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Original Paper

Development of a new high-throughput method to determine the composition of ten monosaccharides including 4-*O*-methyl glucuronic acid from plant cell walls using ultra-performance liquid chromatography

Shingo Sakamoto[1], Kouki Yoshida[2], Satoko Sugihara[3], Nobutaka Mitsuda[1,*]

[1]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan; [2]Technology Center, Taisei Corporation, Nase-cho 344-1, Totsuka-ku, Yokohama, Kanagawa 245-0051, Japan; [3]Technology Center, Nihon Waters K.K., Kita-shinagawa 1-3-12, Shinagawa-ku, Tokyo 140-0001, Japan
*E-mail: nobutaka.mitsuda@aist.go.jp Tel: +81-29-861-2641 Fax: +81-29-861-3026

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Abstract Plant cell walls are an important dietary source for livestock, and could be an enormous resource for production of next-generation bioethanol and more valuable materials. Because polysaccharides are major components of plant cell walls, analysis of their composition is important. In this report, we established a high-throughput method to determine the composition of ten monosaccharides from plant cell walls simultaneously using ultra-performance liquid chromatography with *p*-aminobenzoic ethyl ester-labeling technology. Complete separation of a mixture of internal standards, 2-deoxy-glucose and 3-*O*-methyl glucose, and ten monosaccharides, consisting of seven neutral and three acidic sugars including 4-*O*-methyl-D-glucuronic acid, which are frequently found in plant cell wall polysaccharides, can be obtained within 7 min using this system. Relative standard deviations of retention time and peak area value are lower than 1%. Linearity for broader dynamic ranges (0.02–2000 mg l^{-1}), faster analysis and higher sensitivity than other traditional methods, including one that employs widely used high-performance anion exchange chromatography, are achieved. We evaluated this new method by analyzing the composition of cell walls from three model plants (*Arabidopsis thaliana*, rice and hybrid aspen) and confirmed that the obtained results for most monosaccharides are consistent with those in previous studies. These data suggest that our newly developed system could greatly contribute to the study of plant cell walls, especially research requiring high-throughput analysis.

Key words: 4-*O*-Methyl glucuronic acid, monosaccharide composition, plant cell wall, UPLC.

Plant cell wall polysaccharides are essential compounds for plant bodies, and are an important dietary source for livestock. In addition, it has recently been considered that plant cell walls could be the most abundant terrestrial organic resource for production of next-generation bioethanol and more valuable materials. Plant cell walls contain three major classes of polysaccharides, namely cellulose, hemicellulose and pectin. The composition of polysaccharides varies in each plant species and also in each tissue (Bauer et al. 2006). These polysaccharides are polymers of neutral monosaccharides such as D-glucose (Glc), D-xylose (Xyl), L-fucose (Fuc), L-arabinose (Ara), D-mannose (Man), D-galactose (Gal), L-rhamnose (Rha) and/or acidic monosaccharides such as D-glucuronic acid (GlcA), D-galacturonic acid (GalA), and 4-*O*-methyl-D-glucuronic acid (4-m-GlcA). Neutral monosaccharides are components of cellulose, and the backbones and/or side chains of hemicelluloses, while acidic monosaccharides are components of the pectin backbone or side chains of heteroxylan (Albersheim et al. 2010). Recently, not only cellulose (Somerville 2003) and the backbone of hemicelluloses (Brown et al. 2009; Jensen et al. 2014), but also the modification of GalA residues in pectin of primary cell wall (Hongo et al. 2012) and GlcA/4-m-GlcA side chains of heteroxylan of secondary cell wall (Lee et al. 2012) have been demonstrated to be essential for normal plant growth. Regarding saccharification of plant biomass, the arabinofuranose side chains promote hydrolysis of heteroxylan by some xylanases (Shallom and Shoham 2003). In addition, the

Abbreviations: ABEE, *p*-aminobenzoic ethyl ester; AIR, alcohol insoluble residue; Ara, L-arabinose; Fuc, L-fucose; GC, gas liquid chromatography; GC-MS, gas liquid chromatography-mass spectometry; 2-d-Glc, 2-deoxy-glucose; Gal, D-galactose; GalA, D-galacturonic acid; HPAEC, high-performance anion exchange chromatography; HPLC, high-performance liquid chromatography; Glc, D-glucose; GlcA, D-glucuronic acid; Man, D-mannose; 3-m-Glc, 3-*O*-methyl glucose; 4-m-GlcA, 4-*O*-methyl-D-glucuronic acid; PA, peak area; PAD, pulsed amperometric detection; RSD, relative standard deviation; TFA, trifluoroacetic acid; UPLC, ultra-performance liquid chromatography; Rha, L-rhamnose; Xyl, D-xylose.

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reduction of the degree of 4-*O*-methylation to GlcA on the glucuronoxylan of *Arabidopsis* mutants was found to increase the release of Xyl during hydrothermal pretreatment of plant cell wall (Urbanowicz et al. 2012). These reports suggest that the profiling of neutral and acidic monosaccharides from total plant cell walls is important for characterizing plant cell wall in the context of growth properties and/or for achieving improved conversion efficiency of the cell wall to ethanol.

To this end, chromatographic analysis is the main technique; gas liquid chromatography (GC) and GC-mass spectrometry (GC-MS) analyses are widely employed to analyze acid hydrolysates of polysaccharides and provide accurate and reproducible data of the composition of neutral and acidic monosaccharides in plant cell walls (Albersheim et al. 1967; Blakeney et al. 1983; Doco et al. 2001; Hoebler et al. 1989). However, this method based on the GC system requires time-consuming derivatization of monosaccharides to alditol acetates or trimethylsilyl ethers (McNeil et al. 1982; Pettolino 2012). On the other hand, high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) had been developed as a method to improve the sensitivity of monosaccharide detection without derivatization and is widely used for the plant cell wall analysis (De Ruiter et al. 1992). A recent study improved the HPAEC-PAD method to quantify two acidic monosaccharides in addition to seven neutral monosaccharides taking only 60 min of analytic time with high resolution (Nagel et al. 2014). However this advanced method has different linearity range for some monosaccharides, such as that for GalA (1.7–128 μmol l^{-1}) is different from those of other sugars (0.28–30.3 μmol l^{-1}, Nagel et al. 2014). In addition, Chong et al. (2013) has reported that the PAD response of 4-m-GlcA is one-third from that of GlcA. These differences necessitate several injections at different concentration levels for the comprehensive quantification of monosaccharides.

A method for high-performance liquid chromatographic (HPLC) analysis of monosaccharides with pre-column derivatization to produce a fluorophore is also commonly employed (Takemoto et al. 1985), which involves *p*-aminobenzoic ethyl ester (ABEE) derivatization of carbohydrates (Matsuura and Imaoka 1988). In the ABEE-labeling method, monosaccharides are derivatized for 1 h and excess reagents are easily removed by chloroform (Yasuno et al. 1999). In addition, the labeling efficiency of ABEE is mostly comparable for a broad range of neutral and acidic saccharide species and ABEE-labeled monosaccharides are efficiently separated in HPLC systems using borate buffer as an eluent, resulting in excellent resolution and wide linearity of 1–1000 pmol at a chromatographic injection for each monosaccharide (Yasuno et al. 1999). This suggests that the ABEE-labeling procedure is one of the most suitable methods for simultaneous determination of neutral and acidic monosaccharides from plant cell walls. However, the total run time of ABEE-labeled monosaccharide separation with HPLC is ca. 75 min for each sample analysis and therefore longer than the HPAEC-PAD method (Nagel et al. 2014). Recently, Kumagai et al. (2012) applied the ultra performance liquid chromatography (UPLC) system, which is a relatively new technique giving new possibilities in liquid chromatography by shortening run time, improving peak resolution, and sensitivity (Swartz 2005), to analyze the composition of trifluoroacetic acid (TFA)-hydrolyzable polysaccharides from Hinoki cypress (*Chamaecyparis obtusa*) slurry. However, chromatographic conditions such as the running time, peak resolutions, and the linearity ranges of each monosaccharide, which are important to evaluate the accuracy of their conditions, remained to be validated. In addition, the amount of 4-m-GlcA in their material was not intended to be analyzed even though Hinoki xylan is enriched with 4-m-GlcA (Ishii et al. 2010). The internal standard which is used for the calibration and estimation of the injection volume and the loss of monosaccharide during the acid hydrolysis (McNeil et al. 1982; Pettolino et al. 2012), also remained to be established (Kumagai et al. 2012). Therefore further development of the chromatographic conditions for UPLC with ABEE-labeling system is still needed for the rapid, accurate and comprehensive quantification of the monosaccharide composition of the plant cell wall including 4-m-GlcA using internal standard.

In this study, we established a system to rapidly analyze major 10 plant cell wall monosaccharides by combining UPLC with the ABEE-labeling technique. We also demonstrated that 2-deoxy-glucose (2-d-Glc) and 3-*O*-methyl glucose (3-m-Glc) can be used as the internal standard in this system. A total run time is shortened to 6.5 min, sensitivity is increased to 2–5 fmol per injection and the linearity range is broadened to 0.02–2000 μg ml^{-1}. We applied this simultaneous monosaccharide determination method to the cell wall samples of inflorescence stem of *Arabidopsis thaliana*, culm of rice, and poplar wood for the method evaluation. We verified that this method allows us a rapid and sensitive determination of 10 cell-wall-composing sugars including 4-m-GlcA in actual biomass samples in reasonable ranges, indicating that this new high-throughput chromatographic method could be an innovative tool for studying plant cell wall composition.

