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Profiling cell wall monosaccharides and nucleotide-sugars from plants

Running Title: Plant carbohydrate analysis

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Significance Statement

Plant cell walls are principally of a complex interlinked matrix of polysaccharides and is an important renewable resource. Plant biomass represent a critical resource for humanity providing us with raw materials for food, clothing, timber, paper, fodder, renewable energy and bioproducts. To effectively utilize biomass for emerging biotechnology applications, a detailed understanding of the glycosyl residue composition is essential. While the development of tailored biomass through metabolic engineering requires the precise measurement of the glycosyl precursors involved in the assembly of the plant cell wall. The following protocols detail procedures for the accurate assessment of cell wall matrix monosaccharides and their activated precursors.

ABSTRACT

The cell wall is an intricate mesh largely composed of polysaccharides that vary in structure and abundance. Apart from cellulose biosynthesis, the assembly of matrix polysaccharides such as pectin and hemicellulose occur in the Golgi apparatus before being transported via

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vesicles to the cell wall. Matrix polysaccharides are biosynthesized from activated precursors or nucleotide sugars. The composition and assembly of the cell wall is an important aspect in plant development and plant biomass utilization. The application of anion-exchange chromatography to determine the monosaccharide composition of the insoluble matrix polysaccharides enables a complete profile of all major sugars in the cell wall from a single run. While porous carbon graphite chromatography and tandem mass spectrometry delivers a sensitive and robust nucleotide sugar profile from plant extracts. Here we describe detailed methodology to quantify nucleotide sugars within the cell and profile the non-cellulosic monosaccharide composition of the cell wall.

Keywords: nucleotide sugars, plant cell wall, monosaccharides

INTRODUCTION

Plant cell walls are a complex extracellular matrix comprised of polysaccharides, phenolics and protein (Keegstra, 2010). This structure determines and maintains the shape of cells, plays a significant role in cell differentiation / expansion as well as serving as a protective barrier (Houston et al., 2016). The matrix polysaccharide component represents the most adaptive structures within the cell wall and is also an important raw material (biomass) employed in a range of economically important processes (Blanch et al., 2008). Thus, the monosaccharide composition of this component can provide insight into how this structure adapts and responds over the life of the plant, can be employed for characterization of cell wall mutants (Rautengarten et al., 2017) and is essential when considering these structures as a raw material (Persil-Cetinkol et al., 2012).

Plant cell wall polysaccharides are highly diverse structures that are biosynthesized by glycosyltransferases from nucleotide sugar substrates. These activated sugar substrates are mainly biosynthesized in the cytosol via a series of interconversion pathways starting at either UDP-glucose or GDP-mannose (Ebert et al., 2015). In plants, more than 16 different nucleotide sugars are required for the synthesis of structurally intricate polysaccharides found in the cell wall (Bar-Peled et al., 2011). Identification and quantification of nucleotide sugars provides important clues about potential polysaccharides found in the cell wall (Rautengarten et al., 2014). The approach also enables precise monitoring of these important precursors when modifying plant polysaccharides by metabolic engineering to develop tailored biomass for biotechnology applications.

The following protocols outline robust quantitative methods for the enrichment, identification and separation of monosaccharides and nucleotide sugars from plant samples. Together they provide a snapshot of the current composition and the potential composition of polysaccharides found in plant cell walls.

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BASIC PROTOCOL 1. EXTRACTION AND QUANTIFICATION OF NUCLEOTIDE SUGARS FROM PLANT MATERIAL BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

Given the complexity of plant metabolism, robust metabolic profiling requires the specific purification of a class of metabolites prior to their separation and detection. This protocol outlines a general plant metabolite extraction method (Arrivault et al., 2009) followed by a sample cleanup method involving a solid phase extraction for polar compounds (Rabina et al., 2001) and finally detection of nucleotide sugars by tandem mass spectrometry using multiple reaction monitoring (MRM).

Materials

Liquid nitrogen

Mortar and pestle or TissueLyser (Qiagen, #85300)

Chloroform / Methanol solution 3:7 (v/v) (see recipe)

Ultrapure water (18.2 MΩ)

Supelclean ENVI-Carb SPE Tube, 250 mg bed wt. volume 3 mL (Sigma-Aldrich, #57088)

10 mM ammonium bicarbonate (NH_4HCO_3) (see recipe)

80 % acetonitrile (v/v), 0.1 % trifluoroacetic acid (v/v) (TFA)

25 % acetonitrile (v/v)

100 mM triethylamine acetic acid, pH 7.0 (TEAA) (see recipe)

50 mM triethylamine acetic acid, pH 7.0 (TEAA) (see recipe)

25 % acetonitrile (v/v), 50 mM TEAA, pH 7.0 (see recipe)

Refrigerated tabletop centrifuge

Freeze dryer

Nucleotide sugar standards: UDP-α-D-xylose, UDP-β-L-arabinopyranose, UDP-α-D-galacturonic acid (Carbosource Services, Complex Carbohydrate Research Center); UDP-α-D-glucuronic acid, UDP-α-D-glucose, UDP-α-D-galactose, UDP-*N*-acetyl-α-D-glucosamine, UDP-*N*-acetyl-α-D-galactosamine, GDP-α-D-mannose, GDP-β-L-fucose, GDP-α-D-glucose, ADP-α-D-glucose (Sigma-Aldrich); UDP-β-L-arabinofuranose (Peptides International). All standards are dissolved in ultrapure water (2.5 µM each) and diluted to the appropriate concentrations to enable loading of 2.5, 5, 10 and 20 pmol for quantitation.

Buffer B: acetonitrile 100 % (v/v), LC-MS grade (Sigma Aldrich, #1000292500)

Buffer A: 0.3 % formic acid (v/v), pH 9.0 (see recipe)

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Hypercarb porous graphire carbon (PGC) column, 150 x 1 mm, particle size 5 µm (Thermo Fisher Scientific, #35005-151030)

High Performance Liquid Chromatography (HPLC) system capable of 50 to 200 µL min^{-1}

Tandem mass spectrometry with multiple reaction monitoring (MRM) capabilities, such as a triple quadrupole (QqQ) mass spectrometer

Metabolite extraction from plant material

The chloroform / methanol extraction method results in a reproducible recovery of nucleotide sugars from plant material, specifically for UDP-Glc and ADP-Glc (Arrivault et al., 2009).

