Xyl
2,3,4,6-Me_4-Gal		15.8	T-Gal [e]
3,4-Me_2-Xyl	18.7	13.1	1,2-Xyl [e]
2,3,6-Me_3-Glc	20.8	15.3	1,4-Glc
2,3-Me_2-Glc	48.6	40.5	1,4,6-Glc

[a] 2,3,4-Me_3-Xyl = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, etc. [b] Values corrected using the molar response factors given by Sweet et al.[11]. [c] All samples prepared by K dimsyl methylation, CF_3CO_2H hydrolysis, and reduction with $NaBD_4$ in NH_3. [d] Acetylation conditions: A, 100 μL CF_3SO_3H, 2 h, 3×1 mL extraction; B, Ac_2O–pyridine, 120°C, 20 min. [e] These components could be resolved on a 30-m OV225 column (see Table VI) using the following oven program after on-column injection: the temperature was maintained at 55°C for 1 min then raised at 5°C/min to 140°C where it was maintained for 140 min and then raised at 1°C/min to 210°C for 40 min.

lysed acetylation resulted in no further significant underestimation of terminal glucosyl groups. This contrasts with the almost total degradation of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol derived from former galactosyl groups reported above.

The deficiencies inherent in acid-catalysed acetylation meant that we returned, in the first instance, to the pyridine-catalysed procedure[12] described in Scheme 2 (despite its procedural inconveniences) and thus used the derivatisation methods detailed for H and I in Table III. However, when a series of analyses of a particular polysaccharide type are planned, we now compare (as in the resistant starch case) the data from pyridine catalysis with that obtained by 1-methylimidazole catalysis[2,5]. If the two sets are comparable, we adopt the latter technique

TABLE V

Comparative methylation data for maize resistant starch obtained using a variety of acetylation conditions

Methylated alditol [a]	Molar composition (mol%)[b,c]				Linkage
	A[d]	B	C	D	
2,3,4,6-Me_4-Glc	1.6	3.8	4.3	2.4	Terminal
2,3,6-Me_3-Glc	96.1	94.2	93.4	94.8	1,4
2,6-Me_2-Glc	0.2	0.2	0.2	0.2	1,3,4
3,6-Me_2-Glc	0.2	trace	0.2	0.4	1,2,4
2,3-Me_2-Glc	1.9	1.7	1.6	2.2	1,4,6
Glc	trace	trace	0.3	trace	1,2,3,4,6

[a] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol etc. [b] Values corrected using the molar response factors given by Sweet et al.[11]. The data presented are the average of duplicate derivatisations. [c] Material was permethylated using a modification[1,2] of the method of Ciucanu and Kerek[20]. [d] Acetylation conditions: A, $HClO_4$–Ac_2O, 5 min (Method 1, Scheme 2); B, pyridine–Ac_2O, 121°C after removal of borate (Method 2, Scheme 2); C, 1-methylimidazole–Ac_2O–water (see text); D, $HClO_4$–Ac_2O, 2 h.

on the grounds of its relative simplicity and its ability to acetylate under much milder conditions. In systems where the procedure is known to underestimate particular residues, [e.g., (1 → 3)-linked glucosyl residues in cereal glycans[3]] or for single analyses, we now remove borate by coevaporation with methanol[9] followed by 1-methylimidazole-catalysed acetylation.

