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Short communication

# Quantitation of 4-*O*-methylglucuronic acid from plant cell walls

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ABSTRACT

The accuracy of commercial α-D-glucopyranosyl uronic acid (GlcA) as a calibration standard for the determination of the 4-*O*-methyl-α-D-glucopyranosyl uronic acid (meGlcA) content in plant materials was studied. A batch of meGlcA standard was purified from commercial birch xylan and quantified using nuclear magnetic resonance spectroscopy. Both commercial GlcA and the purified meGlcA were used as standards for the quantitation of meGlcA in *Arabidopsis thaliana* stems, as well as wood and wheat straw samples using acid methanolysis and gas chromatography (GC). The GlcA standard was partially lactonized during acid methanolysis, thus yielding six glycoside peaks in GC. If all six GlcA-derived peaks were included in the GlcA calibration curve, the calculated meGlcA content was underestimated by 30% compared with that obtained using the purified meGlcA as a standard. The meGlcA content was best estimated by including either the two main GlcA peaks or only peaks corresponding to pyranosides and furanosides of GlcA in the calibration curve.

## 1. Introduction

Glucuronoxylans (GX) consist of linear (1→4)-linked β-D-xylopyranosyl (Xyl*p*) backbones and are randomly substituted by (1→2)-linked α-D-glucopyranosyl uronic acid (GlcA) and/or 4-*O*-methyl-α-D-glucopyranosyl uronic acid (meGlcA) (Aspinall, 1980). Xylans in angiosperms (19–35% of dry weight) are mainly substituted by meGlcA in every 4–16 Xyl*p* residues (Ebringerová & Heinze, 2000). In some cases, both GlcA and meGlcA residues are found – for example, in kenaf (Komiyama et al., 2009) and *Arabidopsis thaliana* (Peña et al., 2007). The latter dicot species is an important plant model for GX biosynthesis studies. Xylans are less abundant in gymnosperms (8–14%), and the average molar ratio of meGlcA: Xyl*p* is 1.4:10 (Jacobs, Larsson, & Dahlman, 2001). Xylans are also abundant in the lignified tissues of cereals and grasses, where both meGlcA and GlcA substitutions have been found (Aspinall, 1980; Yoshida et al., 1990).

Uronic acids present in plant materials are commonly quantified using a colorimetric assay (Filisetti-Cozzi & Carpita, 1991); however, in addition to GlcA, pectin-derived galacturonic acid reacts in this method and, therefore, a more specific approach is needed. Proton nuclear magnetic resonance spectroscopy ($^1$H NMR) can be employed to determine molar ratio of structural components in polysaccharides (Evtuguin, Tomas, Silva, & Pascoal Neto, 2003; Teleman, Lundqvist, Tjerneld, Stålbrand, & Dahlman, 2000) that must first isolate from whole plant materials. Gas chromatography (GC) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) are commonly used for the quantitation of monosaccharides. Traditionally, plant polysaccharides are first depolymerized by sulfuric acid, but the α-(1→2) linkage between meGlcA/GlcA and Xyl*p* residue is unexceptionally stable against acid hydrolysis (Tenkanen, Hausalo, Siika-Aho, Buchert, & Viikari, 1995). On the other hand, extensive hydrolysis results in decarboxylation of uronic acid (Leschinsky, Sixta, & Patt, 2009; Testova, Chong, Tenkanen, & Sixta, 2011). Enzymatic hydrolysis can effectively liberate meGlcA/GlcA from xylans; however, this method is more suitable for isolated polysaccharides than for whole plant materials (Buchert et al., 1993; Dahlman, Jacobs, Liljenberg, & Olsson, 2000; Tenkanen et al., 1999). In comparison, acid methanolysis that depolymerizes mainly non-cellulosic polysaccharides is effective to liberate meGlcA/GlcA that present in plant materials without prior delignification (Sundberg, Sundberg, Lillandt, & Holmbom, 1996; Willför et al., 2009). For the quantitation of uronic acids, calibration standards are needed; however, meGlcA is not commercially available and thus GlcA is normally used as a calibration standard for the determination of meGlcA content. To our knowledge, there are no reports available to evaluate the accuracy of GlcA employed as the calibration standard for this purpose.