1. Thoroughly grind plant material using mortar and pestle placed in liquid nitrogen or by using a TissueLyser with racks precooled in liquid nitrogen

> *Freezing the tissue in liquid nitrogen before homogenization improves cell breakage by growth of ice crystals which rupture individual cells.*

2. Weigh about 50 mg frozen plant material into a 1.5 mL tube

> *While specialty plasticware to reduce plasticizers that interfere with mass spectrometric detection can be employed, the mass range used for nucleotide sugar detection is in the range of 535 – 604 m/z. This is out of range for common plastic derived contaminants.*

3. Add 600 µL ice-cold chloroform / methanol and vortex thoroughly, place tubes at -20 °C for 2 h

> *By addition of ice-cold methanol and chloroform metabolism is quenched immediately. Lipids and proteins are removed.*

4. Add 400 µL ice-cold water and vortex thoroughly

5. Centrifuge at 20,000 x *g* at 4 °C for 5 min and collect upper phase in a 15 mL tube placed on ice

> *It is important not to transfer liquid from the lower phase (chloroform) or interphase.*

6. Repeat steps 4 and 5 twice and combine supernatants with previously collected aqueous phase

7. Add 3 mL ice-cold water to the combined phases

> *Reduce the methanol concentration in order to prevent spills and loss of material during freeze drying.*

8. Punch a hole into the lid of the 15 mL tube and freeze in liquid nitrogen, freeze dry overnight

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Sample cleanup by solid phase extraction (SPE)

Retention of nucleotide sugars from the chloroform / methanol extraction method using the ENVI-Carb SPE has been experimentally assessed and shows excellent recovery (Ito et al., 2014). Some nucleotide sugars, like UDP-apiose, are unstable at ambient temperature, thus it is important that all solutions are kept on ice and the SPE cleanup is carried out in a cold room.

9. Add 1 mL of ice-cold 10 mM ammonium bicarbonate to the sample, vortex for 10 seconds

10. Equilibrate ENVI-Carb cartridge with 3 mL of ice-cold 80 % acetonitrile, 0.1 % TFA solution followed by 2 mL ice-cold water

> *When pipetting TFA, wear protective equipment (PPE) and perform the step in a fume hood. TFA is extremely volatile so care must be taken when pipetting. Due to its corrosive nature and to reduce contaminants, use of glass pipettes is recommended.*
>
> *Wait until each solution completely passes through the column by gravity flow.*

11. Load the sample onto the column and let it pass through by gravity flow

12. Wash the column sequentially with 2 mL ice-cold water, 2 mL ice-cold 25 % acetonitrile and finally 2 mL ice-cold 50 mM TEAA pH 7.0

> *Wait until each solution completely passes through the column by gravity flow.*

13. Place the SPE cartridge into a fresh 15 mL tube (placed on ice) and elute with 2 mL ice-cold 25 % acetonitrile, 50 mM TEAA pH 7.0 solution

> *Wait until the solution completely passes through the column by gravity flow.*

14. Add 2 mL ice-cold water to the eluate, freeze in liquid nitrogen and freeze dry overnight

15. Resuspend sample in 200 µL ice-cold water and store at -80 °C until analysis by mass spectrometry

Nucleotide sugar detection and quantification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

16. Conduct the separation, detection and quantitation by liquid chromatography-tandem mass spectrometry (LC-MS/MS) set to multiple reaction monitoring (MRM) in negative ion mode.

> *The following steps are specific for LC-MS/MS analysis of nucleotide sugars using a 4000 QTRAP LC/MS/MS system (SCIEX) equipped with a TurboIonSpray source (SCIEX) and an Agilent 1100 Series Capillary LC System using*

17. Set the Agilent 1100 HPLC to run at a flow rate of 50 µL min^{-1}. Run the system in Micro mode with a mix rate of 400 µL min^{-1}

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18. Use a Hypercarb Porous Graphitic Carbon column (150 mm × 1 mm, 5 μm) as the stationary phase and set the column compartment to 50 °C.

A range of analytical Hypercarb Porous Graphitic Carbon columns are available, including options for Ultra-Performance Liquid Chromatography (UPLC) systems. An appropriate flow rate to maintain linear velocity should be selected based on the properties of the column.

19. Keep the samples at 4 °C in the autosampler

20. The following gradient is applied: initial conditions are 95 % Buffer A (0.3 % formic acid, pH 9.0) and 5 % Buffer B (100 % acetonitrile) for 1 min followed by a gradient to 75 % (A, by volume) in 20 min, then 20 % (A, by volume) in 2 min, hold for 2 min before returning to 95 % (A, by volume) in 2 min, hold at 95 % (A, by volume) for 5 min (see Table 1)

[*Table 1 near here]

An alternative HPLC can be employed, however optimization of separation conditions will need to be conducted. This can be undertaken using a mix of nucleotide sugar standards.

21. The 4000 QTRAP is operated in negative ion mode employing the MRM scan type. A declustering potential of -40 volts, entrance potential of -10 volts and a collision cell exit potential of -15 volts is applied. The ion spray voltage is set at -4200 V, source temperature (TEM) at 425 °C, collision gas (CAD) was set to high and source gas 1 (GS1) and 2 (GS2) were both set to 20. A time of 100 ms was applied for each transition with Q1 resolution set to Low and Q3 resolution set to Unit. Compound-dependent collision energy parameters for each nucleotide sugar are outlined in Table 2

[Table 2 near here]

22. Nucleotide sugar standards (2.5, 5, 10 and 20 pmol) are analyzed at the start and end of each batch of samples and are used to quantify nucleotide sugars

23. Raw data are acquired using Analyst 1.5.1 (SCIEX) and peak areas of standards and measured nucleotide sugars calculated using MultiQuant 2.1 (SCIEX)

24. A simple linear regression for each nucleotide sugar is generated from standards using average peak areas (y-axis) and mass (pmol) analyzed (x-axis). This is undertaken automatically by MultiQuant 2.1 (SCIEX).

25. For each sample, the mass of nucleotide sugars (usually fmol to pmol) is estimated and averaged over replicates

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26. Finally calculate pmol mg^{-1} fresh weight of each nucleotide sugar from each sample and compare relative changes between treatments or samples.