Although the underestimation of terminal glucosyl groups in acid-catalysed acetylation of the amylodextrins was reproducible, the low molar percentage of terminal residues in these compounds (and hence the relatively small absolute reduction observed) led us to seek confirmatory evidence from another analysis. In the course of our recent study of methylation techniques, cellobiitol was subjected to methylation analysis[2]. In principle, as an example of a short-chain oligoglucan with a high content of terminal glycosyl groups, this material seemed a suitable system. In practice, the ratio of the two resultant PMAA peak areas (i.e., of 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol from the alditol group to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol from the terminal glucosyl group) was in the range 0.04–0.09 (average 0.07), rather than unity, over the course of ten experiments. The methylation conditions varied from sample to sample[1], but the hydrolyses, reductions, acetylations, and attendant workups were the same (i.e., those of Method 1, Scheme 1); in particular, the acetylations were catalysed with perchloric acid. This imbalance was severe and the opposite of that expected if selective degradation of the PMAA derived from terminal glucosyl groups were the dominant factor. Even so, the result was not unexpected; many workers have commented on the loss of volatile "pentamethyl" derivatives (obtained from the reduced, hexosyl reducing end of an oligosaccharide, as here), although few have sought to quantify the effect or to distinguish between highly methylated alditol acetates, and partially methylated alditols and aldoses. Sandford and Conrad[22] reported 1.8 and 3.0% loss of tetra-*O*-methylglucose after 3 and 4 h, respectively, when evaporated at 50°C at water pump pressure, and no loss after 4 h at 40°C; evaporation at 40°C seems to have become the norm in PMAA derivatisation as a result. The possibility of evaporative or other losses at intermediate stages of the derivatisation process leading to PMAAs has not been investigated. We decided initially to test each stage of cellobiitol derivatisation for evaporative loss and also to study the role of the acetylation regime used. To this end, conditions to minimise evaporative losses were devised ("Special" conditions) and compared with samples derivatised by the method of Harris et al.[3] ("Normal" conditions). The reaction volume of each "Special" stage was minimised and the next derivatisation applied directly, after appropriate adjustment of pH, without evaporation. It was hoped that residual inorganic salts not normally present would not interfere and that the water carried through would be insufficient to prevent complete (acid catalysed[3]) acetylation. This proved to be the case (see below). Each stage had a "Normal" or "Special" variant (see Table VI). Thus, a sample of cellobiitol was permethylated and split into eight portions, each of which was hydrolysed with CF_3CO_2H in the usual way[3] (albeit in a smaller volume of acid than usual — see

TABLE VI

Ratio of 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol obtained in methylation analysis of permethylated cellobiitol under various conditions

Method [a]	$N_1N_2N_3$	$N_1S_2N_3$	$N_1N_2S_3$	$N_1S_2S_3$	$S_1N_2S_3$	$S_1S_2N_3$	$S_1N_2N_3$	$S_1S_2S_3$
Product ratio [c]	0.08	0.15	0.08 (0.19) [b]	0.1	0.06	0.54	0.06	0.35 (0.67) [b]

[a] See text. Cellobiitol-*1-d* (12 mg) was permethylated using a non-oxidative modification[1,2] of the method of Ciucanu and Kerek[21]. The sample was split into 8 and each portion was hydrolysed with 2 M CF_3CO_2H (100 μL) for 1 h at 120°C and cooled on ice. At each stage, the samples were subjected to the appropriate Normal or Special procedure detailed below so as to generate the overall protocols shown in the table. N_1, Evaporate CF_3CO_2H at 40°C. Reduce as normal[3] (20 mg $NaBD_4$ in 1 mL of 2 M NH_3 at 60°C, 1 h). S_1, Do not evaporate. Cool on ice. Add 60 μL 25 M NH_3 (check pH > 7). Then reduce with 25 μL of a solution of $NaBD_4$ in 2 M NH_3 (100 mg/mL) at 60°C 1 h. N_2, Add acetone (500 μL) and evaporate at 40°C. Acetylate by (*i*) add 200 μL AcOH, 1 mL EtOAc, 3 mL Ac_2O (mix); (*ii*) add 100 μL $HClO_4$ (mix, 5 min); (*iii*) cool on ice, add 10 mL water and 200 μL 1-methylimidazole, and leave for 10 min. S_2, Cool on ice. Add 50 μL glacial AcOH. Check pH < 7. Do not evaporate. Then acetylate in same way as N_2. N_3, Extract with 1 mL CH_2Cl_2. Wash with water (3×3 mL). Evaporate at 40°C. S_3, Extract with 100 μL CH_2Cl_2. Wash with 3 mL water and add 100 μL CH_2Cl_2 (twice). Inject a portion of the sample directly onto gas chromatograph. [b] Modified S_3, extract with 3×10 mL CH_2Cl_2. Combine the organic phases, wash with 3×30 mL water, dry with $MgSO_4$, and evaporate to dryness at 30°C. Redissolve in acetone (100 μL) prior to gas chromatography. [c] The data given are the ratios of the peak areas and are uncorrected.

