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Methylation analysis of polysaccharides: Technical advice

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ABSTRACT

Glycosyl linkage (methylation) analysis is used widely for the structural determination of oligo- and poly-saccharides. The procedure involves derivatisation of the individual component sugars of a polysaccharide to partially methylated alditol acetates which are analysed and quantified by gas chromatography-mass spectrometry. The linkage positions for each component sugar can be determined by correctly identifying the partially methylated alditol acetates. Although the methods are well established, there are many technical aspects to this procedure and both careful attention to detail and considerable experience are required to achieve a successful methylation analysis and to correctly interpret the data generated. The aim of this article is to provide the technical details and critical procedural steps necessary for a successful methylation analysis and to assist researchers (a) with interpreting data correctly and (b) in providing the comprehensive data required for reviewers to fully assess the work.

1. Introduction

The study of complex carbohydrate polymers continues to be an increasingly important area of research. Structural characterisation of polysaccharides, proteoglycans and other glycoconjugates, is challenging due to the complexity and diversity of structures. Complete characterisation requires determination of constituent sugar composition, their configuration, anomerisation and ring form, glycosyl linkages and their sequences (Chaplin & Kennedy, 1994). One of the most widely used methods for determining the linkage structure of complex carbohydrates is methylation analysis. The method involves derivatisation of the individual component sugars of a polysaccharide to partially methylated alditol acetates (PMAAs) which are analysed and quantified by gas chromatography-mass spectrometry (GC–MS). Although the methods are well established (Ciucanu & Kerek, 1984; Hakamori, 1964) they require careful attention to detail and considerable experience in interpreting data correctly. There are several reviews on methylation analysis including that by Carpita and Shea (1989), and an excellent Nature Protocols paper on the structural analysis of plant cell wall polysaccharides which provides good detail on the derivatisation protocols required as well as the identification and quantitation of the glycosyl linkages that are typically present in plant cell walls (Pettolino, Walsh, Fincher & Bacic, 2012).

In this article, we discuss technical aspects for preparing and correctly identifying PMAA derivatives by GC–MS, using glycosyl linkages of specific sugars as examples. It is intended to address specific concerns we have regarding methylation analysis data that has been published recently, rather than to review the comprehensive literature on the subject. We outline the methods required to derivatise specific polysaccharides to their corresponding PMAA derivatives that will enable correct unambiguous identification of their linkages. We also provide reference spectra to demonstrate the analysis required to distinguish key derivatives and their diagnostic fragmentation patterns. The aim is to provide technical details and critical procedural steps to assist researchers (a) with the successful methylation analysis of their materials and interpretation of the data, and (b) in providing the comprehensive data required for reviewers to fully assess the work.

2. Preparation of partially methylated alditol acetates

There are a number of methods for preparing PMAA derivatives for analysis by GC–MS, but most of them follow the same basic procedures as summarised in Scheme 1. The precise procedure used depends on the particular characteristics of the polysaccharide being studied and should be assessed before analysis.

Ensuring full methylation of the polysaccharide before proceeding to the subsequent steps is essential to obtaining reliable results. Ideally, complete methylation is confirmed by analysis such as FTIR,

Abbreviations: Ara, arabinose; Xyl, xylose; Rha, rhamnose; Fuc, fucose; Gal, galactose; Man, mannose; Glc, glucose; Hex, hexose; PMAA, partially methylated alditol acetate; GC–MS, gas chromatography–mass spectrometry; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; 2,3,4-tri-*O*-methyl-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4,-tri-*O*-methyl-mannopyranose; etc, et cetera

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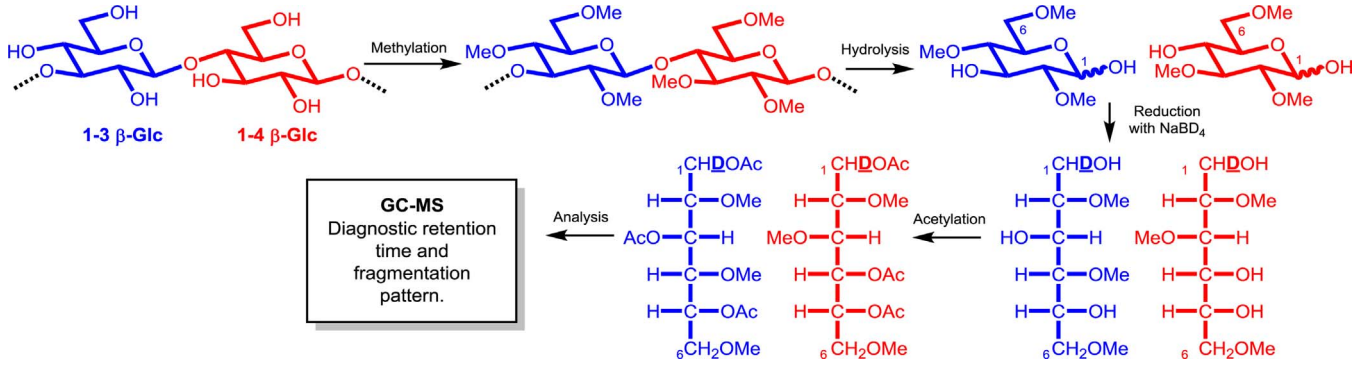

Scheme 1. Preparation of PMAAs.

monitoring the disappearance of a band in the O—H region (3000–3500 cm^{-1}) of the spectrum (Hakamori, 1964). However, this is not always practical (e.g. due to the amount of sample required), so a number of steps can be taken to assist with the complete methylation of samples. The solubility of the polysaccharide in DMSO is a major problem that hinders complete methylation. Whole plant cell walls and some other polysaccharides can be difficult to dissolve fully. In these cases, swelling the samples by heating in DMSO overnight can improve the methylation of free hydroxyl groups. A number of reagents have been used to try to improve the solubility of polysaccharides in DMSO, including 1,1,3,3-tetramethylurea (Narui, Takahashi, Kobayashi & Shibata,1982), *N*-methyl morpholine *N*-oxide (MMNO; Harris, Henry, Blakeney & Stone, 1984) and LiCl in DMSO (Petruš, Gray & BeMiller, 1995). Also, Kim, Reuhs, Michon, Kaiser and Arumugham (2006) improved the methylation of certain polysaccharides by using glycerol to solubilise the polymer prior to methylation. Sulfated polysaccharides, such as carrageenans, are often insoluble in DMSO in their native salt forms and are best converted to triethylammonium salts to increase their solubility prior to methylation (Stevenson & Furneaux, 1991).