## 2. Experimental

### 2.1. Chemicals, enzymes and carbohydrates

Dry methanol was obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetyl chloride and trimethylsilyl chloride were obtained

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from Fluka (St. Louis, MO, USA). Pyridine, $D_2O$, sodium acetate (NaAc), and bis(trimethylsilyl)trifluoroacetamide were obtained from Merck (Darmstadt, Germany). HPLC grade heptane was obtained from Rathburn (Walkerburn, UK). Sodium hydroxide (NaOH) solutions were prepared from 50–52% NaOH (Fluka). Ultraflo L enzyme mixture was from Novozymes (Bagsvaerd, Denmark), and it contains 2900 nkat/ml endo-1,4-β-xylanase activity (Bailey, Biely, & Poutanen, 1992) and 102 nkat/ml α-glucuronidase activity (Siika-aho, Tenkanen, Buchert, Puls, & Viikari, 1994). Dowex 1×2 anion exchange resin was from Sigma, and BioGel P-2 size exclusion resin from Bio-Rad (Hercules, CA, USA).

L(+)-arabinose (Ara) and D(+)-xylose (Xyl) were from Merck. D-Glucuronic acid sodium monohydrate (GlcA), D-sorbitol, and birchwood glucuronoxylan (X0502) were from Sigma–Aldrich, and 1,4-β-D-mannobiose (Man$_2$) was from Megazyme (Bray, Ireland). Powdered wood samples (aspen, birch, spruce) and wheat straw were from Prof. Stefan Willför of Åbo Akademi University, Finland. *A. thaliana* alcohol insoluble residue (AIR) was from Prof. Ewa Mellerowicz of Swedish University of Agricultural Sciences. Sample preparation was according to Chong et al. (2011).

### 2.2. Production of meGlcA

Birch xylan (1 g, 0.5%, w/v) was suspended in 50 mM NaAc buffer pH 5.0. Hydrolysis with Ultraflo L (dosed at 50,000 nkat/g xylan xylanase activity, which contained 170 nkat α-glucuronidase activity/g xylan) was performed at 37 °C for 72 h. Afterwards, the solution was kept in a boiling water bath for 15 min and centrifuged at 18,000 × g to remove insoluble materials. Released meGlcA was purified using anion exchange (Dowex 1×2) and size-exclusion chromatography performed according to Koutaniemi et al. (2012).

### 2.3. NMR spectroscopy

One dimensional (1D) $^1$H NMR was acquired at 25 °C on Varian Unity 500 or 600 spectrometers (Varian NMR Systems, Palo Alto, CA, USA). In 1D $^1$H spectra, a modification of the water-eliminated Fourier transform (WEFT) sequence was used. Chemical shifts were determined relative to an internal acetone standard at 2.225 ppm for $^1$H. For the quantitation of meGlcA, 180, 540, or 990 μg of Man$_2$ were added into tubes, each containing 40 μl of meGlcA and added to total volume of 0.5 ml with $D_2O$. Prior to NMR measurements, samples were exchanged four times with $D_2O$. Recovery test was done in the same way using 500 μg of GlcA.

### 2.4. HPAEC-PAD analyses

The behavior of purified meGlcA and commercial GlcA standards was studied with HPAEC-PAD using instrumentation and conditions described by Rantanen et al. (2007). Shortly, the eluents were A: 1 M NaAc in 100 mM NaOH and B: 100 mM NaOH. The analysis method was started with isocratic 100% B for 15 min, followed by a linear gradient to achieve 12% A and 88% B in 20 min. The second isocratic phase was 12% A and 88% B for 5 min and back to 100% B in 5 min. Total analysis time was 50 min and flow rate was 1 ml/min. The injected amount of meGlcA and GlcA standards was 4 nmol.