> *In general, nucleotide sugar levels are consistent between plant organs (Rautengarten et al., 2014). We have observed relatively minor changes when plants are subjected to abiotic stress conditions. More significant changes in nucleotide sugar levels occur when genetic disruption of a biosynthetic pathway or transport mechanism is undertaken (Zhao et al., 2018).*

BASIC PROTOCOL 2. PLANT CELL WALL MONOSACCHARIDE COMPOSITION ANALYSIS BY HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION (HPAEC-PAD)

Nucleotide sugars are transported into the Golgi where glycosyltransferases catalyze the formation of glycosidic linkages to build polysaccharides. The distinct functions of the individual cell wall polymers and how they adapt during the life of the plant are also largely unknown. Extensive research is required to close this gap in our knowledge and tools to investigate the cell wall composition of for example mutant plants play an important role. One of the most widely applied approaches to profile monosaccharides is to extract alcohol insoluble residue (AIR; considered cell wall material), hydrolyze with TFA and analyze by HPAEC-PAD.

Materials

Liquid nitrogen

Mortar and pestle or TissueLyser (Qiagen, #85300)

Heat block, for 2 mL microfuge tubes

Miracloth (Merck Millipore, # 475855)

Microcentrifuge

ThermoMixer (Eppendorf, #2232000083

Ultrapure water (18.2 MΩ)

100 % ethanol (ACS Reagent)

70 % ethanol (ACS Reagent)

96 % ethanol (ACS Reagent)

chloroform / methanol solution 3:7 (v/v) (see recipe)

acetone (ACS Reagent, Sigma-Aldrich, #179124)

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50 mM cyclohexanedinitrilotetraacetic acid (CDTA) (ACS Reagent, Sigma-Aldrich #319945)

50 mM sodium carbonate (Na_2CO_3) (ACS Reagent, Sigma-Aldrich #223484)

1 N potassium hydroxide (KOH) (ACS Reagent, Sigma-Aldrich #221473)

4 N potassium hydroxide (KOH) (ACS Reagent, Sigma-Aldrich #221473)

50 mM sodium acetate (NaOAc) (ACS Reagent, Sigma-Aldrich #241245), pH 5.5 with acetic acid

acetic acid (ACS Reagent, Sigma-Aldrich #695092)

hydrochloric acid (HCl) (ACS Reagent, Sigma-Aldrich #320331)

200 mM (3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0 (see recipe)

200 mM sodium acetate (NaOAc) (ACS Reagent, Sigma-Aldrich #241245), pH 4.5 with acetic acid

amyloglucosidase (Megazyme, #E-PULBL)

pullunanase (Megazyme, # E-AMGDF)

α-amylase (*Bacillus licheniformis*) (Megazyme, #E-BLAAM)

D-Tube Dialyzer Maxi, MWCO 3.5 kDa (Merck Millipore, #71508)

Freeze dryer

1 mL syringes

trifluoroacetic acid (TFA) (Sigma-Aldrich, #T6508)

Ion Chromatograph (IC) system such as Dionex ICS-6000

Dionex CarboPac PA20 BioLC Analytical 3 X 150 mm column (Thermo Fisher, #060142)

Dionex CarboPac PA20 BioLC guard 3 X 30 mm column (Thermo Fisher, #0601144)

50% sodium hydroxide solution (NaOH) for HPAEC buffer preparation (Sigma-Aldrich, #415413)

Monosaccharide standards: D-xylose (Sigma-Aldrich, #X3877), L-arabinose (Sigma-Aldrich, #A3256), D-glucose (Sigma-Aldrich, # G8270), D-mannose (Sigma-Aldrich, #63582), D-galactose (Sigma-Aldrich, #G0750), L-rhamnose monohydrate (Sigma-Aldrich, #R3875), L-fucose (Sigma-Aldrich, #F2252), D-glucuronic acid (Sigma-Aldrich, #G5269), D-galacturonic acid sodium salt (Sigma-Aldrich, #73960). Prepare the standards in ultrapure water and combine to make a master mix for each concentration (2.5, 5, 10, 25, 50 and 100 μM)

1 mL vials (Agilent, #5182-0567) and 11 mm clear snap, pre-slit caps (Agilent, #5183-4511)

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Screw cap 2 mL microcentrifuge tubes with ethylene propylene O-ring (VWR, #3600-870)

Preparation of alcohol insoluble residue (AIR)

The protocol results an alcohol insoluble residue representing enriched cell wall material. This is accomplished through the homogenization of plant tissue and removal of soluble material with organic solvents. The method has been applied to a wide range of plant materials including woody tissue (Persil-Cetinkol et al., 2012), straw (Costa et al., 2016) and herbaceous plant material (Ebert et al., 2015). For recalcitrant plant tissue like wood and straw, further processing is usually required e.g. milling, screening and more extensive solvent extraction using a Soxhlet extractor to remove contaminants (DeMartini et al., 2013). This protocol details the extraction and analysis of AIR from herbaceous plant material which is well suited to minimal processing. Aqueous acid hydrolysis of AIR with TFA will result in the the majority of non-cellulosic polysaccharides in the AIR being liberated (Selvendran et al., 1990).

1. Thoroughly grind plant material using a mortar and pestle placed in a liquid nitrogen bath or use a TissueLyser with racks precooled in liquid nitrogen or at -80 °C.

> *Freezing the tissue in liquid nitrogen before homogenization improves cell breakage by growth of ice crystals which rupture individual cells.*

2. Weight about 100 mg frozen plant material into a 2 mL screw cap tube.

> *More than 100 mg of material can be processed. However, the number of incubation / washing steps may need to be increased.*

3. Add 1 mL 100 % ethanol and incubate at 100 °C for 30 min, mix the sample frequently using a vortex throughout this incubation step.

> *The addition of ethanol and incubation of the sample at high temperature is used to rapidly quench metabolism. Care should be taken during this step to ensure consistent time and temperature treatments. The procure could result in some damage to glycan structures and needs to be applied consistently across samples. The potential effect of aqueous acid hydrolysis on specific monosaccharides has been previously outlined and should be taken into consideration when interpreting results (Selvendran et al., 1990).*

4. Centrifuge at 20,000 x *g* at room temperature for 10 min and discard liquid.

5. Wash the resultant pellet with 1 mL 70 % ethanol, vortex sample, centrifuge at 20,000 x *g* at room temperature for 5 min and discard liquid. Repeat this step.

6. Wash the pellet with 1 mL chloroform / methanol solution, vortex sample, centrifuge at 20,000 x *g* at room temperature for 5 min and discard liquid.