The methylation reaction is stopped by addition of water and the methylated material is partitioned into chloroform. The appearance of insoluble material at the organic/aqueous interface during this partitioning may indicate the presence of unmethylated material and necessitate a double methylation procedure (Pettolino et al., 2012). The identification of high proportions of fully acetylated derivatives and low terminal to branch point sugars at the end of the methylation analysis is a sign of undermethylation and also indicates that a double methylation of the material is necessary. Methylation analysis of polysaccharides with non-carbohydrate substituents, such as sulfates, will also show low terminal to branch point sugars. In the case of sulphate groups methylation analysis is repeated following solvolytic desulfation of the polysaccharide (Falshaw & Furneaux, 1994), while other substituents require different approaches depending on their chemical nature. Conversely, high terminal to branch points may result from incomplete hydrolysis of the methylated polysaccharide requiring optimisation of hydrolysis conditions (see below). Whilst, fully methylated polysaccharides are commonly isolated by extraction with chloroform, permethylated charged polysaccharides, such as sulfated polysaccharides, do not partition well in to chloroform and must be isolated by dialysis (Falshaw, Bixler & Johndro, 2001). In addition, reversed-phase solid phase extraction (SPE) has been used to purify methylated oligo- and polysaccharides, but this has not been widely adopted (Mort, Parker & Kuo, 1983).

The subsequent hydrolysis, reduction and acetylation steps have been done under various conditions, based mostly on historical protocols that were the method of choice for the individual research group, but also dependent on the polysaccharide being analysed. Our preferred procedure is hydrolysis with 2.5 M TFA (1 h, 121 °C), reduction with 1 M $NaBD_4$ (18 h, 25 °C) and acetylation with acetic anhydride (600 µL), ethyl acetate (200 µL), acetic acid (40 µL) and perchloric acid (60%, 23 µL) (15 min, room temperature) (Harris, Henry, Blakeney & Stone, 1984). However, these conditions are not ideal for all polysaccharides and different conditions are often required to achieve optimal derivatisation. For example, the fructofuranosyl linkages of fructans are acid labile and are hydrolysed with 1 M TFA, for 30 min at 70 °C, with addition of *tert*-butyl alcohol prior to evaporation to minimise decomposition of released residues (Housley, Gibeaut, Carpita, Sims & Pollock, 1991). Determining the ratio of terminal to branch point sugars can assist in optimising hydrolysis conditions, with a 1:1 ratio optimal. Also, certain acetylation conditions can lead to losses of particular PMAA derivatives. Needs and Selvendran (1993) traced the underestimation of terminal galactosyl and glucosyl groups to degradation during a perchloric acid-catalysed acetylation procedure. They found that base-catalysed acetylation gave more reliable results.

3. Identification of linkages using partially methylated alditol acetate standards

Once prepared, PMAAs are identified by GC–MS using a combination of their retention time and mass spectra, with both of these pieces of information required to correctly identify the derivatives and the sugars they are derived from. Ideally, authentic reference standards of each derivative would be synthesised to enable quantitation using response factors generated from calibration curves. However, in most cases this is impractical and has only been done for analysis of specific polysaccharides; Falshaw and Furneaux (1994) determined the response factors using flame ionisation detection (FID) of authentic PMAA standards for quantitative analysis of carrageenans from *Gigartina decipiens*. More commonly, mixed PMAA standards for use as retention time standards are generated by intentionally under-methylating methyl glycosides to provide mixtures of PMAAs of each sugar, representing each possible linkage type anticipated (Doares, Albersheim & Darvill, 1991; Sassaki, Gorin, Souza, Czelusniak & Iacomini, 2005). Specific methods have been described for preparing PMAA standards of arabinofuranose- and galactofuranose-containing structures (He, Guo, Zhang & Huang, 2013; Sassaki, Iacomini & Gorin, 2005). Also, in recent work, we have shown that melt polymerisation of individual monosacccharides can produce highly branched polymers which when fully derivatised may provide a novel way to generate PMAA mixtures of both pyranose and furanose linkages (Daines, Smart, Sims, Tannock & Hinkley, 2015).

Once mixed PMAA standards have been synthesised they are separated by GC and the relative retention times of each derivative determined relative to *myo*-inositol hexaacetate as an internal retention time standard; in certain circumstances *scyllo*-inositol or 2-deoxy-glucose are alternative standards. The choice of GC column is important so as to avoid, as far as possible, co-elution of derivatives; we routinely use a SGE BPX90 column (SGE Analytical Science, Australia), with additional analysis on an Agilent HP5-ms (Agilent Technologies, Santa Clara, CA) where PMAA derivatives co-elute on the BPX90 column. The

Table 1
Relative retention times (relative to *myo*-inositol hexaacetate, 1.00) of partially methylated alditol acetates on a BPX90 capillary column (GC: constant flow 0.6 cm/min; 80 °C, 1 min, 50 °C/min to 130 °C, 3 °C/min to 230 °C, hold 5 min). Similar data for a HP5-ms is shown in parentheses (GC: constant flow 1.0 cm/min; 50 °C, 1 min, 50 °C/min to 130 °C, 3 °C/min to 230 °C, hold 2 min).

Position of *O*-methyl group	Retention time relative to *myo*-inositol hexaacetate Parent monosaccharide						
	Ara	Xyl	Fuc	Rha	Gal	Man	Glc
None	0.792 (0.757)	0.857 (0.780)	0.712 (0.752)	0.697 (0.742)	0.937 (1.031)	0.913 (1.014)	0.962 (1.023)
2-	0.736 (0.684)	0.776 (0.696)	0.673 (0.678)	0.658 (0.674)	0.887 (0.959)	0.866 (0.945)	0.897 (0.953)
3-	0.749 (0.700)	0.772 (0.698)	0.710 (0.707)	0.693 (0.700)	0.932 (0.993)	0.908 (0.977)	0.916 (0.971)
4-	0.746 (0.689)	0.776 (0.696)	0.705 (0.709)	0.678 (0.691)	0.932 (0.993)	0.908 (0.977)	0.941 (0.983)
6-	– (–)	– (–)	– (–)	– (–)	0.823 (.904)	0.802 (.894)	0.850 (.904)
2,3-	0.650 (0.591)	0.677 (0.598)	0.631 (0.607)	0.597 (0.598)	0.849 (0.889)	0.811 (0.874)	0.841 (0.881)
2,4-	0.652 (0.601)	0.650 (0.591)	– (0.623)	0.593 (0.608)	0.852 (0.919)	0.826 (0.901)	0.825 (0.887)
2,5-	0.616 (0.557)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
2,6-	– (–)	– (–)	– (–)	– (–)	0.778 (0.831)	0.762 (0.825)	0.791 (0.833)
3,4-	0.658 (0.599)	0.677 (0.598)	0.641 (0.625)	0.588 (0.593)	0.869 (0.916)	0.853 (0.886)	0.842 (0.884)
3,5-	0.589 (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
3,6-	– (–)	– (–)	– (–)	– (–)	0.807 (0.853)	0.797 (0.851)	0.806 (0.848)
4,6-	– (–)	– (–)	– (–)	– (–)	0.778 (0.847)	0.757 (0.835)	0.797 (0.845)
2,3,4-	0.536 (0.491)	0.543 (0.490)	0.530 (0.523)	0.474 (0.490)	0.770 (0.815)	0.722 (0.779)	0.725 (0.778)
2,3,5-	0.491 (0.451)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
2,3,6-	– (–)	– (–)	– (–)	– (–)	0.729 (0.751)	0.706 (0.752)	0.737 (0.759)
2,4,6-	– (–)	– (–)	– (–)	– (–)	0.702 (0.770)	0.687 (0.760)	0.685 (0.747)
3,4,6-	– (–)	– (–)	– (–)	– (–)	0.722 (0.769)	0.690 (0.743)	0.695 (0.740)
2,3,4,6-	– (–)	– (–)	– (–)	– (–)	0.623 (0.666)	0.588 (0.642)	0.591 (0.640)