### 2.5. Acid methanolysis and GC(–MS) analysis of the meGlcA and GlcA standards

GlcA and meGlcA standards were incubated in 2 M HCl/methanol for 5 h, according to the acid methanolysis method described by Sundberg et al. (1996). An aliquot of the methyl ester methyl glycosides equivalent to 400 nmol was further trimethylsilylated (TMSi) and analyzed with HP 6890N GC equipped with a flame ionization detector (FID) (Agilent Technologies, Waldbronn, Germany). The capillary column used was a DB-1 (30 m, 0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies. Oven temperature profile was as follows: 150 °C (3 min)–1 °C/min–155 °C (1 min)–2 °C/min–200 °C–20 °C/min–325 °C.

For GC–MS analysis, the TMSi derivatives were separated on HP-5 capillary column (30 m, 0.25 mm i.d, 1 μm film thickness) (Agilent Technologies) using the following oven temperature profile: 170 °C (10 min)–4 °C/min–200 °C (10 min).

### 2.6. Analysis of woods and wheat straw

The non-cellulosic polysaccharides in powdered *A. thaliana* AIR, wood, and wheat straw samples were degraded by acid methanolysis and trimethylsilylated for GC analysis as described above. The monosaccharide standards used were Xyl, Ara, GlcA, and the purified meGlcA. Sorbitol was used as an internal standard. The total peak area ratios were obtained from the total sum of peak areas of monosaccharide standards to that of internal standard. Monosaccharide contents were calculated from external calibration curves that were plotted as total peak area ratio against the amount of monosaccharide standards. The monosaccharide composition was calculated from triplicate samples and expressed as % anhydro-monosaccharides of dry weight.

Four calibration curves were plotted from the GlcA standard to calculate the meGlcA content in samples. They were obtained by summing up the total peak area ratios derived from either all six (GlcA6p), four (GlcA4p; excluded peaks corresponding to lactones), two main (GlcA2p; retention time (RT): 20.4 and 28 min), or only the main (GlcA1p; RT: 28 min) GlcA peak(s).

## 3. Results and discussion

### 3.1. Determination of meGlcA concentration using NMR spectroscopy

Concentration of the purified meGlcA (referred to as meGlcA standard below) was determined with $^1$H NMR spectroscopy using Man$_2$ as an internal standard, as the resonances of its anomeric protons were clearly separated from those of meGlcA. The NMR spectra showed two anomeric reducing end resonances from α and β positions at 5.21 and 4.60 ppm for meGlcA and at 5.15 and 4.91 ppm for Man$_2$ (Fig. 1). Resonance at 4.74 ppm originates from the terminal proton of Man$_2$. Signals from other ring protons were found in the region of 3.2–4.1 ppm. $^1$H chemical shift for meGlcA standard is shown in Supplementary Table 1.

For quantitative determination, three samples were measured, each containing different amounts of Man$_2$ in standard

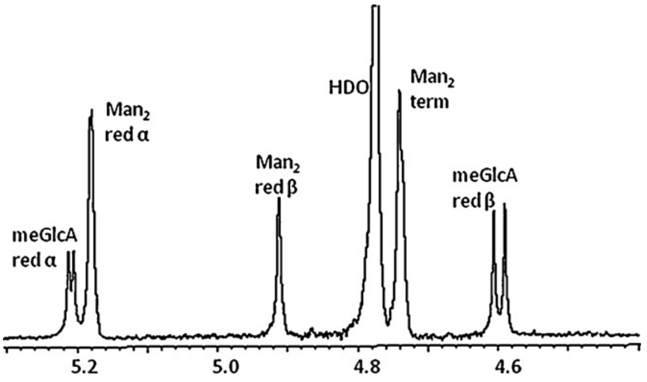

**Fig. 1.** Anomeric region of $^1$H NMR spectrum of the mixture of mannobiose (Man$_2$) and 4-*O*-methyl-α-D-glucopyranosyluronic acid standard (meGlcA). red = reducing end proton; term = terminal proton.