Materials and methods

Chemicals

The standard monosaccharides, Gal, Man, Ara, Xyl, Fuc, and Rha were purchased from Kanto Chemical Inc. (Japan), GlcA,

GalA, and 2-D-Glc were obtained from Sigma-Aldrich Inc. (USA), and Glc and 3-m-Glc was sourced from Wako Pure Chemical Industries, Ltd. (Japan). An ABEE-labeling kit was purchased from J-Oil mills Inc. (Japan). 4-m-GlcA purified from the sap of a lac tree (Kuroyama et al. 2001; Nakamura et al. 1984) was kindly gifted from Prof. Y. Tsumuraya (Saitama University, Japan). Synthetic 4-m-GlcA is also commercially available from Toronto Research Chemicals Inc. (www.trc-canada.com, Cat. #: M308350).

Plant materials

A. thaliana ecotype Columbia-0 were grown in soil at 22°C under a 16 h day (60–70 μmol·m^{-2}·s^{-1})/8 h night cycle after 3 weeks of germination on 1/2 Murashige and skoog medium. Regenerated rice plants (*Oryzae sativa* cv. Nippon-bare) from seed callus (Hiei et al. 1994) were grown in soil for 8 months at 28°C under a 13 h day (130–150 μmol·m^{-2}·s^{-1})/11 h night cycle in a phytotron. Rooted cuttings of hybrid aspen (*Populus tremula*×*Populus tremuloides*) were grown in the greenhouse at ambient temperature under natural light.

Preparation of cell wall residue

The inflorescence stem of 2-month-old *A. thaliana*, the culms of 8-month-old *O. sativa*, was cut into 1 cm-segments and fixed with methanol in a 50 ml-Pyrex glass tube with a screw cap (Asahi Glass Inc., Japan). The fixed samples were treated with methanol twice at 80°C for 10 min, twice with acetone at 70°C for 5 min, and twice with methanol/chloroform (1 : 1, v/v) at 70°C for 5 min and then dried at 65°C for 18 h. The dried tissue segments were powdered with a stainless steel bead (6 mm, Biomedical Science, Japan) and three zirconia beads (3 mm, Nikkato Corp., Japan) using a Shake Master NEO (Biomedical Science Inc., Japan). In case of poplar, xylem tissues were collected from 1–2 years old twigs and then ground into fine powder. The starch in resulting powder of *A. thaliana*, *O. sativa*, and poplar was degraded with α-amylase solution and de-starched residue was prepared as previously described (Sakamoto and Mitsuda 2014). The de-starched residue was designated as alcohol-insoluble residue (AIR).

Sulfuric acid hydrolysis of the cell wall

The AIR was hydrolyzed by the two-step hydrolysis method based on NREL protocol (Sluiter et al. 2008) with slight modification as previously described (Sakamoto and Mitsuda 2014). As a recovery standard, we prepared the authentic monosaccharide-standards including 7 neutral monosaccharides and 3 acidic monosaccharides (20 μg each in a tube, see Figure 1) in a 2 ml-Eppendorf safe-lock microtube and added 1.45 ml of 4% sulfuric acid. After addition of 10 μl of 3-m-Glc solution (1 mg ml^{-1}) as an internal standard, the resulting suspension was autoclaved at 121°C for 1 h and cooled at room temperature. The hydrolysate was neutralized and adjusted to around pH 5.0 with calcium carbonate powder. The supernatant of neutralized hydrolysate was used for ABEE-labeling.

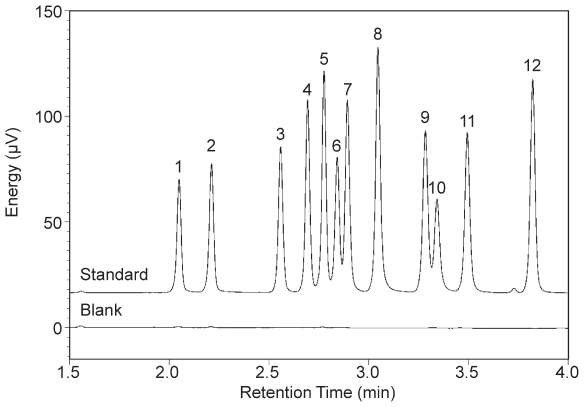

Figure 1. UPLC chromatogram of monosaccharides labeled with ABEE. A monosaccharide mixture (20 μg ml^{-1} of each, Standard) and ultrapure water (Blank) was reacted with ABEE regent and 2 μl of reacted solution containing 0.89 ng of ABEE-labeled monosaccharides was injected for chromatographic analysis. Peak assignment: 1, GlcA; 2, GalA; 3, Gal; 4, Man; 5, Glc; 6, 4-m-GlcA; 7, Ara; 8, Xyl; 9, Fuc; 10, Rha; 11, 2-d-Glc; 12, 3-m-Glc.

Preparation of ABEE-labeled monosaccharides

The acidic and neutral monosaccharides were labeled with an ABEE labeling kit (J-Oil mills Inc., Japan) based on the method of Yasuno et al. (1999). Twenty-microliter of ABEE reagent, which was prepared as described in Yasuno et al. (1999), was added to 5 μl of the neutralized hydrolysates in a 1.5 ml-microtube. The mixture was heated at 80°C for 1 h and then cooled to room temperature. Two hundred-microliter of distilled water and 200 μl of chloroform were added. After vigorous vortexing, the mixture was centrifuged at 20,000×*g* for 1 min at 25°C, and then the upper aqueous phase was collected. This aqueous phase containing ABEE-labeled monosaccharides was filtrated with syringe filter unit (Millex-LG 0.2 μm pore, Millipore Inc., USA) and the resulting solution was analyzed with UPLC system.

UPLC conditions

The chromatographic separation and detection of monosaccharides labeled with ABEE was performed using an ACQUITY UPLC H-Class system (Waters Inc., USA) equipped with ACQUITY UPLC BEH C18 column (100 mm×2.0 mm, id, 1.7 μm particle size, Waters Inc.) and fluorescence detector (ACQUITY UPLC FLR Detector, Waters Inc.). The eluent A was 200 mM potassium borate buffer (pH 8.9). The eluent B was 100% acetonitrile. The column was equilibrated with the mixture of 97% (v/v) eluent A and 3% (v/v) eluent B at a flow rate of 0.7 ml min^{-1}. The elution was started with a linear gradient of eluent B from 3 to 21% at a flow rate of 0.7 ml min^{-1} for 4.0 min. The elusion program including washing and regeneration of the column was shown in gradient profile 4 in Supplementary Table S1. The temperature of column and auto-sampler was maintained at 50°C and 10°C respectively. The ABEE-labeled monosaccharides were detected with 305 nm of emission and 360 nm of excitation using fluorescence detector. The injection volume of all samples was 2 μl.

Table 1. Comparison of assay conditions between a previous study*[1] and this study.

	Kumagai et al. (2012)	This study
Eluent method	Isocratic	One step gradient
Detector	UV	Fluorescence
Column length (mm)	150	100
Initiate buffer composition	A*[2] containing 10% B*[3]	Mixture of 97% A and 3% B
Flow rate (ml/min)	0.3	0.7
Column temperature (°C)	30	50

*[1] Kumagai et al. (2012), *[2] A, 200 mM potassium borate (pH 8.9), *[3] B, 100% acetonitrile.

Determination of peak resolution ($R_{P1, P2}$)

The resolution ($R_{P1, P2}$; Equation 1) between two adjacent peaks (P1 and P2) was deduced from the retention times of the two analytes (RT_{P1}, RT_{P2}) and the baseline widths (W_{P1} and W_{P2}) resulting from the tangents of the inflection points of each peak (Nagel et al. 2014).

$$R_{P1, P2} = 2(RT_{P2} - RT_{P1}) / (W_{P2} + W_{P1}) \quad (1)$$

Results and discussion

Assay condition to separate twelve monosaccharides by UPLC was established

Based on established methodologies for the ABEE labeling of monosaccharides (Kwon and Kim 1993; Yasuno et al. 1997, 1999), Kumagai et al. (2012) determined the monosaccharide composition of slurry of *Chamaecyparis obtusa* (Hinoki cypress) wood after the method transfer from HPLC to UPLC. However, (1) the established column performance such as specificity and stability remained to be validated. (2) 4-m-GlcA, which is present in major side chains of heteroxylan in secondary cell wall of eudicot, was not intended to be analyzed and (3) the internal standard correction was not applied. Because of these reasons, evaluation of the method transfer from HPLC to UPLC is still needed in addition to establishment of the condition to separate 4-m-GlcA, two other acidic monosaccharides, seven neutral monosaccharides and two modified monosaccharides (2-d-Glc or 3-m-Glc) which are not usually detected in the plant cell wall (as the potential internal standards). Using the same chromatographic conditions as those reported previously except for employing a shorter column in this study to shorten the total run time, all compounds were eluted within 10 min of injection in their isocratic elution with 10% acetonitrile (Supplementary Figure S1, isocratic 1). It was found that Glc co-eluted with 4-m-GlcA at 5.02 min under these conditions, although the separation of other monosaccharides was mostly acceptable (Supplementary Figure S1, isocratic 1). In addition, the total run time including wash and regeneration of the column was 25 min. Although this time frame is better than those of GC or HPLC methods, further shortening is desirable. Therefore, we needed to develop the different chromatographic conditions to separate all of the major monosaccharides from the plant cell wall and to shorten run time. Through many trial-and-error procedures, as described in detail in Supplementary Text, we finally established appropriate assay conditions that allowed rapid separation of all the monosaccharides (Figure 1). Under these conditions, the elution-program, the mobile phase, flow rate and column temperature were entirely changed from those of the reported method by Kumagai et al. (2012) (Table 1). Total analysis time was shortened to 6.5 min, including column washing and re-equilibration (Gradient profile 4 in Supplementary Table S1), and 4-m-GlcA was able to be detected simultaneously with the other monosaccharides and two internal standard candidates.

The established method has improved linearity and sensitivity

The reproducibility of the developed method was assessed by carrying out 12 independent injections of a mixture of twelve authentic monosaccharides. As listed in Tables 2 and 3, the relative standard deviation (RSD) values of retention time (RT) and peak area (PA) were less than 1% for all examined monosaccharides. These data indicate that the established system has a sufficient robustness for repeated analyses of chromatographic injection.