above). The samples were cooled in ice. A first set of four were evaporated at 40°C just to dryness and reduced as normal[3] ("N_1") and the second set were made alkaline with ammonia and reduced directly ("S_1"). Half of each set were acetylated normally[3] — see "N_2", Table VI and Method 1, Scheme 2 — and half in a modified fashion — "S_2". One of each pair of the resulting four sets of duplicates was then worked up as normal[3] and half treated in a modified way — "N_3" and "S_3", respectively. In this way, eight samples, each representing a unique derivatisation variant, were generated. The ratio of the derivatives obtained for each is given in Table VI.

Inspection of the data suggested that (*i*) both the modified reduction and acetylation were effective despite the presence first of ammonium trifluoroacetate and then of sodium acetate and borate; (*ii*) both modifications S_1 and S_2 lent improvement, showing that 1,2,3,5,6-penta-*O*-methylglucitol is sufficiently volatile to undergo loss in the conventional procedure; (*iii*) S_3 was detrimental; this was not unexpected given the susceptibility of highly methylated PMAAs to loss from insufficient extraction (see above); the procedure had been adopted to avoid any evaporation at this critical stage; (*iv*) when S_3 was modified (see data in brackets) to include more thorough extraction with dichloromethane, an improvement was observed; however, an equimolar recovery of the two products was still not obtained.

To test whether this residual loss was due to the acetylation conditions used (i.e., if the pentamethyl component was degraded to an even greater extent than the tetramethylated, terminal glucosyl derivative) and/or a volatility loss, the fully modified procedural variant described above (i.e., $S_1S_2S_3$) was applied to cellotri-

itol; this allowed the 1,4-linked glucosyl residue to be used as an intrinsic internal standard. In addition, both cellobiitol and cellotriitol were analysed in an identical manner except that base-catalysed acetylation (namely 1-methylimidazole-catalysed acetylation, without prior removal of borate or evaporation) was substituted for the modified acid-catalysed acetylation (S_2). The results of this are summarised in Table VII. The following points may be made: (*i*) when base-catalysed acetylation was used, an almost equimolar recovery of the expected products was obtained for both cellobiitol and cellotriitol. In fact, the apparent shortfall in the pentamethylglucitol recovery was due, at least in part, to the fact that these data were uncorrected. An effective carbon response factor for this derivative was unavailable; it would act to augment the observed ratio[11]; (*ii*) acid-catalysed acetylation of the cellotriitol-derived partially methylated alditols revealed that both the pentamethyl derivative and the tetramethyl derivative were degraded to a greater extent than the trimethyl derivative of the 1,4-linked residue. The high degree of loss of the pentamethylated product first observed was thus shown to be due to both the acid-catalysed acetylation used and to evaporation and under-extraction. In fact, the apparent recovery of the pentamethyl derivative initially obtained was seen to have been augmented by the (lesser) degradation of the PMAA derived from the terminal glucosyl group. This result demonstrates that the acid-catalysed acetylation procedure of Harris et al.[3] is particularly unsuitable for the analysis of oligoalditols as PMAAs.

Subsequently, an attempt was made to quantify the potential evaporative loss of a range of PMAAs. Representative PMAA mixtures, obtained from each of the oligo- and poly-saccharides discussed above, were subjected to extended evapora-

TABLE VII

Ratio of products obtained from methylation analysis of cellobiitol and cellotriitol under a variety of conditions

Methylated alditol [a]	Cellobiitol (acid-catalysed acetylation) [b]	Cellobiitol (base-catalysed acetylation) [c]	Cellotriitol (acid-catalysed acetylation) [b]	Cellotriitol (base-catalysed acetylation) [c]	Linkage
1,2,3,5,6-Me_5-Glc	0.65 [d]	0.86	0.29	0.78	4-Glcol
2,3,4,6-Me_4-Glc	1	1	0.44 (0.47) [e]	0.92 (0.97) [e]	T-Glc
2,3,6-Me_3-Glc			1	1	1,4-Glc
1,2,3,5,6-Me_5Glc: 2,3,4,6-Me_4-Glc	0.65	0.86	0.66	0.85	4-Glcol: T-Glc