data for each sugar is then used to generate a table of *O*-methyl positions and relative retention times (Table 1). The relative retention times of the derivatives vary depending on the type of GC column and temperature programme (Table 1), but, in general, higher molecular weight, more highly acetylated, PMAA derivatives elute later from most columns. For example, 2,3,4-tri-*O*-methyl-mannitol (derived from 1,6-Man*p*) will not elute before 2,3,4,6-tetra-*O*-methyl-glucitol (terminal Glc*p*), as reported by Mao et al. (2015). The combination of retention time and mass fragmentation data shown in Table 2 of this paper suggest that the peak interpreted as 2,3,4-tri-*O*-methyl-mannitol is more likely to be either 2,3- or 3,4-di-*O*-methyl-pentitol, suggesting the presence of either 1,5-Ara*f*, or 1,4-/1,2-Xyl*p*. Similarly, other papers have reported derivatives eluting in the wrong order for the GC column used. Thus, Chen, Zhang, Fu and Liu (2016) reported 2,3,4-tri-*O*-methyl glucitol (1,6-Glc*p*) eluting before 2,3,5-tri-*O*-methyl-arabinitol (terminal Ara*f*); in this case the retention time and mass fragmentation data indicate that the peak identified as 2,3,4-tri-*O*-methyl glucitol should be 2,3,5-tri-*O*-methyl-arabinitol, but it is not clear what the peak interpreted as 2,3,5-tri-*O*-methyl-arabinitol is. Other examples of erroneous retention time data include 2,4,6-tri-*O*-methyl-mannitol (1,3-Man*p*) and 3,4-di-*O*-methyl-mannitol (1,2,6-Man*p*) eluting before 2,3,4,6-tetra-*O*-methyl-mannitol (terminal Man*p*) (Zhang, Wang, Lai & Wu, 2016); 2,3,4-tri-*O*-methyl-rhamnitol (terminal Rha*p*) eluting later than 2,4,6-tri-*O*-methyl-glucitol (1,3-Glc*p*) (Wu, Zhang, Yu, Lin & Yang, 2015); and 2,3,4,6-tetra-*O*-methyl-glucitol (terminal Glc*p*) eluting later than several tri-*O*-methyl hexitols (Wang, Yang, Zhao, Lu & Wu, 2017). Thus, correct identification of derivatives requires both retention time and mass fragmentation data.

In addition to their relative retention times, PMAA standards also provide electron impact mass spectra for each derivative. There are published spectra (He et al., 2013; Sassaki, Gorin et al., 2005; Sassaki, Iacomini et al., 2005) and online resources (https://www.ccrc.uga.edu/specdb/ms/pmaa/pframe.html) to assist with identification. However, as the precise mass fragmentation patterns obtained are influenced by the tuning of the mass spectrometer and ionisation voltage used, it is recommended to compare experimental data directly with standards run on the same equipment, under the same conditions as samples.

It is important that the mass fragmentation patterns of the PMAAs prepared from samples are compared with those of standards to ensure correct identification of the derivatives, and that the diagnostic mass fragments are reported. These diagnostic fragments are primarily those that result from cleavage of the carbon-carbon bonds of the alditol, and subsequent loss of acetoxyl and methoxyl groups from these fragments (Carpita & Shea, 1989). We have encountered a number of recent papers which either do not show all of the diagnostic fragments, or show mass fragments that do not match those expected for the PMAA derivative identified. Thus, in the analysis of a polysaccharide from *Sargassum fusiforme*, Hu et al. (2016) reported the presence of 1,3- and 1,3,4-Fuc*p* (13.52 and 18.93 min, respectively), but did not show the fragments (*m/z* 131 and 275, respectively) which are diagnostic of the PMAA derivatives of these sugars, thereby raising doubt over their correct identification. The peak at 13.52 min contains a fragment with *m/z* 143 which is diagnostic of 1,4-deoxyHex*p*, rather than 1,3-deoxyHex*p*, whilst the fragmentation pattern of the peak at 18.93, together with elution before 1,4-Gal*p* possibly indicates the presence of a 1,3-Hex*p* residue, although the full data would be required for comprehensive assessment. Similarly, the mass fragmentation pattern for 2,3,6-tri-*O*-methyl glucitol, the PMAA derivative of 1,4-Glc*p* reported by Pan, Wang, Chen, Hu and Zhou (2015) did not include *m/z* 233, with the mass fragments shown (*m/z* 101, 117, 129 and 161) and elution before terminal Glc*p* suggesting that this derivative may actually be 2,3,5-tri-*O*-methyl arabinitol (terminal Ara*f*). Some of the other interpretations based on the fragments shown and the peak retention times in Table 2 of this paper are also open to question. We speculate that peak 2 in the GC, eluting earlier than terminal sugars may be 1,3-Ara*f* rather than 1,6-Gal*p*, peak 3 with *m/z* 143 and 175 is more likely to be a derivative of a deoxyhexose, possibly terminal Rha*p*, rather than terminal Glc*p*, and peak 9 is probably a pentitol pentaacetate, rather than 2,3,4-Glc*p*. To be able to review this data fully and identify the derivatives correctly requires submission of comprehensive methods which include the GC column used and the temperature programme, together with a total ion chromatogram and mass spectra for each of the peaks identified. Ma, Guo, Peterson, Dun and Li (2016) reported the structure of a novel extracellular polysaccharide from a *Phellinus* sp., but the mass fragments shown do not match those in the Complex Carbohydrate Research Center (CCRC) database, with which the data was compared. Other workers have also reported incorrect mass fragmentation patterns for the linkages shown (Hu et al., 2016; Jiang et al., 2016; Shi et al., 2017; Zhang et al., 2017).