Supplementary Table S2 shows that the peak resolution values between each pair of two neighboring monosaccharides in the chromatograph except for "4-m-GlcA/Ara (0.80±0.01)" and "Fuc/Rha (0.82±0.01)" were between 0.99±0.01 and 2.25±0.05, when 0.89 ng/2 μl of sample was injected. This indicates that the separations of all tested monosaccharides were acceptable. Reduction of the injected sample amount from 0.89 ng/2 μl to 0.05 ng/2 μl markedly increased the peak resolution values for five pairs of monosaccharides without affecting those of the other four pairs of monosaccharides (Supplementary Table S3).

We also investigated the relationship between peak area and the concentration of monosaccharide labeled with ABEE to estimate the linearity range for the determination of monosaccharide concentration. As shown in Figure 2, the linearity of GlcA and 4-m-GlcA, for example, in our established method ranged from

Table 2. Reproducibility of retention time of ABEE-labeled monosaccharides using the established separation conditions shown in gradient profile 4 of Table S1.

Monosaccharide*	Retention time (min)
	AVE±SD (RSD %, n=12)
GlcA	2.04±0.004 (0.18)
GalA	2.21±0.003 (0.16)
Gal	2.55±0.004 (0.14)
Man	2.69±0.004 (0.14)
Glc	2.77±0.004 (0.15)
4-m-GlcA	2.83±0.004 (0.15)
Ara	2.89±0.004 (0.13)
Xyl	3.04±0.004 (0.13)
Fuc	3.28±0.004 (0.12)
Rha	3.34±0.004 (0.11)
2-d-Glc	3.49±0.004 (0.10)
3-m-Glc	3.82±0.003 (0.09)

*ABEE-labeled monosaccharide mixture (0.89 ng of each monosaccharide per chromatographic injection) was injected.

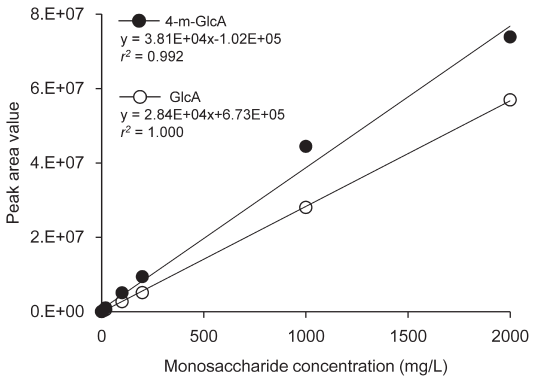

Figure 2. Relationship between area value and monosaccharide concentration of GlcA and 4-m-GlcA. Each concentration (0.02–2000 mg/l) of ABEE-labeled monosaccharide was prepared by dilution. The peak area and concentration of 4-m-GlcA (filled circles) and GlcA (open circles) are plotted.

Table 3. Detected area value of various monosaccharides using the established separation conditions shown in gradient profile 4 of Table S1.

Monosaccharide*	Peak area value ($\mu V \cdot s^{-1} \times 10^4$)
	AVE±SD (RSD %, n=12)
GlcA	83±0.61 (0.74)
GalA	99±0.45 (0.45)
Gal	113±0.32 (0.28)
Man	135±0.39 (0.29)
Glc	142±0.48 (0.34)
4-m-GlcA	75±0.66 (0.88)
Ara	121±1.08 (0.89)
Xyl	207±0.76 (0.37)
Fuc	117±0.43 (0.37)
Rha	58±0.33 (0.54)
2-d-Glc	140±0.56 (0.40)
3-m-Glc	181±0.53 (0.29)

*ABEE-labeled monosaccharide mixture (0.89 ng/2 μl of each monosaccharide) was injected.

0.02 to 2000 μg ml^{-1} (approximately 0.004–400 pmol per chromatographic injection) with a correlation coefficient r^2 value of 0.99 (Supplementary Table S4). This is much better than the previous report in which the detection linearity of detected monosaccharides was 1–1000 pmol per chromatographic injection in HPLC (Yasuno et al. 1999). Our results indicate that the reestablished UPLC method shows markedly improved linearity range (up to 100 fold) and sensitivity (up to 250 fold) for the determination of all examined monosaccharides compared with those of HPLC.

Application of the established method to three different types of plant cell walls produced reasonable results

To evaluate the analytical procedure with the UPLC system in actual plant cell wall samples, we quantified the monosaccharide contents in sulfuric acid-hydrolysate of three types of plant cell walls; namely, inflorescence stem of *A. thaliana*, culm of rice, and woody tissues of poplar stem. According to the recovery rate of sulfuric acid hydrolysis described in Supplementary text, 3-m-Glc is resistant to sulfuric acid hydrolysis, while 2-d-Glc is intensely degraded (Supplementary Table S5). Therefore 3-m-Glc was used for internal standard in sulfuric acid hydrolysate in this application.

As shown in Figure 3A, the chromatogram of cell wall hydrolysate revealed the 2 major peaks (peak No. 5 and 8) and 9 minor but significant peaks (peak No. 1 to 4, 6 to 10, and 12 as an internal standard, See Figure 3A–D), whose elution times were identical to those of monosaccharide standards, in all tested plant tissues. These two major monosaccharides were Glc and Xyl, respectively and other minor monosaccharides are three uronic acids (GalA, GlcA, and 4-m-GlcA) and five neutral sugars (Ara, Fuc, Gal, Man and Rha). The chromatogram also revealed that some unknown peaks eluted between GlcA and GalA around the elution time of 2.2 min (Figure 3A, B). These unknown peaks might be products from incomplete hydrolysis with sulfuric acid, such as substance with aldobiuronic acid structures presumably from arabinogalactan protein (Yates et al. 1996) or glucuronoxylans, which are relatively resistant to acid hydrolysis (Chong et al. 2013; Vignon and Gey 1998).

We calculated the monosaccharide contents in the cell wall of tested plants with the recovery rate (Supplementary Table S6) and compared our data with the previous reports in same or similar plant species (Figure 4). Most monosaccharide contents quantified in this study were in the range of previous studies, except for Rha in *A. thaliana* and GalA in poplar species (Figure 4). Rha content in *A. thaliana* cell wall in this study (20.5±1.5 μg mg^{-1} AIR) was ca. 1.5 to 3 times

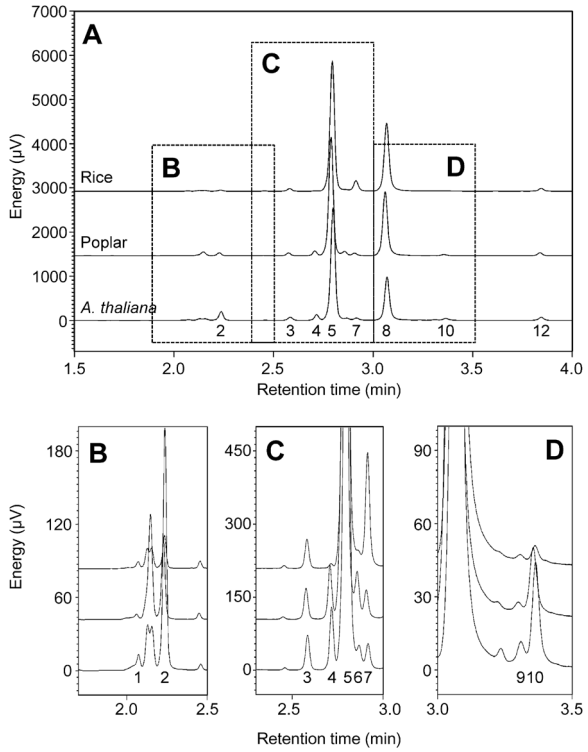

Figure 3. UPLC chromatogram of plant cell wall hydrolyzed by sulfuric acid. Monosaccharide profiles of cell walls from three plants *A. thaliana*, rice culm, and poplar stem are shown. The hydrolysates of cell wall samples were neutralized using calcium carbonate and then the supernatant was labeled with ABEE. Labeled monosaccharide in hydrolysate (2 μl) was injected for chromatographic analysis. Peak assignments are shown in Figure 1. A: Total chromatogram of cell wall acid hydrolysates from three plants. B to D: Expanded chromatograms from (A) to show minor but significantly detected peaks of monosaccharides.

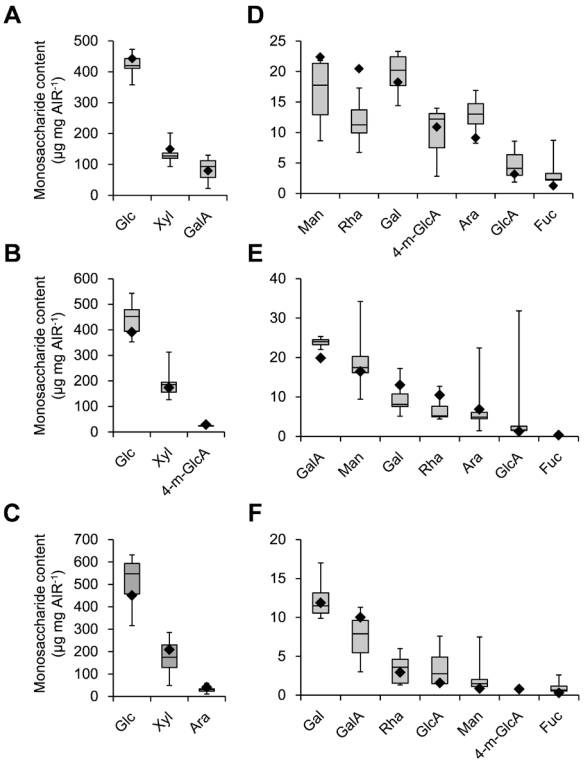

Figure 4. Comparison of monosaccharide compositions of cell walls from stem/culm in *Arabidopsis*, poplar, and rice between this study and previous studies. Contents of three major and seven minor monosaccharides of cell walls were determined for *Arabidopsis* inflorescence stems (A, D), poplar woody stems (B, E), and rice culms (C, F). The positions of monosaccharides are arranged in order of higher amounts determined in this study. The filled diamonds indicate the monosaccharide contents determined in this study (n=4 for *Arabidopsis* and rice, n=2 for poplar). Gray boxplots represent a range of values in previous studies; seven studies of *Arabidopsis*, six studies of poplar species, and six studies of rice, respectively (See supplementary Table S9 in detail). Horizontal bar in the gray box indicates the median of the reported values. Upper and lower hinges of gray box indicate 75%- and 25%-ranges of reported values, respectively. The upper and lower extreme bars of box plot indicate maximum (upper) and minimum (lower) of reported values, respectively.