[a] 1,2,3,5,6-Me_5-Glc = 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol, etc. [b] The fully modified procedure detailed in Table VI was used, i.e., $S_1S_2S_3$ (modified). The data are the average of duplicate derivatisations. [c] The samples were acidolysed and reduced by the modified procedures detailed in Table VI (i.e., S_1S_2) except that acetylation was performed as follows. After addition of acetic acid, add 65 μL of water and cool on ice. Add 300 μL of 1-methylimidazole and 3 mL of Ac_2O, and heat at 30°C for 30 min. Add 10 mL of water and then extract with 3×10 mL of CH_2Cl_2. Combine the organic phases, wash with 3×30 mL of water, dry with $MgSO_4$, and evaporate to dryness at 30°C. Redissolve in acetone (100 μL) prior to gas chromatography. The data are the average of duplicate derivatisations. [d] The data given are uncorrected peak area ratios (unless otherwise stated) relative to the least-volatile expected PMAA present. [e] The data in brackets are the peak area ratios corrected by the response factors of Sweet et al.[11].

tion at 40°C after addition of *myo*-inositol hexaacetate as an internal standard. At intervals, samples were taken and analysed by GLC–MS. Unfortunately, random variations in the data obtained precluded accurate estimation of individual volatility losses from single injection data. It was later found that 4 repeat sample injections gave, typically, a 5% coefficient of variation in peak area[16]. However, the study was sufficient to show that loss of even the more volatile PMAAs would be insignificant in normal practice. For example, the losses of 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol and the corresponding tetra-*O*-methylpentitols were, at worst, of the order of 20 and 10%, respectively, after 2 h. In particular, the loss of the PMAA derived from terminal xylose was insufficient to account for the serious underestimation of this residue always reported in methylation analyses of xyloglucans. Total xylose is often estimated at only 60–70% (for example, see Table IV, column B) of the sugar analysis level[17]. Whether these losses are due to the volatility of the intermediate partially methylated xylose and xylitol or to some other cause has not yet been investigated.

CONCLUSIONS

The simplified procedure of Harris et al.[3] for the methylation analysis of polysaccharides has been tested on a range of branched complex polysaccharides and oligosaccharides. It has several deficiencies: (*i*) terminal galactosyl and glucosyl groups are seriously underestimated if a perchloric acid-catalysed acetylation procedure is used; triflic acid (a safer, non-oxidative alternative to perchloric acid) also leads to underestimation of these groups; (*ii*) other terminal residues are underestimated, to a lesser extent, if the acid-catalysed acetylations are inadvertently extended; (*iii*) highly methylated alditols and aldoses, as well as PMAAs, are prone to loss from evaporation; in particular, 2,3,4,6-tetra-*O*-methylglucose and 1,2,3,5,6-penta-*O*-methylglucitol, intermediates in the derivatisation of cellobiitol, were both more susceptible to evaporative loss than the corresponding PMAAs, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol, under the conditions of Harris et al.[3]; a procedure which avoided intermediate evaporations corrected these losses; (*iv*) highly methylated PMAAs are prone to underextraction into organic solvents, especially from aqueous solutions containing acetic acid; (*v*) GLC samples prepared by the method of Harris et al.[3] are prone to decomposition during storage unless the sample is first washed with water.

EXPERIMENTAL

KFM.—Kiwi fruit mucilage (KFM) was isolated as described by Redgwell[23].

Reagents.—All reagents used were of the highest grade available. Me_2SO was stored over approximately one quarter of its volume of 3A molecular sieves (which had been activated by heating at 300°C overnight) for at least 2 days before use.

Preparation of potassium methylsulfinylmethanide.—Potassium methylsulfinylmethanide (potassium dimsyl) was prepared by the method of Harris et al.[3].