The detection of peaks on GC with unusual or unexpected fragmentation patterns often indicates the presence of co-chromatographing derivatives. In our experience, when analysing the linkage composition of complex polysaccharide mixtures, such as those present in plant cell walls, it is common for some PMAA derivatives to co-elute from the GC column. If the potential derivatives are from different sugars but have the same linkage (i.e. they have the same mass fragments), it is sometimes possible to identify the sugar by reference to constituent sugar analysis data, where only one of the possible sugars is present. However, if both sugars are present then analysis on a second

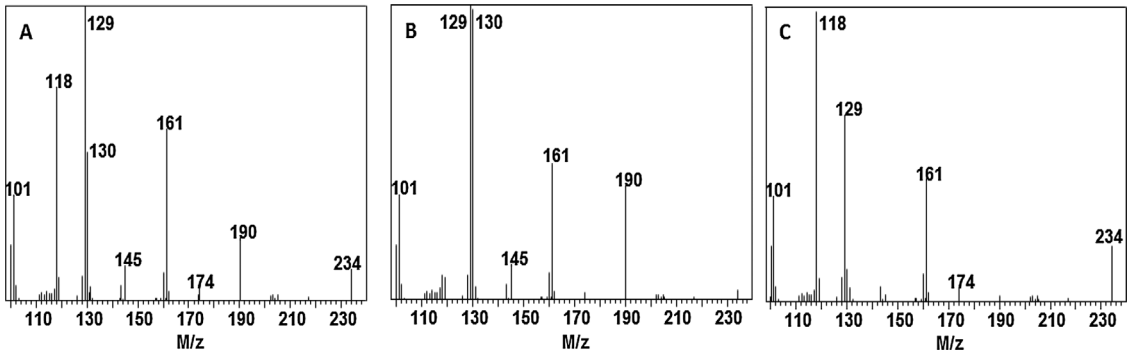

Fig. 1. Mass spectra of (A) 3,4,6-tri-*O*-methyl-Gal*p* (1,2-Gal*p*) and 2,4,6- tri-*O*-methyl-Gal*p* (1,3-Gal*p*), which co-chromatograph on an Agilent HP5-ms column, and (B) 3,4,6- tri-*O*-methyl-Gal*p* and (C) 2,4,6- tri-*O*-methyl-Gal*p* which separate on a SGE BPX90 column.

column with a different polarity is necessary; i.e. 2,4-di-*O*-methyl mannitol and 2,4-di-*O*-methyl glucitol (1,3,6-Man*p* and 1,3,6-Glc*p*) co-chromatograph on a BPX90 column but separate on a HP5-ms column (Table 1). It is also possible to get different derivatives of the same sugar co-chromatographing on a particular column, again requiring analysis on a second column. The mass spectrum shown in Fig. 1A, from a peak eluting from a HP5-ms column, does not match any derivative anticipated, but shows mass fragments of both 3,4,6-tri-*O*-methyl-galactitol (1,2-Gal*p*) and 2,4,6-tri-*O*-methyl-galactitol (1,3-Gal*p*). Whilst it is possible to estimate the proportions of these derivatives by extracting selected ions which are present in one derivative but not the other, analysis using a column with a different polarity, such as a BPX90, which separates 3,4,6-tri-*O*-methyl-galactitol (Fig. 1B) from 2,4,6-tri-*O*-methyl-galactitol (Fig. 1C) should, ideally, be used.

Once each peak in the total ion chromatogram has been assigned to a particular PMAA derivative, and the corresponding sugar and linkage, the mol% of each derivative is calculated by dividing the peak area from the total ion chromatograms by the molecular weight of the PMAA (Pettolino et al., 2012). The results can be expressed either as mol% (our preferred method), or as molar ratios. This method of calculating results gives good data in most cases where a range of sugars with different linkages, with varying rates of hydrolysis and susceptibility to degradation, are present. However, when a high degree of precision is required quantitation using “effective carbon response” following separation by GC and flame ionization detection (FID) is recommended (Sweet, Shapiro & Albersheim, 1975).

4. Labelling of anomeric carbon with deuterium

After acid hydrolysis of methylated polysaccharides, it is important to reduce the resulting monosaccharides with $NaBD_4$ to introduce a deuterium atom at the anomeric carbon. This enables enantiomeric PMAA derivatives, which do not separate on most GC columns, to be distinguished by differences in their mass fragmentation patterns. Such enantiomeric derivatives include 3,4-di-*O*-methyl-xylitol (1,2-Xyl*p*) and 2,3-di-*O*-methyl-xylitol (1,4-Xyl*p*), 4-*O*-methyl-xylitol (1,2,3-Xyl*p*) and 2-*O*-methyl-xylitol (1,3,4-Xyl*p*), and 4-*O*-methyl-hexitol (1,2,3,6-Hex*p*) and 3-*O*-methyl-hexitol (1,2,4,6-Hex*p*). For example, analysis of NZ flax xylan shows the presence of both 1,2-Xyl*p* and 1,4-Xyl*p* (Centanni et al., 2017); the total ion chromatogram (TIC) and mass spectrum (Fig. 2A) shows that the PMAA derivatives for these two linkages, 2,3-di-*O*-methyl-Xyl*p* (1,4-Xyl*p*, Fig. 2C) and 3,4-di-*O*-methyl-Xyl*p* (1,2-Xyl*p*, Fig. 2D), elute as a single peak, but each derivative can be quantified by determining the ratios of the areas of *m/z* 189 and 190 (Fig. 2B). Pei, Wang, Ma and Yan (2015) reported the presence of both 3,4-di-*O*-methyl-Xyl*p* (1,2-Xyl*p*) and 2,3-di-*O*-methyl-Xyl*p* (1,4-Xyl*p*), but reduction with $NaBH_4$ means that these derivatives were indistinguishable from each other. These authors also show 3,4-di-*O*-methyl-Xyl*p* eluting before 2,3-di-*O*-methyl-Xyl*p* on GC, which is highly unlikely. The data shown in Table 2 of this paper does not enable us to speculate the true identity of these peaks. Similarly, Zheng, Dong, Du, Wang and Ding (2015) show the presence of 1,2-Xyl*p* in a neutral polysaccharide from chrysthanthemum flowers, but it is not possible to distinguish between 1,2-Xyl*p*, as reported, and 1,4-Xyl*p* when $NaBH_4$ is used in the reduction step. However, following partial acid hydrolysis of the polysaccharide, the linkage analysis indicates the presence of xyloglucan where α-D-1,2-Xyl*p* would be expected to be present (Fry, 1988), and NMR spectroscopy supports this assignment.

Another example where deuterium labelling at the anomeric carbon allows unequivocal determination of the linkage type is in the analysis of fructans (Bancal, Gibeaut & Carpita, 1993). Reduction of partially methylated fructosyl residues from 2,1-linked fructans (inulin) with $NaBD_4$ yields 3,4,6-tri-*O*-methyl-(2-deuterio)-mannitol and glucitol while similar reduction of 2,6-linked fructans (levan) yields 1,3,4-tri-*O*-methyl-(2-deuterio)-mannitol and glucitol (Fig. 3). The linkages are distinguished by the presence of ions at *m/z* 189 (2,6-Fru*f*) or *m/z* 190 (2,1- Fru*f*) and, if both linkages are present, quantified from the ratio of the areas of these two species.