higher than the previous studies, but relatively close to the data (14.4 and 17.3 μg mg^{-1} AIR) determined by the combination of the two step-hydrolysis with sulfuric acids and the quantification with GC (Lee et al. 2011b, 2012), while the data determined with HPEAC-PAD system (9.6 and 10.6 μg mg^{-1} AIR, Eudes et al. 2012; Iwase et al. 2009) and the data determined by the combination of one-step hydrolysis and GC (6.72 μg mg^{-1} AIR, Persson et al. 2007) were low. Therefore higher content of Rha in *A. thaliana* in this study might be caused by the difference of peak resolution of quantification apparatus (HPAEC or GC) and hydrolysis condition between our study (two-step) and the previous studies.

In case of 4-m-GlcA in rice and Fuc in poplar, few report showed the quantification data though we detected significant peaks in the all tested plant species (peak No. 6 in Figure 3C, No. 9 in Figure 3D, Supplementary Table S6). Leplé et al. (2007) used Fuc as an internal standard for HPAEC analysis of the monosaccharide composition in poplar cell wall, indicating that the Fuc in poplar wood sample is below the detection limit of HPEAC-PAD system. Hence, our improvement of the sensitivity may contribute to more detailed quantification of the monosaccharides in poplar wood sample.

Our analysis of monosaccharide composition of plant cell walls clearly revealed the difference between *A. thaliana*, poplar, and rice as reviewed previously (Albersheim et al. 2010; Carpita and Gibeaut 1993). The contents of Glc, Xyl and GalA were higher than those of other monosaccharides in *Arabidopsis* (Figure 4, Supplementary Table S6), which is consistent with the previous report that cellulose, xyloglucan, xylan and polygalacturonic acid are major cell wall components in eudicot plants (Zablackis et al. 1995). The contents of Glc, Xyl, and 4-m-GlcA were higher than those of other monosaccharides in poplar cell wall, which is also consistent with the previous review in which cellulose

Table 4. Comparison of chromatographic analyses used to determine monosaccharide composition.

Procedure	Derivatization (h)	Analysis (min/run)	Linearity (mg l^{-1})	Reference
UPLC-ABEE	1	6.5	0.02–2000	This study
HPLC-ABEE	1	75	4.5–4500*[1]	Yasuno et al. (1999)
HPEAC-PAD	None	60	0.05–5.5*[2]	Nagel et al. (2014)
			3.3–24.9*[3]	
GC-FID	3	30	0.1–150*[4]	Blakeney et al. (1983)
	3	30	0.3–3.0*[5]	Jones and Albersheim (1972)
GC-MS	17	65	0.6–1500 μg*[6]	Guadalupe et al. (2012)
			4.4–475 μg*[7]	

*[1] Concentration was calculated from the linearity range shown as 1–1000 pmol. *[2] Neutral monosaccharide and GlcA, *[3]: GalA, *[4] Neutral monosaccharides from Jones and Albersheim (1972), *[5] GlcA and GalA, *[6] Monosaccharides except GlcA, *[7] GlcA.

and 4-*O*-methyl-glucurono xylan are described as major components of dicotyledonous woody plants (Sannigrahi et al. 2010). In case of rice cell wall, contents of Glc, Xyl and Ara were relatively high, while the content of GalA was lowest among three tested species, which well describes the characteristics of *Poaceae* cell wall; namely, cellulose, mixed linkage-glucan, and arabinoxylan are major components but polygalacturonic acid is minor (Smith and Harris, 1999).

In terms of GlcA methylation, we found that 95%, 77%, and 30% of GlcA were methylated in poplar, *Arabidopsis*, and rice cell walls, respectively (calculated from Supplementary Table S6). According to previous studies, almost all and 60% of GlcA were described to be methylated in poplar (Lee et al. 2011a, 2012) and *Arabidopsis* (Lee et al. 2012; Zhong et al. 2005), respectively, and therefore our data is consistent with these previous studies. By contrast in case of rice, it is difficult to evaluate this value because few report succeeded to quantify the amount of 4-m-GlcA in rice as mentioned above.

Conclusion

The UPLC system established here greatly improves the throughput and quantification range of seven neutral and three acidic monosaccharides including 4-m-GlcA from plant cell walls in addition to 3-m-Glc as an internal standard. As listed in Table 4, we were able to shorten the total time for the chromatographic analysis of monosaccharides to 6.5 min and improved both the linearity range and detection limit, with a 100-fold increase of the linearity range and 200–500-fold increase of the detection limit compared to those of the HPLC-ABEE method. Furthermore, the developed method gives mostly comparable data for the monosaccharide compositions of plant cell walls with those of previous studies using GC or HPAEC-PAD systems. We believe this new UPLC system will greatly contribute to the study of plant cell walls and assist development of production of bioethanol and other valuable materials.

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References

Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A (2010) *Plant cell walls: from chemistry to biology*. Garland Science, New York, pp 43–118

Albersheim P, Nevins DJ, English PD, Karr A (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. *Carbohydr Res* 5: 340–345

Bauer S, Vasu P, Persson S, Mort AJ, Somerville CR (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc Natl Acad Sci USA* 103: 11417–11422

Biswal AK, Soeno K, Gandla ML, Immerzeel P, Pattathil S, Lucenius J, Serimaa R, Hahn MG, Moritz T, Jönsson LJ, et al. (2014) Aspen pectate lyase *Ptxt*PL1-27 mobilizes matrix polysaccharides from woody tissues and improves saccharification yield. *Biotechnol Biofuels* 7: 11

Blakeney AB, Harris PJ, Henry RJ, Stone BA (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr Res* 113: 291–299

Brown DM, Zhang Z, Stephens E, Dupree P, Turner SR (2009) Characterization of IRX10 and IRX10-like reveals an essential role in glucuronoxylan biosynthesis in Arabidopsis. *Plant J* 57: 732–746

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* 3: 1–30

Chong SL, Koutaniemi S, Virkki L, Pynnönen H, Tuomainen P, Tenkanen M (2013) Quantitation of 4-*O*-methylglucuronic acid from plant cell walls. *Carbohydr Polym* 91: 626–630

De Ruiter GA, Schols HA, Voragen AG, Rombouts FM (1992)

Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal Biochem* 207: 176–185

Doco T, O'Neill MA, Pellerin P (2001) Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydr Polym* 46: 249–259

Eudes A, George A, Mukerjee P, Kim JS, Pollet B, Benke PI, Yang F, Mitra P, Sun L, Cetinkol OP, et al. (2012) Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. *Plant Biotechnol J* 10: 609–620

Guadalupe Z, Martinez-Pinilla O, Garrido A, Carrillo JD, Ayestaran B (2012) Quantitative determination of wine polysaccharides by gas chromatography-mass spectrometry (GC-MS) and size exclusion chromatography (SEC). *Food Chem* 131: 367–374

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6: 271–282

Hoebler C, Barry JL, David A, Delort-Laval J (1989) Rapid acid hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas-liquid chromatography. *J Agric Food Chem* 37: 360–367

Hongo S, Sato K, Yokoyama R, Nishitani K (2012) Demethylesterification of the primary wall by PECTIN METHYLESTERASE35 provides mechanical support to the *Arabidopsis* stem. *Plant Cell* 24: 2624–2634

Ishii T, Konishi T, Yamasaki T, Enomoto A, Yoshida M, Maeda I, Shimizu K (2010) NMR characterization of acidic xylo-oligosaccharides containing two methylglucuronic acid residues from Japanese cedar and Hinoki cypress. *Carbohydr Polym* 81: 964–968

Iwase A, Hideno A, Watanabe K, Mitsuda N, Ohme-Takagi M (2009) A chimeric NST repressor has the potential to improve glucose productivity from plant cell walls. *J Biotechnol* 142: 279–284

Jensen JK, Johnson NR, Wilkerson CG (2014) *Arabidopsis thaliana* IRX10 and two related proteins from psyllium and *Physcomitrella patens* are xylan xylosyltransferases. *Plant J* 80: 207–215

Jones TM, Albersheim P (1972) A gas chromatographic method for the determination of aldose and uronic Acid constituents of plant cell wall polysaccharides. *Plant Physiol* 49: 926–936

Kumagai Y, Usuki H, Yamamoto Y, Yamasato A, Mukaihara T, Hatanaka T (2012) Preparation of hemicellulolic oligosaccharides from *Chamaecyparis obtuse* (hinoki) slurry using commercial enzymes. *Front Chem Sci Eng* 6: 224–231

Kuroyama H, Tsutsui N, Hashimoto Y, Tsumuraya Y (2001) Purification and characterization of a beta-glucuronidase from *Aspergillus niger. Carbohydr Res* 333: 27–39

Kwon H, Kim J (1993) Determination of monosaccharides in glycoproteins by reverse-phase high-performance liquid chromatography. *Anal Biochem* 215: 243–252

Lam TBT, Iiyama K (2000) Characteristics of senescent straw cell walls of dwarf, semidwarf, and normal strains of rice (*Oryza sativa*) plants. *J Wood Sci* 46: 376–380