> *Methanol / chloroform remove protein and lipid from the sample.*

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7. Wash the pellet with 1 mL acetone, vortex sample, centrifuge at 20,000 x *g* at room temperature for 5 min and discard liquid.

The addition of acetone reduces the drying time of samples in the next step.

8. Dry pellet overnight at room temperature or under vacuum.

The dry cell wall samples can be stored at room temperature for several months.

Removal of starch from AIR samples

The removal of starch from plant material prior to carbohydrate analysis is optional, however for photosynthetically-active leaf material, which can accumulate starch it is recommended. If the de-starching-step is omitted, it is general practice to omit glucose from the analysis.

9. Add 150 µL of 50 mM (3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer pH 7.0 containing 3 U/mL α-amylase to 2 - 5 mg AIR, vortex and spin at low speed to ensure sample and buffer are at the bottom of the tube.

Ensure that the entire sample is covered with buffer. The buffer amount used can be increased, if ratios are kept the same, keep in mind the size of the tubes used.

10. Incubate at 85 °C for 10 min.

11. Add 200 µL of 200 mM sodium acetate buffer (NaOAc) pH 4.5 containing 3 U/mL amyloglucosidase and 1 U/mL pullunanase, vortex the sample and spin at low speed to ensure sample and buffer are at the bottom of the tube.

12. Incubate at 50 °C for 120 min.

13. Wash the pellet with 1 mL 96 % ethanol, vortex the sample thoroughly, centrifuge at 20,000 x *g* for 5 min and discard liquid at room temperature.

14. Wash the resultant pellet twice with 1 mL 70 % ethanol.

15. Dry pellet overnight at room temperature or under vacuum.

The dry de-starched residue can be stored at room temperature for several months.

Sequential extraction of cell wall material

The (de-starched) AIR can be subjected to sequential extraction; this separates cell wall material into fractions according to specific characteristics (Rautengarten et al., 2016). Here we described the basic extraction technique. However, further extraction steps can be performed.

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16. Weigh around 10 to 15 mg of AIR into a 2 mL screw cap tube.

The above procedure (100 mg tissue in 2 mL tubes) will result in around 2 – 5 mg AIR. Sequential extraction results in sample fractionation and requires more starting material. This can be accomplished by scaling-up the preparation of AIR or combining and weighing AIR from replicate 100 mg extractions (3 to 5 samples).

17. Add 1 mL 50 mM CDTA and shake for 2 h at room temperature at maximum speed in a ThermoMixer (or similar).

It is important that the 50 mM CDTA solution is freshly prepared. Depending on the amount of starting material (AIR) the volume of the solutions should be scaled up to ensure that the entire AIR sample is covered with buffer.

18. Centrifuge at 20,000 x *g* for 10 min at room temperature, filter the supernatant using Miracloth (or similar), collect supernatant into a fresh tube and store at 4 °C.

After the CDTA extraction the sample is generally not easily collected at the bottom of the tube, to avoid contamination with AIR particles a filtration step is highly recommended. Use a syringe (1 mL) to force the supernatant through a piece of pre-wetted Miracloth.

19. Add another 1 mL of 50 mM CDTA and shake at 4 °C overnight, briefly centrifuge and combine supernatants.

This fraction contains the CDTA-soluble pectic polysaccharides.

20. Repeat steps 17 to 19 by sequentially extracting the sample using 50 mM Na_2CO_3, then 1 N KOH, and finally 4 N KOH, respectively. This includes 2 h room temperature incubation (step 17), 10 min centrifuge step without filtration (step 18) and incubation in 1 mL buffer at 4 °C overnight (step 19).

These sequential steps result in a carbonate-soluble pectin enriched fraction and two hemicellulose enriched KOH fractions. To avoid contamination the entire fraction needs to be transferred into a new tube and a filtration step is highly recommended for each fraction. The insoluble pellet can be used for the determination of crystalline cellulose content by *acid hydrolysis (Turner et al., 1997), which is not part of this protocol.*

21. For CDTA fractions, dialyze against 5 L of 50 mM NaOAc pH 5.2 - 5.5 overnight followed by 3 x 1 L H_2O, 2 h each at 4 °C.

The volume of sodium acetate solution and water used for dialysis depends on the number of samples. We recommend the use of at least 1 L per 1-2 samples

22. For the other fractions, neutralize the Na_2CO_3 fraction with 6.7 µL acetic acid (glacial), 1 N and 4 N KOH fractions with 171 µL and 684 µL concentrated HCl, respectively.

Neutralization of the samples is necessary to prevent damage to the dialysis membrane.

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23. Dialyze theses fractions (Na_2CO_3, 1 N KOH, and 4 N KOH) at 4 °C over two days against 1 L water (change the water at least 3 times).

The volume of water used for dialysis depends on the number of samples. We recommend the use of at least 1 L per 1-2 samples.

24. A total of four dialyzed fractions (CDTA, Na_2CO_3, 1 N KOH, and 4 N KOH) are generated per sample. Freeze dry all fractions prior to processing with TFA.

TFA hydrolysis

To generate monosaccharides (single sugar units) of the cell wall material, AIR preparations (de-starched AIR) and fractions need to undergo acid hydrolysis. TFA is generally regarded as the most advantageous acid for this process as it results in uniform release of monosaccharides from glycoconjugates compared to hydrochloric or sulfuric acid (Manzi, 1995).

25. Resuspend each fraction in 850 µL water and transfer into a screw cap tube.

Always use 2 mL screw cap tubes (with O-rings) to avoid sample loss during the hydrolysis step.

26. Add 150 µL TFA and vortex vigorously.

Always wear suitable protective equipment (PPE) when pipetting TFA into the sample lysate. Perform this step in a fume hood. Make sure to quickly pipet the TFA into the solution as it is extremely volatile. Due to its corrosive nature and to reduce contaminants, use of glass pipettes is recommended.

27. Incubate at 120 °C for exactly 1 h, cool down for about 10 min at room temperature and shortly spin samples to collect all material at the bottom of the tube.

This incubation step should be performed in a fume hood.

28. Evaporate the TFA solution in a vacuum concentrator overnight.

29. Resuspend each fraction in 1 mL water (or less depending on the pellet size of each fraction), shake at max speed for 30 min

If the pellets do not dissolve well in water incubate them at 40 °C and continue shaking.