Preparation of "resistant starches".—Samples of resistant starch were prepared as previously described[2].

Methylations of KFM and tamarind xyloglucan with potassium methylsulfinylmethanide and methyl iodide.—A modification of the method of Harris et al.[3] was used as previously described[2].

Methylations of resistant starch, cellobiitol, and cellotriitol with sodium hydroxide and methyl iodide.—A non-oxidative modification[1,2] of the procedure of Ciucanu and Kerek[21] was used as previously described.

Carboxyl reduction of KFM.—Permethylated KFM was carboxyl-reduced by a modification[2] of the method of Lindberg[9] as previously described.

Preparation of partially methylated aldoses from methylated carbohydrates.—Methylated carbohydrates were converted into partially methylated alditols, using the procedure of Harris et al.[3] except that polysaccharides were isolated by dialysis after methylation[8]. (Methylated oligosaccharides were isolated by extraction[3].)

Conversion of partially methylated aldoses into PMAAs.—This was achieved in a variety of ways (see text). *1.* Perchloric acid-catalysed acetylation and borodeuteride reduction were performed as described by Harris et al.[3] except that on occasion the acetylation period was extended to monitor the degradation of certain sugar derivatives (see text) and the mixture was extracted three times with CH_2Cl_2 (1 mL) to ensure complete recovery of terminal pentosyl derivatives. *2.* Triflic acid-catalysed acetylation and borodeuteride reduction were performed as described by Harris et al.[3] except that (*i*) on occasion the acetylation period was extended to monitor the degradation of certain sugar derivatives (see text); (*ii*) 100 μL of triflic acid was added instead of 100 μL of 72% perchloric acid to the vigorously stirred solution; and (*iii*) the mixture was extracted three times with CH_2Cl_2 (1 mL) to ensure complete recovery of terminal pentosyl derivatives. *3.* Pyridine-catalysed acetylation and borodeuteride reduction were performed by a slight modification[12] of the method of Lindberg[9] except that, after toluene co-evaporation, the acetylated samples were dissolved in CH_2Cl_2 (1 mL), and the solutions were washed with water (3 × 3 mL) and evaporated to dryness at 40°C. *4.* 1-Methylimidazole-catalysed acetylation and borodeuteride reduction with borate removal. Borodeuteride reduction was performed by the method of Harris et al.[3]. After acetone addition and evaporation, borate was removed by the method of Lindberg[9]. Acetylation was performed by a slight modification[2] of the method of Englyst and Cummings[5]. The mixture was cooled on ice and water (300 μL), and 1-methylimidazole (450 μL) and Ac_2O (3 mL) were added. The mixture was vortex-mixed, heated at 30°C for 30 min, and then cooled. Water (5 mL) was added, and the mixture was vortexed and after 5 min extracted with CH_2Cl_2 (3 × 1 mL). The organic extracts were combined, washed with water (3 × 3 mL), and evaporated to dryness at 40°C. *5.* 1-Methylimidazole-catalysed acetylation and borodeuteride reduction with borate removal were performed as described in the preceding paragraph except that borate was not removed prior to acetylation.