5. Reduction of uronic acids prior to methylation

Many polysaccharides from plants, macroalgae and other sources contain acidic sugars, which need to be reduced to their equivalent neutral sugars to enable their linkages to be determined efficiently. Acidic sugars, such as uronic acids, can be reduced after methylation using lithium triethylborodeuteride (York, Darvil, McNeil, Stevenson & Albersheim, 1986), or prior to methylation (Kim & Carpita, 1992). Galacturonic acid (GalA) in plant pectic polysaccharides may be present as the free acid, or as the methylester. Selective reduction of methylesterified uronic acids and subsequent reduction of carbodiimide-activated free uronic acids, prior to methylation, allows simultaneous determination of uronic acid linkages and the degree of methylesterification (Kim & Carpita, 1992; Sims & Bacic, 1995; Pettolino, Walsh, Fincher & Bacic, 2012). To ensure complete reduction of all uronic acid residues repeated reductions may be necessary (Du et al., 2015; Maas et al., 2012; Nep et al., 2016). We monitor the extent of carboxyl-reduction by determining constituent sugar composition by high-performance anion-exchange chromatography (HPAEC) after hydrolysis of the polysaccharides to their component monosaccharides, where, as the uronic acid peak decreases (Fig. 4A) the equivalent neutral sugar peak increases (Fig. 4B). In a recent study a total of four reductions was required to reduce the uronic acid content of a polysaccharide extracted from sesamum leaves to < 5% of the native polysaccharide (Nep et al., 2016).

The derivatives of identically linked neutral sugars and carboxyl-reduced uronic acids co-chromatograph on both high and low polarity GC columns, and thus their identification requires extraction of specific ions from the total ion chromatogram. In the characterisation of a polysaccharide from fruits of *Zizyphus jujube* Li, Ai, Yang, Liu and Shan (2013) showed 2,3,6-tri-*O*-methyl-6,6-dideuteriogalactitol (1,4-Gal*p*A)

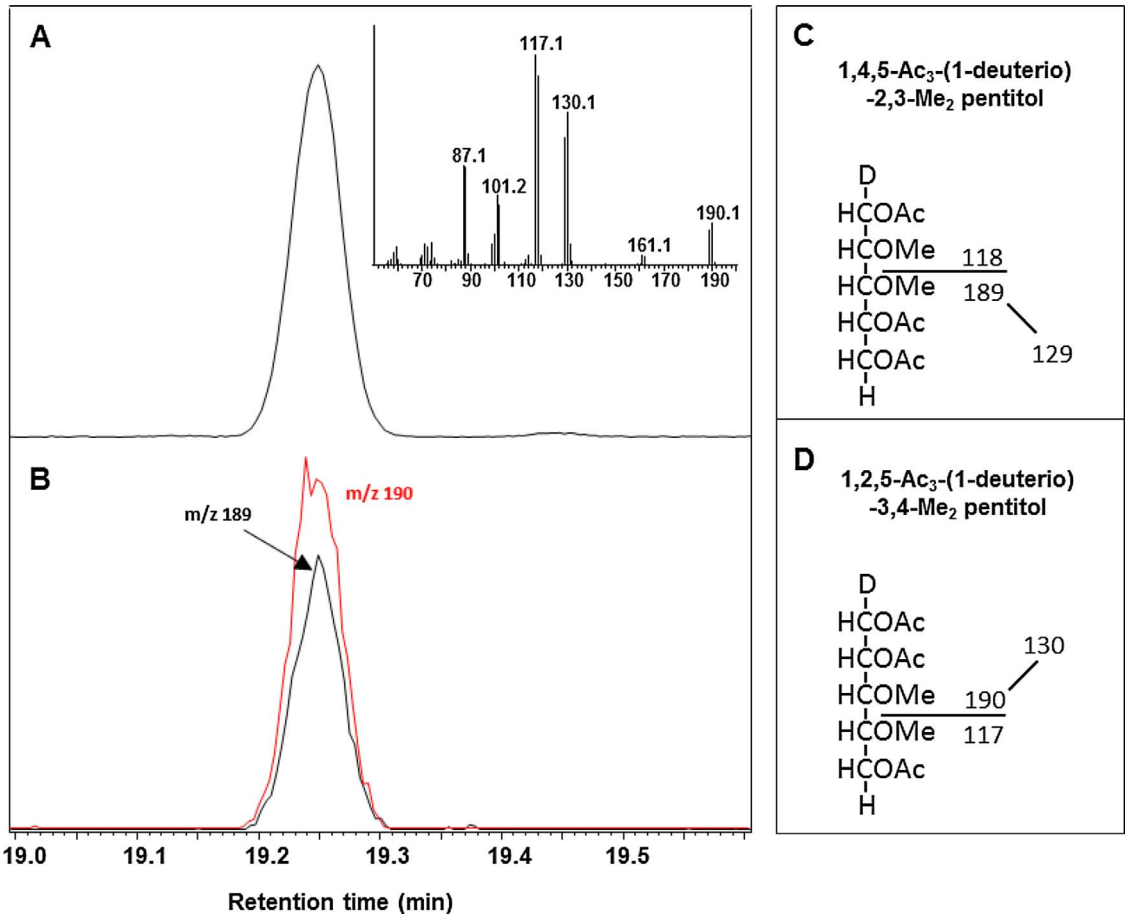

Fig. 2. Total ion chromatogram and mass spectrum of co-eluting PMAA derivatives, 3,4-di-*O*-methyl-xylitol (1,2-Xyl*p*) and 2,3-di-*O*-methyl-xylitol (1,4-Xyl*p*), from the glycosyl linkage analysis of NZ flax xylan (A). Linkages are quantified from the ratio of *m/z* 189 (2,3-di-*O*-methyl-xylitol, C) to *m/z* 190 (3,4-di-*O*-methyl-xylitol, D) (B).

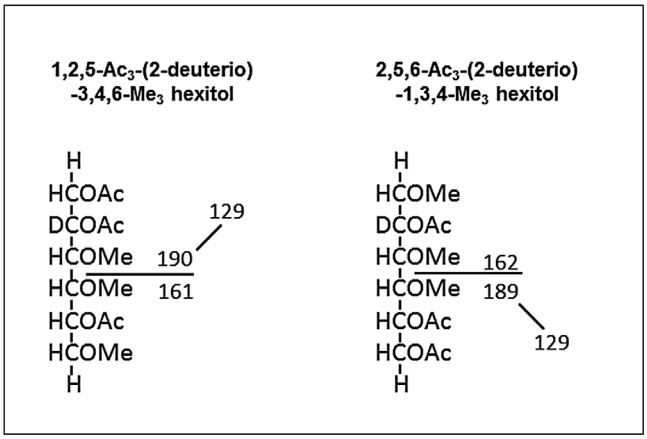

Fig. 3. Diagnostic fragmentations of PMAA derivatives of 2,1-linked fructans (1,2,5-tri-*O*-acetyl-[2-deuterio]-3,4,6-tri-*O*-methyl hexitol) and 2,6-linked fructans (2,5,6-tri-*O*-acetyl-[2-deuterio]-1,3,4-tri-*O*-methyl hexitol).

eluting over three minutes earlier than 2,3,6-tri-*O*-methyl-galactitol (1,4-Gal*p*), suggesting that some derivatives were identified incorrectly, although insufficient data is presented in Table 1 of this paper to allow correct identifications to be made. Fig. 5A shows a selected region of a TIC from the glycosyl linkage analysis of a pectic fraction of gold kiwifruit (Sauvageau, Hinkley, Carnachan & Sims, 2010). Following reduction of both methyl esterified and free uronic acids with $NaBD_4$, the areas of *m/z* 233:235 are used to calculate the proportions 1,4-Gal*p* and total 1,4-Gal*p*A (Fig. 5B); reduction of esterified uronic acids with $NaBD_4$ and the free uronic acids with $NaBH_4$ is used to calculate the proportion of esterified 1,4-Gal*p*A (Fig. 5C). Reduction of both esterified and free uronic acids with $NaBH_4$ shows mostly *m/z* 233 for the 1,4-Gal*p*/1,4-Gal*p*A peak, with the small proportion of *m/z* 235 (1.5% of *m/z* 233) representing the contribution from the natural abundance of the ^{13}C isotope (Fig. 5D).