**Table 1**
Anhydro-monosaccharide compositions (% dry weight) present in powdered *A. thaliana* AIR, wood (aspen, birch, and spruce) and wheat straw. The monosaccharides were released from powdered samples by acid methanolysis and further trimethylsilylated for GC analysis. The 4-*O*-methylglucuronic acid (meGlcA) content was determined using linear equations created from purified meGlcA (a) and commercial glucuronic acid (GlcA; b, c, d, and e).

| | Anhydro-monosaccharide compositions (% dry weight) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Xylose | Arabinose | GlcA | meGlcA | | | | |
| | | | | (a) | (b) GlcA6p | (c) GlcA4p | (d) GlcA2p | (e) GlcA1p |
| *A. thaliana* | 13.0 ± 0.2 | 1.4 ± 0.0 | 0.87 ± 0.04 | 1.3 ± 0.0 | 0.9 ± 0.0 | 1.4 ± 0.0 | 1.2 ± 0.0 | 2.2 ± 0.1 |
| Aspen | 18.0 ± 0.5 | 0.41 ± 0.01 | 0.12 ± 0.01 | 2.2 ± 0.1 | 1.5 ± 0.1 | 2.3 ± 0.1 | 2.0 ± 0.1 | 3.6 ± 0.2 |
| Birch | 27.0 ± 1.9 | 0.49 ± 0.06 | ND | 2.2 ± 0.2 | 1.5 ± 0.1 | 2.3 ± 0.2 | 2.0 ± 0.2 | 3.3 ± 0.3 |
| Spruce | 7.3 ± 0.5 | 1.5 ± 0.1 | 0.53 ± 0.02 | 2.2 ± 0.1 | 1.5 ± 0.1 | 2.3 ± 0.1 | 2.0 ± 0.1 | 3.3 ± 0.2 |
| Wheat straw | 23.4 ± 0.2 | 3.2 ± 0.1 | 0.44 ± 0.0 | 1.2 ± 0.1 | 0.8 ± 0.1 | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.9 ± 0.2 |

ND, not detected from GC analysis.

meGlcA solution. Signals from anomeric reducing end protons of meGlcA and $Man_2$ were integrated, and the amount of meGlcA was calculated based on the molar ratio of $Man_2$ and meGlcA. The sum integral of both α and β reducing end signals was used for calculation. MeGlcA content in the NMR sample determined from three parallel measurements was 497 ± 3.1 μg, which corresponds to 5.3 mg/ml (acid form) in the original purified preparation. The accuracy of meGlcA quantitation was tested with commercial GlcA, for which the anomeric reducing end resonances were detected at 5.27 and 4.68 ppm (data not shown). The quantified yield from 500 μg of GlcA was 509 μg, validating the procedure.

### 3.2. Acid methanolysis and GC analysis of meGlcA and GlcA

Acid methanolysis of a given monosaccharide yields several methyl glycosides due to anomerization and ring isomerization (De Ruiter, Schols, Voragen, & Rombouts, 1992; Sundberg et al., 1996). When separated on GC, the TMSi derivatives of meGlcA and GlcA yielded two and six peaks, respectively (Fig. 2a). The furanosides