Lee C, Teng Q, Huang W, Zhong R, Ye ZH (2009) Down-regulation of PoGT47C expression in poplar results in a reduced glucuronoxylan content and an increased wood digestibility by cellulose. *Plant Cell Physiol* 50: 1075–1089

Lee C, Teng Q, Zhong R, Ye ZH (2011a) Molecular dissection of xylan biosynthesis during wood formation in poplar. *Mol Plant* 4: 730–747

Lee C, Teng Q, Zhong R, Ye ZH (2011b) The four *Arabidopsis* reduced wall acetylation genes are expressed in secondary wall-containing cells and required for the acetylation of xylan. *Plant Cell Physiol* 52: 1289–1301

Lee C, Teng Q, Zhong R, Yuan Y, Haghighat M, Ye ZH (2012) Three *Arabidopsis* DUF579 domain-Containing GXM proteins are methyltransferases catalyzing 4-*O*-methylation of glucuronic acid on xylan. *Plant Cell Physiol* 53: 1934–1949

Leplé JC, Dauwe R, Morreel K, Storme V, Lapierre C, Pollet B, Naumann A, Kang KY, Kim H, Ruel K, et al. (2007) Downregulation of cinnamoyl-coenzyme A reductase in poplar: multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. *Plant Cell* 19: 3669–3691

Li Y, Qian Q, Zhou Y, Yan M, Sun L, Zhang M, Fu Z, Wang Y, Han B, Pang X, et al. (2003) *BRITTLE CULM1*, which encodes a COBRA-like protein, affects the mechanical properties of rice plants. *Plant Cell* 15: 2020–2031

Li M, Xiong G, Li R, Cui J, Tang D, Zhang B, Pauly M, Cheng Z, Zhou Y (2009) Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and plant growth. *Plant J* 60: 1055–1069

Matsuura F, Imaoka A (1988) Chromatographic separation of asparagine-linked oligosaccharides labeled with an ultravioletabsorbing compound, *p*-aminobenzoic acid ethyl ester. *Glycoconj J* 5: 13–26

McNeil M, Darvill AG, Aman P, Franzén LE, Albersheim P (1982) Structural analysis of complex carbohydrates using high-performance liquid chromatography, gas chromatography, and mass spectrometry. *Method Enzymol* 83: 3–45

Nagel A, Sirisakulwat S, Carle R, Neidhart S (2014) An acetate-hydroxide gradient for the quantitation of the neutral sugar and uronic acid profile of pectins by HPAEC-PAD without postcolumn pH adjustment. *J Agric Food Chem* 62: 2037–2048

Nakamura K, Tsumuraya Y, Hashimoto Y, Yamamoto S (1984) Arabinogalactan-proteins reacting with eel anti-H agglutinin from leaves of cruciferous plants. *Agric Biol Chem* 48: 753–760

Persson S, Caffall KH, Freshour G, Hilley MT, Bauer S, Poindexter P, Hahn MG, Mohnen D, Somerville C (2007) The *Arabidopsis irregular xylem8* mutant is deficient in glucuronoxylan and homogalacturonan, which are essential for secondary cell wall integrity. *Plant Cell* 19: 237–255

Pettolino FA, Walsh C, Fincher GB, Bacic A (2012) Determining the polysaccharide composition of plant cell walls. *Nat Protoc* 7: 1590–1607

Sakamoto S, Mitsuda N (2014) Reconstitution of a secondary cell wall in a secondary cell wall-deficient Arabidopsis mutant. *Plant Cell Physiol*, in press

Sannigrahi P, Ragauskas AJ, Tuskan GA (2010) Poplar as a feedstock for biofuels: A review of compositional characteristics. *Biofuel Bioprod Biorefin* 4: 209–226

Shallom D, Shoham Y (2003) Microbial hemicellulases. *Curr Opin Microbiol* 6: 219–228

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass, Laboratory analytical procedure (LAP). *National Renewable Energy Laboratory Technical Report* NREL/TP-510-42618

Smith BG, Harris PJ (1999) The polysaccharide composition of Poales cell walls: Poaceae cell walls are not unique. *Biochem Syst Ecol* 27: 33–53

Somerville C (2003) Cellulose synthesis in higher plants. *Annu Rev Cell Dev Biol* 22: 53–78

Swartz ME (2005) UPLC™: an introduction and review. *J Liq Chromatogr* 28: 1253–1263

Takemoto H, Hase S, Ikenaka T (1985) Microquantitative analysis of neutral and amino sugars as fluorescent pyridylamino derivatives by high-performance liquid chromatography. *Anal Biochem* 145: 245–250

Urbanowicz BR, Peña MJ, Ratnaparkhe S, Avci U, Backe J, Steet HF, Foston M, Li H, O'Neill MA, Ragauskas AJ, et al. (2012) 4-*O*-methylation of glucuronic acid in *Arabidopsis* glucuronoxylan is catalyzed by a domain of unknown function family 579 protein. *Proc Natl Acad Sci USA* 109: 14253–14258

Vega-Sánchez ME, Verhertbruggen Y, Christensen U, Chen X, Sharma V, Varanasi P, Jobling SA, Talbot M, White RG, Joo M, et al. (2012) Loss of *cellulose synthase-Like F6* function affects mixed-linkage glucan deposition, cell wall mechanical properties, and defense responses in vegetative tissues of rice. *Plant Physiol* 159: 56–69

Vignon MR, Gey C (1998) Isolation, ^{1}H and ^{13}C NMR studies of (4-*O*-methyl-D-glucurono)-D-xylans from luffa fruit fibres, jute bast fibres and mucilage of quince tree seeds. *Carbohydr Res* 307: 107–111

Willför S, Sundberg A, Pranovich A, Holmbom B (2005) Polysaccharides in some industrially important hardwood species. *Wood Sci Technol* 39: 601–617

Yasuno S, Murata T, Kokubo K, Yamaguchi T, Kamei M (1997) Two-mode analysis by high-performance liquid chromatography of *p*-aminobenzoic ethyl ester-derivatized monosaccharides. *Biosci Biotechnol Biochem* 61: 1944–1946

Yasuno S, Kokubo K, Kamei M (1999) New method for determining the sugar composition of glycoproteins, glycolipids, and oligosaccharides by high-performance liquid chromatography. *Biosci Biotechnol Biochem* 63: 1353–1359

Yates EA, Valdor JF, Haslam SM, Morris HR, Dell A, Mackie W, Knox JP (1996) Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies. *Glycobiology* 6: 131–139

Zablackis E, Huang J, Müller B, Darvill AG, Albersheim P (1995) Characterization of the cell-wall polysaccharides of *Arabidopsis thaliana* leaves. *Plant Physiol* 107: 1129–1138

Zhang SJ, Song XQ, Yu BS, Zhang BC, Sun CQ, Knox JP, Zhou YH (2012) Identification of quantitative trait loci affecting hemicellulose characteristics based on cell wall composition in a wild and cultivated rice species. *Mol Plant* 5: 162–175

Zhong R, Peña MJ, Zhou GK, Nairn CJ, Wood-Jones A, Richardson EA, Morrison WH 3rd, Darvill AG, York WS, Ye ZH (2005) *Arabidopsis fragile fiber8*, which encodes a putative glucuronyltransferase, is essential for normal secondary wall synthesis. *Plant Cell* 17: 3390–3408

Zhou Y, Li S, Qian Q, Zeng D, Zhang M, Guo L, Liu X, Zhang B, Deng L, Liu X, et al. (2009) BC10, a DUF266-containing and golgi-located type II membrane protein, is required for cell-wall biosynthesis in rice (*Oryza sativa* L.). *Plant J* 57: 446–462

Supplementary text

Improvement of method for monosaccharides separation

Because Glc co-eluted with 4-m-GlcA in the same assay condition as the one previously established (Kumagai et al. 2012) and we considered total analysis time should be shorter, we needed to modify the assay conditions of chromatography to separate all and to shorten run time more.

As the first trial, we slightly reduced the ratio of acetonitrile to borate buffer in the mobile phase from 10 % to 9 % or 8% for the isocratic elution, respectively, in order to increase the hydrophobic interactions between the solid phase and monosaccharides. This reduction of acetonitrile-ratio significantly improved the separation of Glc and 4-m-GlcA even in the 9% of acetonitrile (Supplementary Figure S1, isocratic 2). As shown in Figure S1, all tested monosaccharides were well separated in the mobile phase containing 8 % of acetonitrile (Supplementary Figure S1, isocratic 3) and the resolution values between two adjacent peaks of monosaccharides were more than 1.5 except for the set of GlcA and GalA (resolution value were 1.33) and the set of Glc and 4-m-GlcA (resolution value were 1.13, Supplementary Table S8). However, the drawback of the reducing ratio of acetonitrile was the longer elution time taken more than 20 min.

As the second trial, we adopted the gradient separation rather than the isocratic separation in the original paper (Kumagai et al. 2012). Advantages of gradient separation are well known (Schellinger and Carr 2006) including improved sensitivity due to lower chromatographic dilution and reduced time for the analysis (Guillarme et al. 2008). The first gradient condition was in 10-30 % acetonitrile in 5.5 min (Table S1, gradient profile 1). The mobile phase flow rate (0.3 ml min^{-1}) and the column temperature (30ºC) were the same as the original isocratic separation, respectively. As shown in Figure S2B, the condition of "gradient profile 1" reduced the elution time for Man to 2-d-Glc. Total elution time was within 5 min. The separation of Glc and 4-m-GlcA was improved from the original isocratic condition (compared with Supplementary Figure S1, isocratic 1), while Xyl co-eluted with Fuc in the gradient condition, unfortunately. The gradient separation increased the peak heights of all monosaccharides which were more than 2 folds of the original isocratic condition (Supplementary Figure S2A, isocratic 1).