6. Endogenous *O*-methylation or *O*-acetylation of polysaccharides

Some sugars are endogenously *O*-methylated or *O*-acetylated and determination of their linkages requires the use of labelled reagents during derivatisation. A particular example of *O*-methylation is 4-*O*-methyl glucuronic acid (4-*O*-MeGlcA) that is found in some plant cell wall polysaccharides (Penã et al., 2016). To identify the *O*-methyl positions, carboxyl reduced polysaccharide is methylated using CD_3I instead of CH_3I, to distinguish endogenous $-OCH_3$ groups and the resulting mass fragmentation patterns used to determine their location (Carpita and Shea, 1989). Ho, Zou, Aslaksen, Wangensteen & Barsett (2016) reported the presence of both terminally-linked GlcA and 4-*O*-MeGlcA in pectic polysaccharides from elderflowers; however, without a description of how the linkages were distinguished these results are questionable.

Many polysaccharides are naturally *O*-acetylated, but the basic conditions commonly used for methylation cleave these base-labile substituents and information on their position is lost. Several authors have used a mild methylation, under neutral conditions to methylate oligo- and poly-saccharides without removal of *O*-acetyl groups (Prehm, 1980), although care is required to ensure full methylation. The location of *O*-acetyl groups on acetan (Ojinnaka et al., 1996) and an *O*-

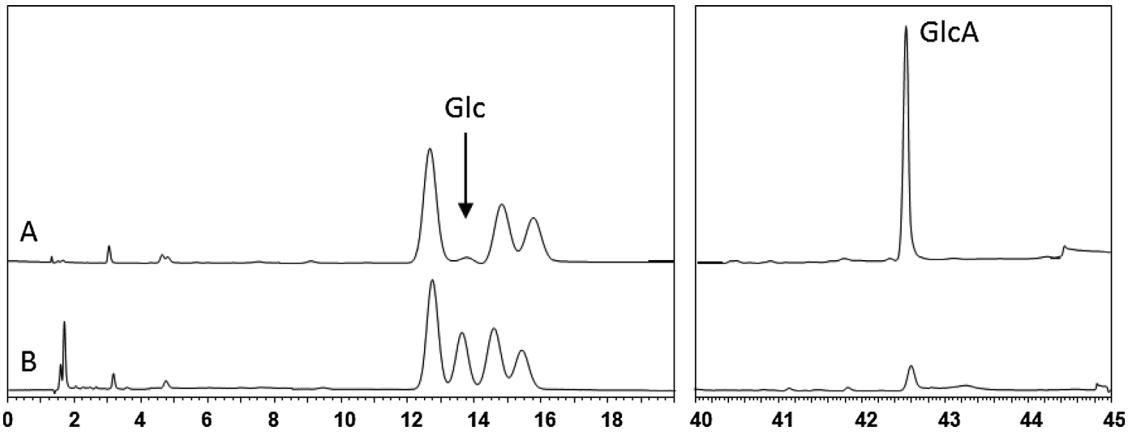

Fig. 4. High-performance anion-exchange chromatograms of native (A) and four times carboxyl-reduced (B) sesamum gum polysaccharide, showing reduction of glucuronic acid (GlcA) to glucose (Glc).

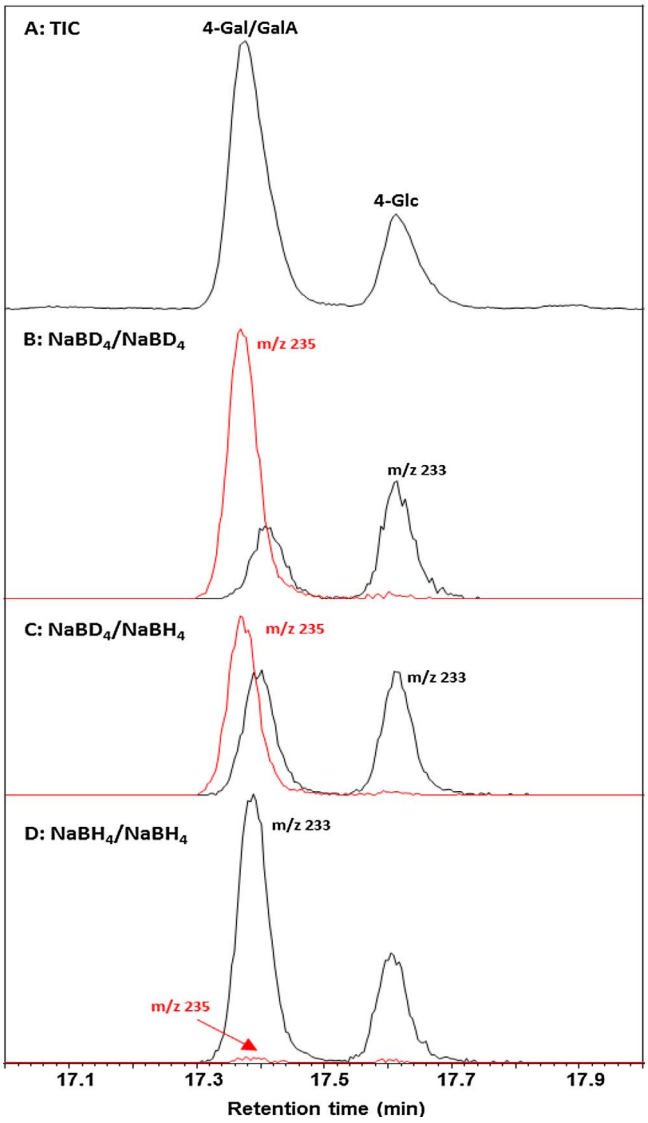

Fig. 5. Selected region of a total ion chromatogram (A) and extracted ion chromatograms (B, C and D) of a carboxyl reduced pectin-rich fraction from gold kiwifruit. See text for details.

polysaccharide isolated from *Salmonella paratyphi* A (Ravenscroft et al., 2015) have been determined by comparing the results of linkage analysis using mild methylation conditions with those of base-catalysed methylation methods. In other work, Sims, Munro, Currie, Craik and Bacic (1996) used mild methylation, followed by remethylation under basic conditions with CD_3I to enable the location of the *O*-acetyl groups to be determined by a 3 atomic mass unit increase in the specific mass fragments.