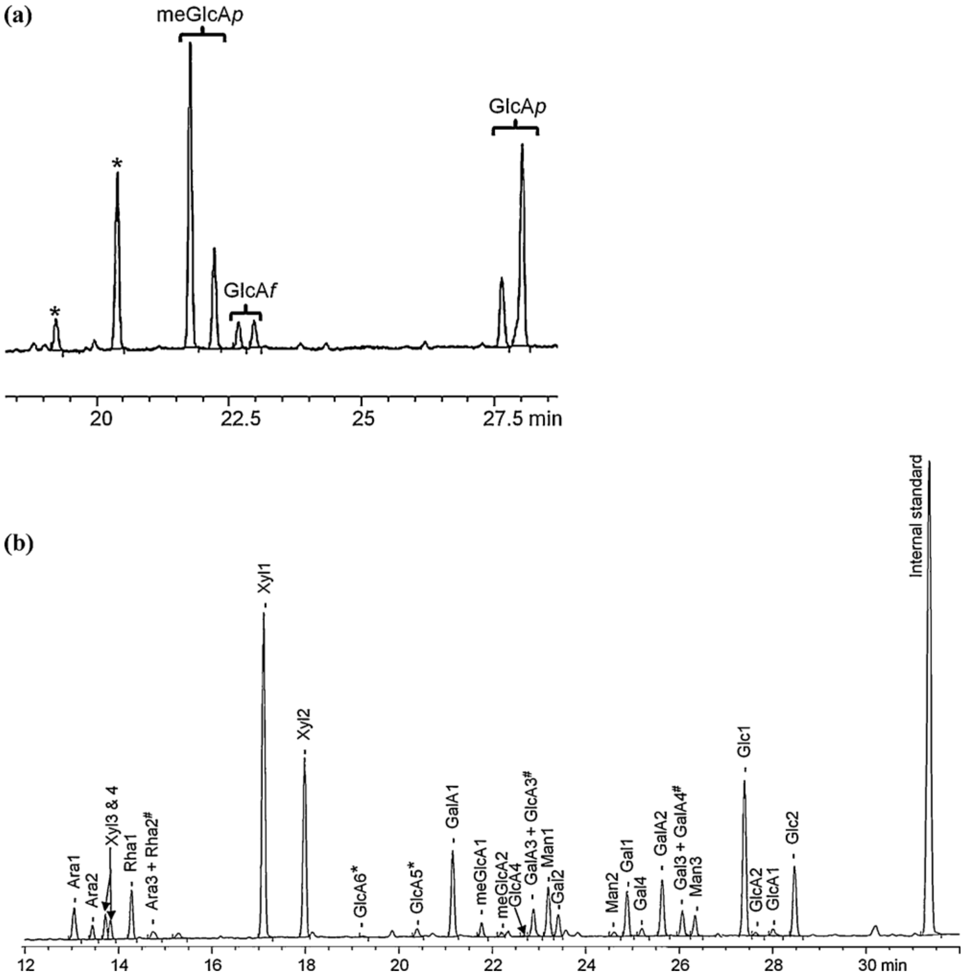

**Fig. 2.** GC chromatogram showing (a) isomeric forms of 4-*O*-methylglucuronic acid (meGlcA*p*) and glucuronic acid (GlcA*f*, GlcA*p*) standards yielded from acid methanolysis. Glucurono-6,3-lactones derived from GlcA were labeled with (*). Injected amounts were 400 nmol. (b) Separation of derivatized monosaccharides released from *A. thaliana* by acid methanolysis. Peaks that were labeled with (#) representing combination of two glycosides. Ara, arabinose; Xyl, xylose; Rha, rhamnose; GlcA, glucuronic acid; meGlcA, 4-*O*-methylglucuronic acid; GalA, galacturonic acid; Man, mannose; Gal, galactose; Glc, glucose.

and pyranosides derived from meGlcA and GlcA were identified by comparing their corresponding electron impact (EI)–MS spectra (data not shown) with previously published data (Bleton, Mejanelle, Sansoulet, Goursaud, & Tchapla, 1996). Only two pyranosides were derived from meGlcA (Fig. 2a) since the methoxy group at C-4 position prevents the formation of furanosides. In contrast, both furanosides and pyranosides with additional two peaks corresponding to glucurono-6,3-lactone (labeled with (*)) were obtained from GlcA (Fig. 2a). The total peak area of meGlcA obtained was 26% less than that of GlcA when 400 nmol of standards were compared. The kinetic formation of the α- and β-glycosides was not studied in this work; thus, the annotation of α- and β-anomers was not attempted.