Another gradient condition (Supplementary Table S1, gradient profile 2) was 3-30 % acetonitrile in 5.5 min. In this condition, the initial acetonitrile dosage was reduced but the gradient time was kept in order to increase the hydrophobic interactions between solid phase and monosaccharides. We expected that the gradient profile 2

might improve the separation of monosaccharides like our first trial described above. However, the reduction of the initial acetonitrile dosage from 10 to 3 % was not effective for the improvement of separation between Xyl and Fuc. Instead, this reduction of the initial gradient concentration delayed the elution time of all monosaccharides (Supplementary Figure S2C). Taken together, the modification of the original isocratic method to gradient separation appeared effective for the increase of the peak height of monosaccharides, however, did not improve the separation of monosaccharides (especially, Xyl and Fuc) substantially.

In general, the height of theoretical plate of 1.7 μm superficially porous particle phases of ACQUITY UPLC BEH C18 column is optimum around the flow rate 0.65 ml/min, according to the Van Deemter plot (Petersson et al. 2008). This suggests that the increase of the flow rate might improve the separation of all monosaccharides. However, the column pressure was ca. 8000 Psi at 0.3 mL min^{-1} and 30 ºC in the original isocratic separation and it seemed difficult to increase the flow rate at the same buffer-salt concentration and temperature. Therefore, we tried to decrease the concentration of potassium borate buffer in the mobile phase from 200 to 100 mM, since higher concentration of potassium-borate salts may cause an increase of column pressure. However, lower salt concentration of buffers increased the retention time of all monosaccharides. Several peaks became unclear, resulting in six broad peaks consisted of tested 8 monosaccharides (Glc, Gal, Man, Ara, Xyl, Fuc, Rha, GalA) in the chromatogram (data not shown). This result suggests that the relatively higher concentration of potassium borate may be critical both for the short retention time and good resolution of monosaccharides in the reverse phase column chromatography such as C18. Finally, we increased the column temperature from 30 ºC to 50 ºC to maintain the column pressure under the limit of the system at flow rate 0.7 mL min^{-1} to perform the gradient profile 3 in Supplementary Table S1.

As shown in Figure 1, the increase of both flow rate and column temperature significantly enhanced separations between each pair of two monosaccharides, that is “Man/Glc”, “Glc/4-m-GlcA”, “4-m-GlcA/Ara”, “Ara/Xyl” and “Xyl/Fuc”. The resolution between Fuc and Rha got worse but it was still acceptable (resolution value: 0.82 ± 0.01). The gradient profile 3 gave baseline resolution of tested monosaccharides except for 4-m-GlcA, Ara, Fuc and Rha. In addition, this chromatographic condition significantly decreased the elution time of all tested monosaccharides from 4-6 min shown in Fig S2C to 2-4 min. Based on these results, we slightly modified the gradient condition from 3-30 % acetonitrile for 5.5 min to 3-21 % for 4 min to establish new gradient profile without affecting the chromatogram obtained in gradient 3

(Supplementary Table S1, gradient profile 4). Total analysis time of this condition is 6.5 min, including the time for column wash and re-equilibration.

Estimation of recovery rate of authentic monosaccharides after sulfuric acid hydrolysis

Although acid hydrolysis is generally performed before the determination of monosaccharide composition in plant cell walls by chromatographic analysis, partial loss of saccharides during hydrolysis and neutralization is a well-known unavoidable problem (De Ruiter et al. 1992; Willför et al. 2009). However, if recovery rate of each monosaccharide is stable after sulfuric acid hydrolysis in the established system, we could estimate original amount from detected values. Therefore we examined the recovery rates of authentic monosaccharides after sulfuric acid hydrolysis according to the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al. 2008). Although the decreases in the peak areas of each monosaccharide were observed after the hydrolysis with 4 % sulfuric acid, most of authentic monosaccharides could be detected. As shown in Supplementary Table S5, the significant losses of all acidic monosaccharides ($p < 0.01$) and xylose ($p < 0.05$) were observed during the post-hydrolysis step.

As a validation of internal standards, 2-d-Glc, was intensely degraded to 2 % of the initial level during the 4% sulfuric acid hydrolysis as previously reported (Hellerqvist et al. 1990). On the other hand, the loss of 3-m-Glc was not statically significant after the sulfuric acid hydrolysis, indicating that 3-m-Glc is more suitable as the internal standard for the analysis of monosaccharide including the step of post-hydrolysis with 4 % sulfuric acid.

Supplementary references

Guillarme D, Nguyen DT, Rudaz S Veuthey JL (2008) Method transfer for fast liquid chromatography in pharmaceutical analysis: application to short columns packed with small particle. Part II: gradient experiments. *Eur J Pharm Biopharm* 68: 430–440

Hellerqvist CG, Sweetman BJ (1990) *Mass spectrometry of carbohydrates*. In C. H. Smelter & J. Throck Watson (Eds.), Methods of biochemical analysis (pp. 91–143). Hoboken, NJ: Wiley-Interscience

Petersson P, Frank A., Heaton J Euerby MR (2008) Maximizing peak capacity and

separation speed in liquid chromatography. *J. Sep. Sci.* 31: 2346–2357

Schellinger AP and Carr PW (2006) Isocratic and gradient elution chromatography: a comparison in terms of speed, retention reproducibility and quantitation. *J Chromatogr A* 1109: 253–266

Willför S, Pranovich A, Tamminen T, Puls J, Laine C, Suurnäkki A, Saake B, Uotila K, Simolin H, Hemming J, et al. (2009) Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides. *Ind. Crops Prod.* 29: 571–580

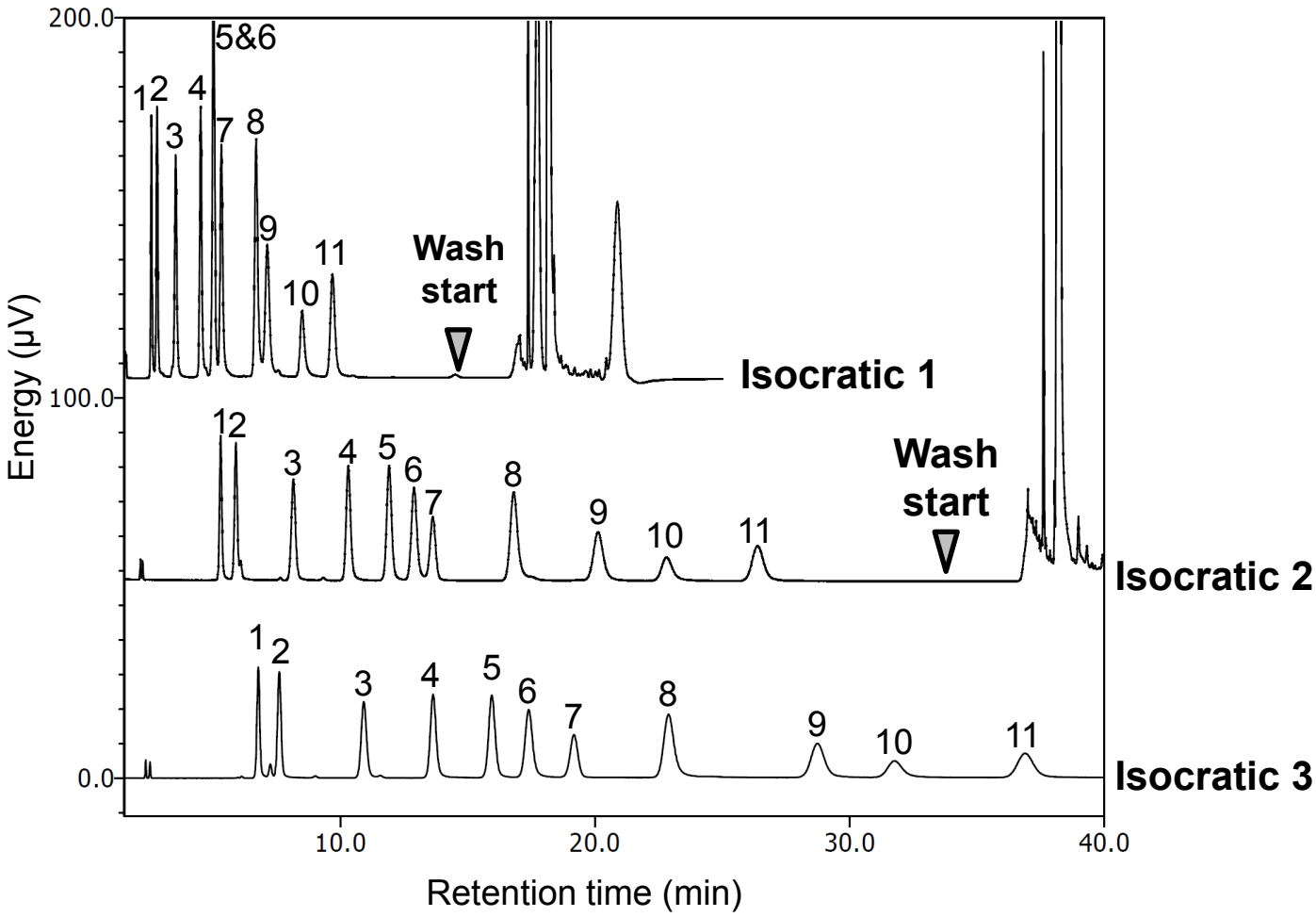

Figure S1 The improvement of isocratic separations of monosaccharide labeled with ABEE by UPLC system (First trial, see supplementary text.). Isocratic 1 (Original condition of Kumagai et al. 2012 except the length of column [100 mm type has been used in this study.]): Mobile phase is 200 mM potassium borate buffer (pH 8.9) containing 10% acetonitrile. Isocratic 2:Mobile phase is the same buffer containing 9% acetonitrile. Isocratic 3:Mobile phase is the same buffer containing 8% acetonitrile. The flow rate (0.3 ml min^{-1}) and column temperature (30°C) were the same among three conditions. Monosaccharides mixture (20 μg ml^{-1} each) was reacted with ABEE and 2 μL of reacted solution including 0.89 ng of each ABEE labeled monosaccharides was injected on a chromatographic analysis. Peak assignment: 1, D-glucuronic acid (GlcA); 2, D-galacturonic acid (GalA); 3, D-galactose (Gal); 4, D-mannose (Man); 5, D-glucose (Glc); 6, 4-O-methyl-glucuronic acid (4-m-GlcA); 7, L-arabinose (Ara); 8, D-xylose (Xyl); 9, L-fucose (Fuc); 10, L-rhamnose (Rha); 11, 2-deoxy-glucose (2-d-Glc).