30. Centrifuge at 20,000 x *g* for 10 min and transfer supernatants into fresh tubes.

Always pipette the supernatant from the top and make sure to avoid any pellet or floating particles as particles could get stuck in the tubing during the HPAEC analysis and can cause instrument defects. It is optional but recommended to filtrate the samples prior to analysis by HPAEC-PAD. All samples can now be stored at -80 °C until analysis by HPAEC-PAD.

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Cell wall monosaccharide composition analysis by HPAEC-PAD

31. Dilute samples appropriately.

> *Dilution depends on the condition and sensitivity of your instrument. We have found that 2 mg of TFA hydrolyzed AIR diluted in 1 mL of water, a 1:10 dilution works well for HPAEC-PAD analysis.*

32. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) can be performed using a Dionex ICS 5000 ion chromatograph (Thermo Fisher) or similar instrument.

33. Run the HPAEC at a flow rate of 400 µL / min using a Dionex CarboPac PA20 column (150 mm x 3 mm) connected to a Dionex CarboPac PA20 BioLC guard (30 mm x 3 mm), as the stationary phase. Set the temperature of the column compartment to 30 °C and keep samples at 10 °C in the autosampler.

34. The following gradient is applied using buffer A (ultrapure water), buffer B (0.1 M NaOH) and buffer C (1 M NaOH). Condition the system with 0.01 M NaOH for 10 min, followed by sample injection (20 µL) and separation of neutral monosaccharides (xylose, arabinose, galactose, rhamnose, fucose) by isocratic elution at 0.01 M NaOH for 15 min. This is followed by an increase in eluent strength to 0.45 M NaOH for 20 min to separate acidic monosaccharides (glucuronic acid, galacturonic acid) (Table 3).

[*Table 3 near here]

> *Note that this gradient does not separate mannose and xylose; since mannose is a minor sugar in plant tissue the peak is often considered the xylose peak. Running the samples using different isocratic conditions enables data for both these sugar residues. The separation of mannose and xylose can be achieved by reducing the concentration of eluent down to 1.5 mM KOH.*

35. A series of monosaccharide standards (2.5, 5, 10, 25, 50 and 100 µM) are run at the commencement, in the middle and at the end of a batch to enable quantitation.

36. After analysis of standards and samples, extract peak areas using the Chromeleon Chromatography Data System Software.

37. A simple linear regression for each monosaccharide is initially generated from the standards using peak area (y-axis) and mass (pmol) analyzed (x-axis). This is generally done automatically by the Chromeleon Chromatography Data System Software.

38. For each sample, the mass of the eight monosaccharides is determined.

39. Data is generally presented as mole percent (mol %). This is accomplished by calculating the proportion of each of the eight monosaccharides identified in each sample to compare relative changes between treatments or samples.

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Data can also be displayed as absolute values (µg/mg AIR). To determine µg/mg values AIR, multiply the amount of each monosaccharide calculated by the Chromeleon Chromatography Data System Software with its respective molecular weight (i.e. Fucose 164.16 g) and factor in any dilution employed e.g. 20 µL loaded from 1 mL. Lastly, normalize by the mass of AIR (µg) used in each sample..

REAGENTS AND SOLUTIONS

Use ultrapure water (18.2 MΩ) in all recipes and protocol steps

Chloroform / Methanol 3:7 (v:v) solution

Premix 15 mL chloroform with 35 mL methanol in 50 mL tube and store at 4 °C

Ammonium bicarbonate solution

Prepare a 1 M stock solution in water and store in aliquots at -20 °C, use stock solution to freshly prepare a 10 mM solution in water

Triethylamine acetic acid (TEAA), pH 7.0

Prepare a 100 mM stock solution by adding 7 mL triethylamine (Sigma Aldrich, #90338) to 400 mL water, adjust to pH 7.0 with 10 % acetic acid and then the volume to 500 mL, aliquot into 50 mL tubes and store at -20 °C

Prepare in fume hood and slowly adjust the pH to 7.0.

0.3 % Formic acid (v/v), pH 9.0

Add 3 mL formic acid to 800 mL LC-MS grade water; slowly adjust the pH to 9.0 with ammonium hydroxide solution (28 %) (Sigma Aldrich, #338818)

Prepare in a fume hood

200 mM MOPS buffer, pH 7.0

For 1L, combine 41.86 g 3-(N-Morpholino)propanesulfonic acid (Sigma Aldrich, #M1254), 4.1 g sodium acetate (Sigma-Aldrich #241245) and 3.72 g ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (Sigma Aldrich, #E4884) Add ultrapure water and adjust to pH 7.0 with NaOH.

Sodium hydroxide (NaOH)

Use 52.3 mL of 50% sodium hydroxide and dilute to 1 L with deionized water to prepare 1 M NaOH. Dilute the 1 M NaOH solution to 100 mM and 450 mM NaOH respectively, degas both solutions and store in plastic bottles blanketed with helium or nitrogen.

Always use 50% w/w sodium hydroxide solution (do not use sodium hydroxide pellets, since they are coated with a layer of carbonate and do not pipette the required aliquot

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from the top of the bottle where sodium carbonate may have been formed) for preparation (Sigma Aldrich, #415413), keep the eluent blanketed with helium or nitrogen to avoid carbon dioxide contamination from the air.

COMMENTARY

Background Information

Nucleotide sugar measurements

The importance of nucleotide sugars in glycosylation processes along with their biosynthetic proximity to primary metabolism has resulted in numerous approaches to detect and quantify these metabolites from biological samples. A significant challenge has been the confident separation and identification of different isomers among the many nucleotide sugars found in plants. Initial chromatographic approaches to identify these molecules from biological samples employed paper- and thin layer chromatography (Bochner et al., 1982; Carminatti et al., 1965). However, these methods suffer from limitations in resolution, making the separation of nucleotide sugars and nucleotides difficult. With the development of advanced separation techniques, such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) a range of new separation approaches were pursued for the analysis of nucleotide sugars. These include anion exchange (Hull et al., 1994), ion-pairing reversed phase chromatography (Ryll et al., 1991) as well as capillary zone electrophoresis (Lehmann et al., 2000). However, these early separations approaches were hampered by limited detection techniques (e.g. ultraviolet detectors) that make unambiguous assignment of metabolites from complex biological samples difficult. While the adoption of nucleotide sugar cleanup methods greatly improved resolution with these traditional detection systems (Nakajima et al., 2010), the adoption of mass spectrometry (LC-MS) and the ability to assign mass has enabled the assignment of nucleotide sugars without standards (Pabst et al., 2010), which is particularly useful for plant samples. A series of LC-MS nucleotide sugar detection and quantitation approaches from complex biological samples have now been conducted including CE-MS (Soo et al., 2004), hydrophilic interaction LC-MS/MS (Ito et al., 2014), ion-pairing reversed phase LC-MS/MS (Turnock et al., 2007), anion-exchange LC-MS/MS (Alonso et al., 2010) and porous graphitic carbon (PGC) LC-MS/MS (Pabst et al., 2010). Moreover, the implementation of MS-based targeted quantification approaches (MRM) for many of these approaches has further improved specificity of these methods for identification and quantification.