### 3.3. Compositional analysis of A. thaliana AIR, wood and wheat straw samples

Monosaccharides liberated from plant materials during acid methanolysis yielded complex mixtures of furanosides and pyranosides that were difficult to separate on GC. An example of the GC chromatogram representing the separation of the *A. thaliana* acid methanolysate is shown in Fig. 2(b). All four Xyl*p* and Xyl*f*, and the two meGlcA*p* were used for the calculation of Xyl and meGlcA contents. Only the first two Ara glycosides (RT: 13 and 13.5 min) were included in the calculation for Ara content, since the third Ara glycoside (RT: 14.75 min) was not well resolved from its adjacent rhamnose glycoside. For the calculation of GlcA content, peaks corresponding to the α- and β-GlcA*f* were excluded since one of these peaks (RT: 23 min) was overlapping with the galacturonic acid glycoside.

The Xyl, Ara, GlcA and meGlcA anhydro-monosaccharide contents are shown in Table 1. For angiosperms, hardwoods contained a higher amount of xylan than the herbaceous *A. thaliana* did: Xyl content in aspen and birch wood was 18% and 27% of dry weight, respectively, while Xyl content in *A. thaliana* was only 13%. Softwood contained less xylan, and Xyl determined in spruce wood was only 7%. For monocots, 23% of dry weight was Xyl in wheat straw. The determined Ara content ranged from 0.4 to 3.2% % dry weight, while the amount of GlcA ranged from 0 to 0.87% dry weight in all samples. The calculated molar ratios of Xyl residues relative to Ara, GlcA, and meGlcA residues are shown in Supplementary Table 2.

The calculated amounts of meGlcA differed between calibration curves based on purified meGlcA and commercial GlcA standards. With the purified meGlcA standard, the average meGlcA contents were 1.3% of dry weight for *A. thaliana*, 2.2% for aspen, 2.2% for birch, 2.2% for spruce, and 1.2% for wheat straw, from which the average meGlcA substitution per xylose residues can be calculated as 1:14, 1:11, 1:17, 1:5, and 1:27, respectively. Calibration curves calculated from the GlcA standard had different slopes compared to the curve based on meGlcA (Fig. 3). When the meGlcA contents were calculated from GlcA standard by summing up the total peak area of all six peaks (Table 1; GlcA6p) in the calibration curve, the average meGlcA yields were reduced by approximately 30% across all samples. This underestimation would give lower meGlcA substitution per xylose residues: 1:20 in *A. thaliana*, 1:17 in aspen, 1:25 in birch, 1:7 in spruce, and 1:40 in wheat straw. Higher FID response for derivatized GlcA than that for meGlcA contributed to the underestimation of meGlcA. The difference in detector response could be due to the methoxy group at C-4 position of meGlcA, which precludes replacement by trimethylsilylation, thus reducing detection sensitivity in GC. In addition, the glucurono-6,3-lactone formed during acid methanolysis may also possess different detector response than the derivatized GlcA. Another way for calculating the amount of meGlcA in samples was to omit the peaks corresponding to the lactones. The resulting meGlcA yields were found close to the ones calculated from the purified meGlcA standard with only 2–8% overestimation (GlcA4p; Table 1). On the other hand, if the two main peaks that correspond to lactone (RT: 20.4 min) and GlcA*p* (RT: 28 min) were selected to calculate meGlcA content, since these two peaks were most visible on GC chromatogram, the resulting meGlcA yields were approximately 10% (GlcA2p; Table 1) less than those calculated from purified meGlcA standard. If only the main GlcA peak (RT: 28 min) was chosen to create the calibration curve, the calculated meGlcA yields were overestimated by 50–70% (GlcA1p; Table 1).