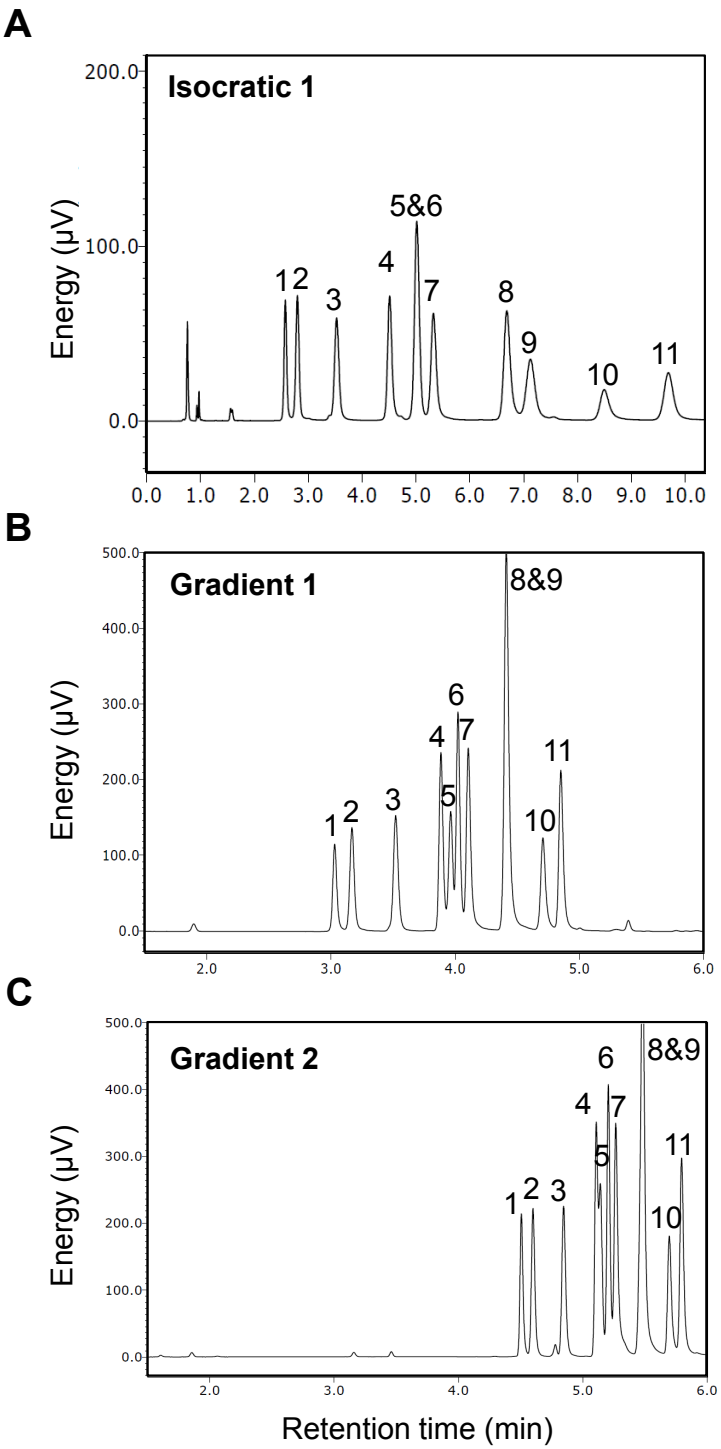

Figure S2 Newly developed gradient separations of monosaccharide labeled with ABEE by UPLC system (Second tiral, see supplementary text). A:A chromatogram of original isocratic condition of Kumagai et al. 2012 except the length of column (100 mm type has been used in this study (Isocratic 1). Mobile phase is 200 mM potassium borate buffer (pH 8.9) containing 10% acetonitrile. B: A chromatogram based on the gradient profile 1 which was in 10-30 % acetonitrile in 5.5 min (See Table S3). C: A chromatogram based on the gradient profile 2 which was 3-30 % acetonitrile in 7.5 min. (See Table S3). The flow rate (0.3 ml min^{-1}) and column temperature (30°C) were the same among three conditions. Monosaccharides mixture (20 μg ml^{-1} each) was reacted with ABEE and 2 μL of reacted solution including 0.89 ng of each ABEE labeled monosaccharides was injected on a chromatographic analysis. Peak assignment: See Figure S1.

Supplementary Table S1 Gradient profiles

Gradient profile 1

			Eluent dosage (%)	
Stage	Step	Time (min)	Eluent A	Eluent B
Initiation			90	10
Injection		0.00	90	10
Elution	onset gradient	0.00	90	10
	end gradient	+5.50	70	30
Wash	onset isocratic	+5.51	70	30
	end isocratic	+7.50	70	30
Regeneration	restart isocratic	+7.51	90	10
	run end	+9.50	90	10

Eluent A and B are 200 mM potassium borate (pH 8.9) and 100 % acetonitrile, respectively. Flow rate is 0.3 mL/ min. The temperature of column and sample are kept at 30°C and 10°C, respectively. Injection volume is 2 μL for all sample.

Gradient profile 2

			Eluent dosage (%)	
Stage	Step	Time (min)	Eluent A	Eluent B
Initiation			97	3
Injection		0.00	97	3
Elution	onset gradient	0.00	97	3
	end gradient	+5.50	70	30
Wash	onset isocratic	+5.51	70	30
	end isocratic	+7.50	70	30
Regeneration	restart isocratic	+7.51	97	3
	run end	+9.50	97	3

Eluent A and B are 200 mM potassium borate (pH 8.9) and 100 % acetonitrile, respectively. Flow rate is 0.3 mL/ min. The temperature of column and sample are kept at 30°C and 10°C, respectively. Injection volume is 2 μL for all sample.

Gradient profile 3

			Eluent dosage (%)	
Stage	Step	Time (min)	Eluent A	Eluent B
Initiation			97	3
Injection		0.00	97	3
Elution	onset gradient	0.00	97	3
	end gradient	+5.50	70	30
Wash	onset isocratic	+5.51	70	30
	end isocratic	+7.50	70	30
Regeneration	restart isocratic	+7.51	97	3
	run end	+9.50	97	3

Eluent A and B are 200 mM potassium borate (pH 8.9) and 100 % acetonitrile, respectively. Flow rate is 0.7 mL min^{-1}. The temperature of column and sample are kept at 50°C and 10°C, respectively. Injection volume is 2 µL for all sample.

Gradient profile 4

			Eluent dosage (%)	
Stage	Step	Time (min)	Eluent A	Eluent B
Initiation			97	3
Injection		0.00	97	3
Elution	onset gradient	0.00	97	3
	end gradient	+4.00	79	21
Wash	onset isocratic	+4.01	70	30
	end isocratic	+5.00	70	30
Regeneration	restart isocratic	+5.01	97	3
	run end	+6.50	97	3

Eluent A and B are 200 mM potassium borate (pH 8.9) and 100 % acetonitrile, respectively. Flow rate is 0.3 mL min^{-1}. The temperature of column and sample are kept at 30°C and 10°C, respectively. Injection volume is 2 µL for all sample.

Supplementary Table S2 Resolution value of each pair of monosaccharides in the established separation condition shown in gradient profile 4 of Supplementary table S1

Pair of monosaccharides[*1]	Resolution value AVE ± SD (RSD%, n = 12)
GlcA/GalA	0.99 ± 0.01 (1.47)
GalA/Gal	2.25 ± 0.05 (2.12)
Gal/Man	1.18 ± 0.02 (1.78)
Man/Glc	1.03 ± 0.01 (0.73)
Glc/4-m-GlcA	1.06 ± 0.01 (1.08)
4-m-GlcA/Ara	0.80 ± 0.01 (1.09)
Ara/Xyl	1.16 ± 0.12 (10.3)
Xyl/Fuc	1.70 ± 0.18 (10.3)
Fuc/Rha	0.82 ± 0.01 (0.72)
Rha/2-d-Glc	1.28 ± 0.08 (6.55)

*1: ABEE labeled monosaccharides mixture (0.89 ng/2μL of each monosaccharide) was injected.

Supplementary Table S3 Effect of sample amount on resolution values

	Sample amount (ng/2μL)			
Monosaccharide pair	0.89	0.45	0.09	0.05
GlcA/GalA	1.30	1.59	2.13	2.56
GalA/Gal	3.06	3.42	4.73	5.31
Gal/Man	1.49	1.50	1.64	1.81
Man/Glc	1.13	1.13	1.13	1.15
Glc/4-m-GlcA	1.05	1.07	1.06	1.15
4-m-GlcA/Ara	0.80	0.80	0.80	0.79
Ara/Xyl	1.36	1.21	1.97	2.39
Xyl/Fuc	1.67	1.49	2.28	2.39
Fuc/Rha	0.73	0.74	0.75	0.75

Supplementary Table S4 Correlation coefficients of area values and monosaccharide concentrations in the established separation condition shown in gradient profile 4 of Supplementary table S1

Monosaccharide	Linearity range (mg L^{-1})*1	Correlation coeffient , r^2	Equation Slope	Intercept
GlcA	0.02-2000	1.000	2.84E+0.4	-1.02E+05
GalA	0.02-2000	0.998	4.20E+0.4	4.76E+05
Gal	0.02-2000	0.997	4.21E+0.4	5.06E+05
Man	0.02-2000	0.994	4.88E+0.4	8.93E+05
Glc	0.02-2000	0.978	4.38E+0.4	1.53E+05
4-m-GlcA	0.02-2000	0.992	3.81E+0.4	6.73E+05
Ara	0.02-2000	0.997	4.39E+0.4	7.31E+05
Xyl	0.02-2000	0.993	7.66E+0.4	1.72E+0.6
Fuc	0.02-2000	0.996	4.83E+0.4	7.64E+05
Rha	0.02-2000	0.998	3.16E+0.4	4.49E+05

*1:For each analysis, calibration lines through 12 standard concentration were produced by simple linear regression.