Conversion of permethylated cellobiitol and cellotriitol into PMAAs without re-

moval of trifluoroacetate and borate salts prior to acetylation.—This was achieved in two ways (see text): *1.* Perchloric acid-catalysed acetylation. A modification of the procedure of Harris et al.[3] was used. Permethylated oligoalditol (1.5 mg) was hydrolysed with 2 M CF_3CO_2H (100 μL) for 1 h at 121°C. The mixture was cooled on ice, 25 M aq NH_3 (60 μL) was added, and the pH was checked to be > 7. A solution of sodium borodeuteride in 2 M NH_3 (25 μL, 100 mg/mL) was added, and the mixture was heated at 60°C for 1 h and then cooled on ice. Glacial AcOH (50 μL) was added and the pH was checked to be < 7. Glacial AcOH (200 μL), EtOAc (1 mL), Ac_2O (3 mL), and 72% $HClO_4$ (100 μL) were added and the mixture was vortex-mixed. After 5 min, the mixture was cooled on ice, water (10 mL) and 1-methylimidazole (200 μL) were added, and the solution was left for 10 min and then extracted with CH_2Cl_2 (3×10 mL). The organic extracts were combined, washed with water (3×30 mL), dried with $MgSO_4$, and evaporated to dryness at 30°C. The samples were redissolved in acetone (100 μL) prior to GLC–MS. *2.* 1-Methylimidazole-catalysed acetylation. Samples of permethylated oligoalditol were hydrolysed and reduced as described in the preceding paragraph. Glacial AcOH (50 μL) and water (65 μL) were added to the cooled reduced solution. The mixture was recooled, 1-methylimidazole (300 μL) and Ac_2O (3 mL) were added, and the mixture was heated at 30°C for 30 min. Water (10 mL) was added and the mixture extracted with CH_2Cl_2 (3×10 mL). The organic phases were combined, washed with water (3×30 mL), dried with $MgSO_4$, and evaporated to dryness at 30°C. The samples were redissolved in acetone (0.1 mL) prior to GLC–MS.

Gas chromatography and mass spectrometry of PMAAs.—PMAAs were separated and analysed as previously described[1,2].

REFERENCES

1 P.W. Needs and R.R. Selvendran, *Carbohydr. Res.*, 245 (1993) 1–10.
2 P.W. Needs and R.R. Selvendran, *Phytochem. Anal.*, 4 (1993) 210–216.
3 P.J. Harris, R.J. Henry, A.B. Blakeney, and B.A. Stone, *Carbohydr. Res.*, 127 (1984) 59–73.
4 A.B. Blakeney, P.J. Harris, R.J. Henry, and B.A. Stone, *Carbohydr. Res.*, 113 (1983) 291–299.
5 H.N. Englyst and J.H. Cummings, *Analyst*, 103 (1984) 937–942.
6 J.N. BeMiller, *Adv. Carbohydr. Chem.*, 22 (1967) 25–108.
7 F.M. Rombouts, *Occurrence and Properties of Bacterial Pectate Lyases*, Centre for Agricultural Publishing and Documentation, Wageningen, Netherlands, 1967, pp 9–12.
8 R.J. Redgwell, M.A. O'Neill, R.R. Selvendran, and K.J. Parsley, *Carbohydr. Res.*, 153 (1986) 97–106.
9 B. Lindberg, *Methods Enzymol.*, 28 (1972) 178–195.
10 B. Lindberg and J. Lönngren, *Methods Enzymol.*, 50 (1978) 3–33.
11 D.P. Sweet, R.H. Shapiro, and P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217–225.
12 R.R. Selvendran, J.F. March, and S.G. Ring, *Anal. Biochem.*, 96 (1979) 282–292.
13 M. Abdel-Akher, J.K. Hamilton, and F. Smith, *J. Am. Chem. Soc.*, 73 (1951) 4691–4692.
14 A.K. Furr (Ed.), *C.R.C. Handbook of Laboratory Safety*, 3rd ed., CRC Press, Boca Raton, FL, 1990, pp 273–284.
15 J.C. Schumacher (Ed.), *Perchlorates: Their Properties, Manufacture and Uses*, American Chemical Society Monograph, Ser. No. 146, Reinhold, New York, 1960.
16 P.W. Needs and R.R. Selvendran, unpublished results.

17 M.A. O'Neill and R.R. Selvendran, *Carbohydr. Res.*, 145 (1985) 45–58.
18 P. Ryden and R.R. Selvendran, unpublished results.
19 P.L. Durette, P. Köll, H. Meyborg, and H. Paulsen, *Chem. Ber.*, 106 (1973) 2333–2338.
20 R.M. Faulks, S. Southon, and G. Livesey, *Br. J. Nutr.*, 61 (1989) 291–300.
21 I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1990) 209–217.
22 P.A. Sandford and H.E. Conrad, *Biochemistry*, 5 (1966) 1508–1517.
23 R.J. Redgwell, *Phytochemistry*, 22 (1983) 951–956.