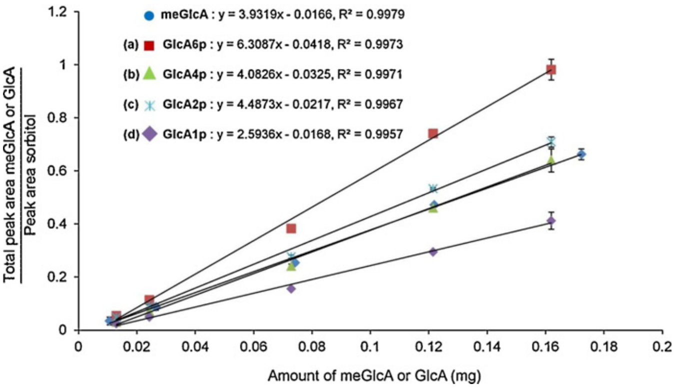

**Fig. 3.** Total peak area ratios at five concentration levels derived from the TMSi derivatives of meGlcA (● meGlcA) and commercial GlcA standards versus amount of monosaccharides in milligrams. The total peak area ratios were obtained from the total sum of peak areas of monosaccharide standards to that of sorbitol. Linear equations and their corresponding $R^2$ values are indicated. The linear equations for GlcA were obtained by summing up the total peak area ratios of: (a) all six (■ GlcA6p), (b) four (excluded lactone derivatives; ▲ GlcA4p), (c) the two main (✱ GlcA2p) and (d) the main (◆ GlcA1p) peak(s). The total peak area ratios used were calculated as means of triplicate samples.

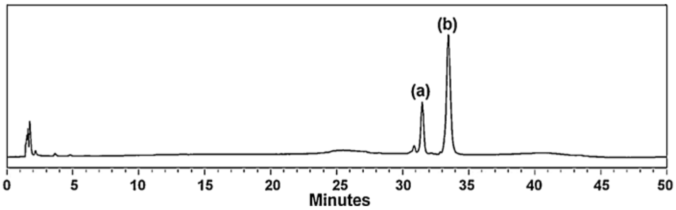

**Fig. 4.** HPAEC-PAD chromatogram of (a) 4-*O*-methylglucuronic acid (meGlcA) and (b) glucuronic acid (GlcA). Injected amount was 4 nmol.

### 3.4. HPAEC-PAD analysis of GlcA and meGlcA standards

Amounts of purified meGlcA and commercial GlcA equivalent to 4 nmol were separated on the same run in HPAEC-PAD (Fig. 4). The peak area and height of meGlcA were only 31% and 43% to that of GlcA, respectively. The lower detector response observed from meGlcA indicated that the calculated meGlcA values based on GlcA standard can potentially be underestimated also by using HPAEC-PAD if an authentic meGlcA standard is not used. Compared to xylose, meGlcA has a significantly lower response in the HPAEC-PAD analysis. Therefore, neutral and acidic monosaccharides are commonly quantified applying separate runs in HPAEC-PAD (Tenkanen & Siika-aho, 2000).

## 4. Conclusion

For acid methanolysis and GC analysis, meGlcA content was best estimated using calibration curves based on only the four peaks corresponding to GlcA*p* and GlcA*f* (GlcA4p), respectively; or the two main GlcA peaks (GlcA2p) from chromatograms. The deviation of calculated meGlcA yield from the actual value based on GlcA4p and Glc2p calibration curves were +2% to +8% and −10%, respectively. The GlcA2p calibration curve is more favorable if the acid methanolysate sample contained complex mixture of monosaccharides, since it was not possible to resolve the GlcA*f* peak from its adjacent galacturonic acid glycoside. However, the use of fresh reagents and the same type of column and separation conditions both between and within experiments is essential for the reproducibility of the method. The calculated meGlcA content, if based on a GlcA standard, can also be underestimated in HPAEC-PAD. The current interest in plant cell walls, for example, in the applied biorefinery field, as well as the advances in xylan biosynthesis studies, has increased the need for a higher accuracy for the quantitation of both GlcA and meGlcA, as well as other uronic acids, in different plant extracts and hydrolysates.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.08.078.

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