Supplementary Table S5 Recovery rates of authentic monosaccharides after sulfuric acid treatment

Monosaccharide	Unhydrolyzed AVE ± SD (n=6)	H_2SO_4 hydrolyzed AVE ± SD (n=6)
GlcA	100 ± 2.2	55 ± 4.0*
GalA	100 ± 1.3	65 ± 4.7*
Gal	100 ± 0.7	100 ± 6.6
Man	100 ± 0.6	99 ± 6.5
Glc	100 ± 0.8	103 ± 6.8
4-m-GlcA	100 ± 1.3	50 ± 3.5*
Ara	100 ± 1.1	100 ± 6.8
Xyl	100 ± 1.7	91 ± 6.4*
Fuc	100 ± 2.3	99 ± 6.8
Rha	100 ± 2.9	97± 6.7
2-d-Glc	100 ± 0.8	2 ± 1.0*
3-m-Glc	100 ± 1.8	97 ± 6.6

Recovery rate was calculated with area values of each monosaccharide peak with and without acid hydrolysis. Authentic monosaccharides were hydrolyzed with sulfuric acid at 121 °C for 1 h and then neutralized with calcium carbonate. Asterisks mean that significant decrease compared with unhydrolysed monosaccharide sample.

Supplementary Table S6 Total monosaccharide compositions of various plant cell walls[*1]

Plant	*A. thaliana*	Rice	Poplar
Genotype/Cv.[*2]	Colombia-0	Nipponbare	Hybrid aspen T89
Tissue	Inflorescence stem	Culm	Stem
Monosaccharide (μg mg AIR^{-1})			
GlcA	3.17 ± 0.19	1.58 ± 0.19	1.27 ± 0.17
GalA	79.5 ± 8.6	10.0 ± 1.3	19.9 ± 1.3
Gal	18.3 ± 1.8	11.9 ± 1.0	13.0 ± 0.8
Man	22.4 ± 0.6	0.9 ± 0.1	16.5 ± 1.4
Glc	443 ± 7	451 ± 29	391 ± 16
4-m-GlcA	10.9 ± 0.6	0.8 ± 0.3	29.1 ± 1.7
Ara	9.1 ± 1.6	41.7 ± 4.0	6.9 ± 0.5
Xyl	150 ± 5	208 ± 19	174 ± 6
Fuc	1.27 ± 0.15	0.31 ± 0.02	0.34 ± 0.03
Rha	20.5 ± 1.5	2.9 ± 0.1	10.5 ± 0.5
Total detected	758 ± 10	729 ± 51	662 ± 14

[*]1: Data are presented as mean (μg mg AIR^{-1}) ± SD (n = 4, except n = 2 for poplar). These values were calculated with adjustment using the recovery standard. (See the representative recovery rates in Supplementary Table S5).*2:Cultivar

Supplementary Table S7 Retention time of each isocratic condition

Isocratic No.	1	2	3
Eluent A (%)	90	91	92
Eluent B (%)	10	9	8
GlcA	2.57	5.30	6.78
GalA	2.79	5.89	7.60
Gal	3.53	8.15	10.92
Man	4.51	10.31	13.64
Glc	5.02	11.91	15.95
4-m-GlcA	5.02*	12.89	17.40
Ara	5.33	13.69	19.17
Xyl	6.69	16.80	22.88
Fuc	7.13	20.12	28.73
Rha	8.50	22.28	31.76
2-d-Glc	9.69	26.39	36.90

Flow rates are 0.3 mL min^{-1}. Asterisk means co-elute with glucose.

Supplementary Table S8 Resolution value of each isocratic condition

Isocratic No.	1	2	3
Eluent A (%)	90	91	92
Eluent B (%)	10	9	8
GlcA/GalA	0.76	1.27	1.33
GalA/Gal	1.58	3.66	4.57
Gal/Man	1.82	2.42	3.06
Man/Glc	1.16	1.76	1.98
Glc/4-m-GlcA	-*1	1.13	1.13
4-m-GlcA/Ara	0.60*1	0.94	1.59
Ara/Xyl	2.39	3.46	2.98
Xyl/Fuc	0.86	2.80	3.91
Fuc/Rha	2.07	2.17	2.01
Rha/2-d-Glc	1.55	2.72	3.26

Flow rates are 0.3 mL min^{-1}. *1: Glc and 4-m-GlcA co-eluted in the isocratic condition No.1 (Supplementary Figure S2 isocratic 1).

Table S9 List of previous reports describing monosaccharide compositions in the cell wall from Arabidopsis, poplar and rice used for the comparison with this study in Figure

Plant species / Tissues	Methods for liberation of monosaccharides	Quantification Appratus	Author	Reference
Arabidopsis				
10 week old stems	Total values after the fractionation of pectin, hemicellulose and cellulose, Hyrdolyzed with TFA or sulfuric acid	GC-MS	Zhong et al. 2005	Plant Cell 17: 3390-3408 (Table 4)
7 week old stems	1step hyrolysis with 1M sulfuric acid	GC	Persson et al. 2007	Plant Cell 19: 237–255 (Table 1)
Inflorescence stems	2 step hyrolysis with sulfuric acid	HPAEC	Iwase et al. 2009	J Biotech 142: 279–284 (Table 1)
10 week old stems	2 step hyrolysis with sulfuric acid	GC	Lee et al. 2011	Plant cell physiol.52: 1289–1301 (Table1)
8-week-old stems	2 step hyrolysis with sulfuric acid	HPAEC	Eudes et al. 2012	Plant Biotechnol.J. 10, pp. 609–620 (Table 6)
10 week old stems	Sulfuric acid hydrolysis for neutral sugars, Metanolysis for acidic sugars	GC for neutral sugars, GC-electron impact-MS for acidic sugars	Lee et al. 2012	Plant cell physiol 53: 1934–1949 (Figure 4 and Table 1)
Arabidopsis AIR	Methanolysis for 4-m-GlcA analysis	GC	Chong et al. 2013	Carbohydr. Polymers 91: 626– 630 (Table 1)
Poplar				
Populus deltoides, P.grandidentata, P.temula, P.tremuloides (Heart-, sap-, stem-wood)	Methanolysis of non-cellulosic polysaccharides, two step sulfuric acid hydrolysis of cellulose	GC	Willfȍr et al. 2005	Wood Sci Technol 39:601-617 Tables 2 and 3
Hybrid Aspen clone T89	5%KOH-, 24%KOH-soluble fractions and insoluble residue were treated with 2 step sulfuric acid hydrolysis	HPAEC	Leple´2007	Plant Cell 19: 3669–3691 (Table 3)
Hybrid poplar	1N or 4N KOH-soluble fractions and insoluble residues treated with 2 step sulfuric acid hydrolysis	GC	Lee et al. 2009	Plant cell physiol. 50: 1075–1089 (Table 2)
Hybrid Poplar Caudina DN34	2 step hydrolysis with sulfuric acid	HPLC equipped with refractive index detector	Sannigrahi et al. 2010	Biofuels, Bioprod. Bioref.4:209-226 (Table 7)
Hybrid poplar	2 step hydrolysis with sulfuric acid	GC	Lee et al. 2011	Molecular Plant 4:730–747 (Table 3)
Hybrid Aspen clone T89	Hydrolysis of non-cellulosic polysaccharides with acetic acid:nitric acid:water (8:1:2) Hydrolysis of cellulose with 72% sulfuric acid	GC for neutral sugars from non-cellulosic analysis, Anthrone method for celllulose analysis	Biswal et al. 2014	Biotechnol. for biofuels 7:11, 1-13 (Table 1)
Oryzae sativa				
Internode of *Oryza sativa* (cv..Taichung 65)	Two step hydrolysis with sulfuric acid	GC and GC-MS	Lam and Iiyama (2000)	J. Wood Sci. 46:376-380 (Table 3)
Culms of *Oryza sativa*	Two step hydrolysis with sulfuric acid	GC	Li et al. (2003)	Plant Cell 15 2020-2031 (Table 1)
Culms of *Oryza sativa*	Two step hydrolysis with sulfuric acid	GC	Zhou et al. (2009)	Plant J 57 446-462 (Table 2)
Culms of *Oryza sativa*	Methanolysis for the analysis of non-cellulosic polysaccharides, 72% Sulfuric acid hydrolysis for the cellulose analysis	GC-MS after the Methanolysis Anthrone assay after the sulfuric acid hydrolysis	Li et al (2009)	Plant J 60 1055-1069 (Table 1)
Second internodes of *Oryza sativa* cv.Nipponbare	TFA hydrolysis for the analysis of non-cellulosic polysaccharides 2 step- Sulfuric acid hydrolysis for the cellulose analysis	HPAEC	Vega-Sa´nchez et al. (2012)	Plant Physiol. 159:56-69 (Tables 3 and 4)
Second internodes of Yuanji (*Oryza rufipogon* Griff) or Teqing (*Oryza sativa* spp)	TFA hydrolysis for the analysis of non-cellulosic polysaccharides 72% Sulfuric acid hydrolysis for the cellulose analysis	GC-MS after the TFA hydrolysis, Anthrone assay after the sulfuric acid hydrolysis	Zhang et al. (2012)	Mol. Plant 5:162-175 (Table 1)