The limited availability of CE-MS instruments along with the complexity of coupling anion-exchange to an MS makes the adoption of LC-MS-based approaches a more likely option when seeking to profile nucleotide sugars. The selection of which stationary phase is dictated by both the reproducible detection over many hundreds of samples and by the consistent separation of all nucleotide sugars in a given sample. The application of hydrophilic interaction chromatography is challenging due to sample solubility in high organic solvents used in the mobile phase (Ruta et al., 2010). The method is also unable to reliably separate the isomers of UDP-Gal / UDP-Glc or UDP-GlcNAc / UDP-GalNAc (Ito et al., 2014), two important nucleotide sugars in plants. Ion-pairing reversed phase chromatography reportedly separates all nucleotide sugars found in biological samples (Kochanowski et al., 2006;

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Nakajima et al., 2010; Turnock et al., 2007), although baseline resolution can often be an issue, especially without a sample cleanup step. Stationary phase stabilization when using ion-pairing can also be an issue and likely explains variations in baseline resolution. In our hands, we were unable to satisfactorily separate the isomers UDP-GlcNAc / UDP-GalNAc when using ion-pairing reversed phase chromatography and experienced baseline resolution issues and retention time shifts. By contrast, we have found that the application of porous graphitic carbon a reliable and robust separation technique that is highly compatible with MS. The approach generates baseline separation of all reported plant nucleotide sugars and we have not observed the reported retention time instabilities (Behmuller et al., 2014). The stability of porous graphitic carbon we have observed over many hundreds of samples may be because we do not regenerate the column with trifluoroacetic acid, as has been reported for this technique (Pabst et al., 2010). While porous graphitic carbon is unable to separate the isomers of UDP-GlcNAc / UDP-GalNAc, there is no evidence of the latter nucleotide sugar occurring in plants, meaning that the technique is able to quantify all reported plant nucleotide sugars.

Cell wall monosaccharide composition analysis

For the analysis of the sugar composition of most plant cell wall polymers, apart from cellulose, hydrolysis of cell wall material (AIR) with trifluoroacetic acid is a common starting point. The subsequent separation of resultant monosaccharides has historically been undertaken by a handful of analytical methods. Traditionally the two most widely used methods for sugar composition analysis are the alditol acetate (Englyst et al., 1982) and the trimethylsilyl (TMS) (Sweeley et al., 1963) methods. The alditol acetate method can be coupled to the analysis of total uronic acid content (Blumenkrantz et al., 1973; Filisetti-Cozzi et al., 1991; van den Hoogen et al., 1998) or alternatively alditol acetates can be detected by GC-MS after a carbodiimide reduction (Taylor et al., 1972) of the uronic acids. The most recent method developed to separate and quantify monosaccharides after hydrolysis is to undertake high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). This method has now been widely embraced because of its simplicity, straightforward application and fast sample preparation (Oxenboll Sorensen et al., 2000).

While all the above-mentioned methods can be used to detect and quantify low amounts of major neutral sugars (fucose, rhamnose, arabinose, galactose, xylose, glucose and mannose), the evaluation of uronic acids (glucuronic acid and galacturonic acid) is only possible using TMS, carbodiimide or HPAEC-PAD. Consequently, to profile the nine major neutral and acidic sugars present in plant biomass, the application of one of these three methods is recommended. A recent study provides a detailed comparison of these methods and clearly demonstrates that all three deliver comparable quantitative results for both neutral and acidic sugar content (Biswal et al., 2017), although the TMS method resulted in a slightly higher yield. The authors mention that the abundance ranking of the less abundant sugars such as rhamnose, mannose, and fucose is often reversed during anion exchange when compared to the other methods and it does not easily enable analysis by mass spectrometry. However, it requires the least amount of sample preparation time since the TFA-hydrolyzed sugars can be directly separated by anion exchange and detected by electrochemical detection. This

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contrasts with the other methods that involve laborious sample preparation steps and time-consuming derivatization of the samples for GC-MS analysis.

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Critical Parameters and Troubleshooting

Nucleotide sugar measurements

1. The nucleotide sugar extraction protocol should be adjusted to no more than 50 mg (fresh weight) of thoroughly ground plant material. If more than 50 mg of material is employed, the extraction buffer volume needs to be adjusted accordingly.
2. The success of metabolite extraction procedures can be highly dependent on the tissue to be analyzed. We have successfully employed this extraction protocol on a variety of organ types (roots, leaves, flowers) as well as multiple plant species including *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Physcomitrella patens* and *Selaginella moellendorffii*. However, particularly recalcitrant plant tissues may require optimization.
3. During metabolite extraction (methanol / chloroform) be careful when taking the upper phase (methanol) containing polar metabolites. Try not to disturb the interphase and lower phase (chloroform) as they contain contaminating protein and lipids.
4. While it is possible to use general purpose reagents for mass spectrometry (e.g. acetonitrile) and in-house ultrapure water, we have found that contamination issues with these reagents can result in wasted time and the unnecessary acquisition of consumables (e.g. analytical columns and guard columns). Consequently, we recommend the purchase of LC-MS grade acetonitrile and water and high-grade formic acid.
5. The utilization of the SPE cleanup step is an important step in the removal of contaminants that cause matrix effects during mass spectrometry. While it is possible to eliminate this step if processing hundreds of samples, separation of nucleotide sugars will be impacted, the lifetime of the PGC column will be reduced and the sensitivity of the mass spectrometer will be impacted.