7. Conclusions

In this article we have highlighted the technical aspects of glycosyl linkage (methylation) analysis, particularly the preparation of PMAA derivatives and correctly interpreting and reporting the results. To ensure that data submitted for publication can be assessed fully and meets editorial requirements, we propose that authors should include the following information:

(1) Details of the methods used, including details of the GC column and oven temperature programme.
(2) Total ion chromatograms with peaks labelled appropriately.
(3) Mass spectra of peaks with interpretation of the mass fragmentation patterns.
(4) Tabulated data, presented as molar proportions (mol% or molar ratios).

The recent work of Guo et al. (2016) is a good example of a paper in which such comprehensive data has been provided. They provide details of the GC column used and the oven temperature programme; Table 2 of their results presents the retention times of the peaks, the diagnostic fragment ions, the PMAAs and the deduced sugar linkages, and their molar ratios; the supplementary Fig. S1 of their paper shows the total ion chromatogram and mass spectra for each of the peaks along with full interpretations of the mass fragmentation data.

We suggest that there should be discussions throughout the wider community of experts in this field to agree on minimum requirements for journal submissions in which glycosyl linkage analysis data is presented. We propose that journals require authors to submit comprehensive data as given above to enable reviewers who are experts in polysaccharide analysis to fully assess the data and welcome comments and opinion from other researchers in this field.

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References

Bancal, P., Gibeaut, D. M., & Carpita, N. C. (1993). Analytical methods for the

determination of fructan structure and biosynthesis. In M. Suzuki, & N. J. Chatterton (Eds.). *Science and technology of fructans* (pp. 83–118). Boca Raton: CRC.
Carpita, N. C., & Shea, E. M. (1989). Linkage structure of carbohydrates by gas chromatography–mass spectrometry (GC–MS) of partially methylated alditol acetates. In C. J. Bierman, & G. D. McGinnis (Eds.). *Analysis of carbohydrates by GLC and MS* (pp. 157–216). Boca Raton: CRC.
Centanni, M., Hutchison, J. C., Carnachan, S. M., Daines, A. M., Kelly, W. J., Tannock, G. W., et al. (2017). Differential growth of bowel commensal *Bacteroides* species on plant xylans of differing structural complexity. *Carbohydrate Polymers, 157*, 1374–1382.
Chaplin, M. F., & Kennedy, J. F. (Eds.). (1994). *Carbohydrate analysis, a practical approach* (2nd ed.). New York: Oxford University Press Inc.
Chen, C., Zhang, B., Fu, X., & Liu, R. H. (2016). A novel polysaccharide isolated from mulberry fruits (*Murus alba* L.) and its selenide derivative: Structural characterization and biological activities. *Food and Function, 7*, 2886–2897.
Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research, 131*, 209–217.
Daines, A. M., Smart, Z., Sims, I. M., Tannock, G. W., & Hinkley, S. F. (2015). Synthesis, characterisation and microbial utilisation of amorphous polysugars from lactose. *Carbohydrate Polymers, 117*, 223–229.
Doares, S. H., Albersheim, P., & Darvill, A. G. (1991). An improved method for the preparation of standards for glycosyl-linkage analysis of complex carbohydrates. *Carbohydrate Research, 210*, 311–317.
Du, X., Zhang, Y., Mu, H., Lv, Z., Yang, Y., & Zhang, J. (2015). Structural elucidation and antioxidant activity of a novel polysaccharide (TAPB1) from *Tremella aurantialba*. *Food Hydrocolloids, 43*, 459–464.
Falshaw, R., & Furneaux, R. H. (1994). Carrageenan from the tetrasporic stage of *Gigartina decipiens* (Gigartinaceae, Rhodophyta). *Carbohydrate Research, 252*, 171–182.
Falshaw, R., Bixler, H. J., & Johndro, K. (2001). Structure and performance of commercial kappa-2 carraageenan extracts: I Structure analysis. *Food Hydrocolloids, 15*, 441–452.
Fry, S. C. (1988). *The growing plant cell wall: Chemical and metabolic analysis.* New Jersey: The Blackburn Press.
Guo, R., Ai, L., Cao, N., Ma, J., Wu, J., & Sun, X. (2016). Physicochemical properties and structural characterization of a galactomannan from *Sophora alopecuroides* L seeds. *Carbohydrate Polymers, 140*, 451–460.
Hakamori, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *The Journal of Biochemistry, 55*, 205–208.
Harris, P. J., Henry, R. J., Blakeney, A. B., & Stone, B. A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research, 127*, 59–73.
He, J., Guo, Y., Zhang, L., & Huang, L. (2013). A facile method for the synthesis of partially *O*-methylated alditol acetate standards for GC–MS analysis of galactofuranose-containing structures. *Carbohydrate Research, 379*, 18–20.
Ho, G. T. T., Zou, Y.-F., Aslaksen, T. H., Wangensteen, H., & Barsett, H. (2016). Structural characterization of bioactive pectic polysaccharides from elderflowers (*Sambuci flos*). *Carbohydrate Polymers, 135*, 128–137.
Housley, T. L., Gibeaut, D. M., Carpita, N. C., Sims, I., & Pollock, C. J. (1991). Fructosyl transfer from sucrose and oligosaccharides during fructan synthesis in excised leaves of *Lolium temulentum* L. *New Phytologist, 119*, 491–497.
Hu, P., Li, Z., Chen, M., Sun, Z., Ling, Y., Jiang, J., et al. (2016). Structural elucidation and protective role of a polysaccharide from *Sargassum fusiforme* on ameliorating learning and memory deficiencies in mice. *Carbohydrate Polymers, 139*, 150–158.
Jiang, J., Kong, F., Li, N., Zhang, D., Yan, C., & Lv, H. (2016). Purification, structural characterization and in vitro antioxidant activity of a novel polysaccharide from Boshuzhi. *Carbohydrate Polymers, 147*, 365–371.
Kim, J.-B., & Carpita, N. C. (1992). Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiology, 98*, 646–653.
Kim, J. S., Reuhs, B. L., Michon, F., Kaiser, R. E., & Arumugham, R. G. (2006). Addition of glycerol for improved methylation linkage analysis of polysaccharides. *Carbohydrate Research, 341*, 1061–1064.
Li, J., Ai, L., Yang, Q., Liu, Y., & Shan, L. (2013). Isolation and structural characterization of a polysaccharide from fruits of *Zizyphus jujube* cv. *Junzao*. *International Journal of Biological Macromolecules, 55*, 83–87.
Ma, X-k., Guo, D.d, Peterson, E. C., Dun, Y., & Li, D.y. (2016). Structural characterization and anti-aging activity of a novel extracellular polysaccharide from fungus *Phellinus* sp. in a mammalian system. *Food and Function, 7*, 3468–3479.
Maas, N. C., Gracher, A. H. P., Sassaki, G. L., Gorin, P. A. J., Iacomini, M., & Cipriani, T. R. (2012). Sulfation pattern of citrus pectin and its carboxy-reduced derivatives: Influence on anticoagulant and antithrombotic effects. *Carbohydrate Polymers, 89*, 1081–1087.
Mao, G.-H., Ren, Y., Feng, W.-W., Li, Q., Wu, H.-Y., Jin, D., et al. (2015). Antitumor and immunomodulatory activity of a water soluble polysaccharide from *Grifola frondosa*. *Carbohydrate Polymers, 134*, 406–412.
Mort, A. J., Parker, S., & Kuo, M. S. (1983). Recovery of methylated saccharides from methylation reaction mixtures using Sep-Pak C18 cartridges. *Analytical Biochemistry, 133*, 380–384.
Narui, T., Takahashi, K., Kobayashi, M., & Shibata, S. (1982). Permethylation of polysaccharides by a modified Hakomori method. *Carbohydrate Research, 103*, 293–295.
Needs, P. W., & Selvendran, R. R. (1993). A critical assessment of a one-tube procedure for the linkage analysis of polysaccharides as partially methylated alditol acetates. *Carbohydrate Research, 254*, 229–244.
Nep, E. I., Carnachan, S. M., Ngwuluka, N. C., Kontogiorgos, V., Morris, G. A., Sims, I. M., et al. (2016). Structural characterisation and rheological properties of a polysaccharide from sesame leaves (*Sesamum radiatum* Schumach. & Thonn.). *Carbohydrate Polymers, 152*, 541–547.
Ojinnaka, C., Jay, A. J., Colquhoun, I. J., Brownsey, G. J., Morris, E. R., & Morris, V. J. (1996). Structure and conformation of acetan polysaccharide. *International Journal of Biological Macromolecules, 19*, 149–156.
Pan, D., Wang, L., Chen, C., Hu, B., & Zhou, P. (2015). Isolation and characterization of a hyperbranched proteoglycan from *Ganoderma lucidum* for anti-diabetes. *Carbohydrate Polymers, 117*, 106–114.
Pei, J.-J., Wang, Z.-B., Ma, H.-L., & &Yan, J.-K. (2015). Structural features and antitumor activity of a novel polysaccharide from alkaline extract of *Phellinus linteus* mycelia. *Carbohydrate Polymers, 115*, 472–477.
Penã, M. J., Kulkarni, A. R., Backe, J., Boyd, M., O'Neill, M. A., & York, W. S. (2016). Structural diversity of xylans in the cell walls of monocots. *Planta, 244*, 589–606.
Petruš, L., Gray, D. G., & BeMiller, J. N. (1995). Homogeneous alkylation of cellulose in lithium chloride/dimethyl sulfoxide solvent with dimsyl sodium activation. A proposal for the mechanism of cellulose dissolution in LiCl/Me_2SO. *Carbohydrate Research, 268*, 319–323.
Pettolino, F. A., Walsh, C., Fincher, G. B., & Bacic, A. (2012). Determining the polysaccharide composition of plant cell walls. *Nature Protocols, 7*, 1590–1607.
Prehm, P. (1980). Methylation of carbohydrates by methyl trifluoromethanesulfonate in trimethyl phosphate. *Carbohydrate Research, 78*, 372–374.
Ravenscroft, N., Cescutti, P., Gavini, M., Stefanetti, G., MacLennan, C. A., Martin, L. B., et al. (2015). Structural analysis of the O-acetylated O-polysaccharide isolated from *Salmonella paratyphi* A and used for vaccine preparation. *Carbohydrate Research, 404*, 108–116.
Sassaki, G. L., Gorin, P. A., Souza, L. M., Czelusniak, P. A., & Iacomini, M. (2005). Rapid synthesis of partially O-methylated alditol acetate standards for GC–MS: Some relative activities of hydroxyl groups of methyl glycopyranosides on Purdie methylation. *Carbohydrate Research, 340*, 731–739.
Sassaki, G. L., Iacomini, M., & Gorin, P. A. J. (2005). Methylation-GC–MS analysis of arabinofuranose- and galactofuranose-containing structures: Rapid synthesis of partially *O*-methylated alditol acetate standards. *Annals of the Brazilian Academy of Sciences, 77*, 223–234.
Sauvageau, J., Hinkley, S. F., Carnachan, S. M., & Sims, I. M. (2010). Characterisation of polysaccharides from gold kiwifruit (*Actinidia chinensis* Planch. 'Hort16A'). *Carbohydrate Polymers, 82*, 1110–1115.
Shi, X.-D., Nie, S.-P., Yin, J.-Y., Que, Z.-Q., Zhang, L.-J., & Huang, X.-J. (2017). Polysaccharide from leaf skin of Aloe barbadensis Miller: Part I. Extraction, fractionation, physicochemical properties and structural characterization. *Food Hydrocolloids, 73*, 176–183.
Sims, I. M., & Bacic, A. (1995). Extracellular polysaccharides from suspension cultures of *Nicotiana plumbaginifolia*. *Phytochemistry, 38*, 1397–1405.
Sims, I. M., Munro, S. L. A., Currie, G., Craik, D., & Bacic, A. (1996). Structural characterisation of xyloglucan secreted by suspension-cultured cells of *Nicotiana plumbaginifolia*. *Carbohydrate Research, 293*, 147–172.
Stevenson, T. T., & Furneaux, R. H. (1991). Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydrate Research, 210*, 277–298.
Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative aanalysis by various g.l.c response factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research, 40*, 217–225.
Wang, M., Yang, X., Zhao, J., Lu, C., & Wu, W. (2017). Structural characterization and macrophage immunomodulatory activity of a novel polysaccharide from *Smilax glabra* Roxb. *Carbohydrate Polymers, 156*, 390–402.
Wu, M., Zhang, F., Yu, Z., Lin, J., & Yang, L. (2015). Chemical characterization and in vitro antitumor activity of a single-component polysaccharide from *Taxus chinensis* Var. *mairei*. *Carbohydrate Polymers, 133*, 294–301.
York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1986). Isolation and characterization of plant cell walls and cell wall component. *Methods in Enzymology, 118*, 3–40.
Zhang, M., Wang, G., Lai, F., & Wu, H. (2016). Structural characterization and immunolomodulatory activity of a novel polysaccharide from *Lepidium mynenii*. *Journal of Agricultural and Food Chemistry, 64*, 1921–1931.
Zhang, Z.-P., Shen, C.-C., Gao, F.-L., Wei, H., Ren, D.-F., & Lu, J. (2017). Isolation, Purification and structural characterization of two novel water-soluble polysaccharides from *Anredera cordifolia*. *Molecules, 22*, 1276.
Zheng, C., Dong, Q., Du, Z., Wang, P., & Ding, K. (2015). Structural elucidation of a polysaccharide from *Chrysanthemum morifolium* flowers with anti-angiogenic activity. *International Journal of Biological Macromolecules, 79*, 674–680.