Cell wall monosaccharide composition analysis

1. If samples are subjected to a sequential extraction prior to analysis by anion exchange, the dialysis steps are extremely important since high concentrations of anions (e.g. carbonate, chloride) and salts need to be avoided in samples analyzed by ion exchange chromatography.
2. The TFA hydrolysis step is critical to liberate monosaccharides from AIR prioir to their analysis. When performing the hydrolysis step, it is important to monitor sample incubation times and temperature. Furthermore, TFA is highly volatile so ensure that each sample is provided with the same volume of TFA. The TFA should be directly

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pipetted into the sample avoiding disruption onto the tube wall. A filter tip should be employed to reduce contamination and ultimately the destruction of the pipette. The age of the TFA is also an important consideration and we have observed performance differences between TFA batches. Collectively, these factors can critically affect the hydrolysis efficiency and it is therefore recommended that only samples that have undergone sample preparation at the same time are used for comparative analysis.

3. For the HPAEC-PAD analysis, several factors should be monitored. As outlined above, it is critical that NaOH-containing buffers be maintained under a flow of nitrogen or helium gas. This prevents absorption of carbon dioxide and conversion of NaOH into carbonate ions which will result in retention time shifts and baseline drifting. The column should be equilibrated before each run and it is recommended that a blank sample be used each time a new sequence is started.

Anticipated results

Nucleotide sugar measurements

The protocol should generate baseline separation of plant nucleotide sugars that can be readily quantified (fmol to pmol mg^{-1} fresh weight) using a series of standards at known concentrations (Figure 1). The approach will generate a relative abundance for each nucleotide sugar in a sample and relative changes can be used to compare between samples. If using a different LC-MS/MS setup, some optimization will likely need to be undertaken to ensure separation of the nucleotide sugars. This should be initially undertaken using a mix of nucleotide sugar standards.

[*Figure 1 near here]

Cell wall monosaccharide composition analysis

The method will result in the separation of major monosaccharides from matrix polysaccharides of the plant cell wall. The approach will enable the reliable quantitation of theses sugars over multiple samples in conjunction with a series of standards of known concentrations (Figure 2). The approach will generate a relative abundance for each sugars in a sample and relative changes can be used to compare between samples. The reduced sample processing over other methods makes this technique highly reproducible when considerations outlined in this protocol are taken into account.

[*Figure 2 near here]

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Time Considerations

Nucleotide sugar measurements

The basic nucleotide sugar extraction protocol can be completed in three days starting from plant material. Much of this time is waiting for samples to freeze dry overnight. The times indicated are dependent on the number of samples being processed. A suggested timeline is:

Day 1: Grind plant material and extract metabolites, 2 h at -20 °C. (total 3 h), freeze dry overnight.

Day 2: Solid phase extraction to clean-up sample (total 1 h), freeze dry overnight.

Day 3: Analysis by LC-MS/MS (total for each sample 35 min), however, you must also consider column equilibration and standards for each batch (total of around 6 h for 5 to 10 samples).

Cell wall monosaccharide composition analysis

Assuming plant material has been collected, the time from cell wall extraction to sample analysis is 4 days. This is dependent on whether the starch removal step is included, if not the process takes 3 days. However, this process is largely incubation and drying times rather than experimental time. If samples are subjected to a sequential extraction an extra 5 to 6 more days are required since the fractionation protocol includes four overnight incubation steps followed by four overnight dialysis steps. A suggested timeline for the basic protocol is:

Day 1: Extract AIR (total 1 h) but depends on the number of samples to be processed. Dry samples overnight.

Day 2: Removal of starch form samples (total 3 h), dry samples overnight.

Day 3: TFA hydrolysis (total 1 h), dry samples overnight.

Day 4: Analysis of samples by anion exchange (each sample 45 min)

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LITERATURE CITED

Accepted Manuscript

Alonso, A. P., Piasecki, R. J., Wang, Y., LaClair, R. W., & Shachar-Hill, Y. (2010). Quantifying the labeling and the levels of plant cell wall precursors using ion chromatography tandem mass spectrometry. *Plant Physiol, 153*(3), 915-924. doi:10.1104/pp.110.155713

Arrivault, S., Guenther, M., Ivakov, A., Feil, R., Vosloh, D., van Dongen, J. T., Sulpice, R., & Stitt, M. (2009). Use of reverse-phase liquid chromatography, linked to tandem mass spectrometry, to profile the Calvin cycle and other metabolic intermediates in Arabidopsis rosettes at different carbon dioxide concentrations. *Plant J, 59*(5), 826-839. doi:10.1111/j.1365-313X.2009.03902.x

Bar-Peled, M., & O'Neill, M. A. (2011). Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling. *Annu Rev Plant Biol, 62*, 127-155. doi:10.1146/annurev-arplant-042110-103918

Behmuller, R., Forstenlehner, I. C., Tenhaken, R., & Huber, C. G. (2014). Quantitative HPLC-MS analysis of nucleotide sugars in plant cells following off-line SPE sample preparation. *Anal Bioanal Chem, 406*(13), 3229-3237. doi:10.1007/s00216-014-7746-3

Biswal, A. K., Tan, L., Atmodjo, M. A., DeMartini, J., Gelineo-Albersheim, I., Hunt, K., Black, I. M., Mohanty, S. S., Ryno, D., Wyman, C. E., & Mohnen, D. (2017). Comparison of four glycosyl residue composition methods for effectiveness in detecting sugars from cell walls of dicot and grass tissues. *Biotechnol Biofuels, 10*, 182. doi:10.1186/s13068-017-0866-1

Blanch, H. W., Adams, P. D., Andrews-Cramer, K. M., Frommer, W. B., Simmons, B. A., & Keasling, J. D. (2008). Addressing the need for alternative transportation fuels: the Joint BioEnergy Institute. *ACS Chem Biol, 3*(1), 17-20. doi:10.1021/cb700267s

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Anal Biochem, 54*(2), 484-489.

Bochner, B. R., & Ames, B. N. (1982). Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. *J Biol Chem, 257*(16), 9759-9769.

Carminatti, H., Passeron, S., Dankert, M., & Recondo, E. (1965). Separation of sugar nucleotides, phosphoric esters and free sugars by paper chromatography with solvents containing borates of organic bases. *J Chromatogr, 18*, 342-348.

Costa, T. H. F., Vega-Sánchez, M. E., Milagres, A. M. F., Scheller, H. V., & Ferraz, A. (2016). Tissue-specific distribution of hemicelluloses in six different sugarcane hybrids as related to cell wall recalcitrance. *Biotechnol Biofuels, 9*(1), 99. doi:10.1186/s13068-016-0513-2

DeMartini, J. D., Pattathil, S., Miller, J. S., Li, H., Hahn, M. G., & Wyman, C. E. (2013). Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass. *Energy & Environmental Science, 6*(3), 898-909. doi:10.1039/C3EE23801F

Ebert, B., Rautengarten, C., Guo, X., Xiong, G., Solomon, P. S., Smith-Moritz, A. M., Herter, T., Chan, L. J. G., Adams, P. D., Petzold, C. J., Pauly, M., Willats, W. G. T., Heazlewood, J. L., & Scheller, H. V. (2015). Identification and characterization of a

This article is protected by copyright. All rights reserved.

Golgi localized UDP-xylose transporter family from Arabidopsis. *The Plant Cell, 27*(4), 1218-1227. doi:10.1105/tpc.114.133827
Englyst, H., Wiggins, H. S., & Cummings, J. H. (1982). Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst, 107*(1272), 307-318.
Filisetti-Cozzi, T. M., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Anal Biochem, 197*(1), 157-162.
Houston, K., Tucker, M. R., Chowdhury, J., Shirley, N., & Little, A. (2016). The Plant Cell Wall: A Complex and Dynamic Structure As Revealed by the Responses of Genes under Stress Conditions. *Front Plant Sci, 7*, 984. doi:10.3389/fpls.2016.00984
Hull, S. R., & Montgomery, R. (1994). Separation and analysis of 4'-epimeric UDP-sugars, nucleotides, and sugar phosphates by anion-exchange high-performance liquid chromatography with conductimetric detection. *Anal Biochem, 222*(1), 49-54. doi:10.1006/abio.1994.1452
Ito, J., Herter, T., Baidoo, E. E., Lao, J., Vega-Sanchez, M. E., Michelle Smith-Moritz, A., Adams, P. D., Keasling, J. D., Usadel, B., Petzold, C. J., & Heazlewood, J. L. (2014). Analysis of plant nucleotide sugars by hydrophilic interaction liquid chromatography and tandem mass spectrometry. *Anal Biochem, 448*, 14-22. doi:10.1016/j.ab.2013.11.026
Keegstra, K. (2010). Plant cell walls. *Plant Physiol, 154*(2), 483-486. doi:10.1104/pp.110.161240
Kochanowski, N., Blanchard, F., Cacan, R., Chirat, F., Guedon, E., Marc, A., & Goergen, J. L. (2006). Intracellular nucleotide and nucleotide sugar contents of cultured CHO cells determined by a fast, sensitive, and high-resolution ion-pair RP-HPLC. *Anal Biochem, 348*(2), 243-251. doi:10.1016/j.ab.2005.10.027
Lehmann, R., Huber, M., Beck, A., Schindera, T., Rinkler, T., Houdali, B., Weigert, C., Haring, H. U., Voelter, W., & Schleicher, E. D. (2000). Simultaneous, quantitative analysis of UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, UDP-glucose and UDP-galactose in human peripheral blood cells, muscle biopsies and cultured mesangial cells by capillary zone electrophoresis. *Electrophoresis, 21*(14), 3010-3015. doi:10.1002/1522-2683(20000801)21:14<3010::AID-ELPS3010>3.0.CO;2-C
Manzi, A. (1995). Acid hydrolysis for release of monosaccharides. *Current Protocols in Molecular Biology, 32*(1), 17.16.11-17.16.11. doi:10.1002/0471142727.mb1716s32
Nakajima, K., Kitazume, S., Angata, T., Fujinawa, R., Ohtsubo, K., Miyoshi, E., & Taniguchi, N. (2010). Simultaneous determination of nucleotide sugars with ion-pair reversed-phase HPLC. *Glycobiology, 20*(7), 865-871. doi:10.1093/glycob/cwq044
Oxenboll Sorensen, S., Pauly, M., Bush, M., Skjot, M., McCann, M. C., Borkhardt, B., & Ulvskov, P. (2000). Pectin engineering: modification of potato pectin by in vivo expression of an endo-1,4-beta-D-galactanase. *Proc Natl Acad Sci U S A, 97*(13), 7639-7644. doi:10.1073/pnas.130568297
Pabst, M., Grass, J., Fischl, R., Leonard, R., Jin, C., Hinterkorner, G., Borth, N., & Altmann, F. (2010). Nucleotide and nucleotide sugar analysis by liquid chromatography-electrospray ionization-mass spectrometry on surface-conditioned porous graphitic carbon. *Anal Chem, 82*(23), 9782-9788. doi:10.1021/ac101975k
Persil-Cetinkol, Ö., Smith-Moritz, A. M., Cheng, G., Lao, J., George, A., Hong, K., Henry, R., Simmons, B., Heazlewood, J. L., & Holmes, B. M. (2012). Structural and

This article is protected by copyright. All rights reserved.

Author Manuscript

chemical characterization of hardwood from tree species with applications as bioenergy feedstocks. *PLOS ONE, 7*(12), e52820. doi:10.1371/journal.pone.0052820

Rabina, J., Maki, M., Savilahti, E. M., Jarvinen, N., Penttila, L., & Renkonen, R. (2001). Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. *Glycoconj J, 18*(10), 799-805.

Rautengarten, C., Birdseye, D., Pattathil, S., McFarlane, H. E., Saez-Aguayo, S., Orellana, A., Persson, S., Hahn, M. G., Scheller, H. V., Heazlewood, J. L., & Ebert, B. (2017). The elaborate route for UDP-arabinose delivery into the Golgi of plants. *Proc Natl Acad Sci U S A, 114*(16), 4261–4266. doi:10.1073/pnas.1701894114

Rautengarten, C., Ebert, B., Liu, L., Stonebloom, S., Smith-Moritz, A. M., Pauly, M., Orellana, A., Scheller, H. V., & Heazlewood, J. L. (2016). The Arabidopsis Golgi-localized GDP-L-fucose transporter is required for plant development. *Nat Commun, 7*, 12119. doi:10.1038/ncomms12119

Rautengarten, C., Ebert, B., Moreno, I., Temple, H., Herter, T., Link, B., Doñas-Cofre, D., Moreno, A., Saéz-Aguayo, S., Blanco, F., Mortimer, J. C., Schultink, A., Reiter, W. D., Dupree, P., Pauly, M., Heazlewood, J. L., Scheller, H. V., & Orellana, A. (2014). The Golgi localized bifunctional UDP-rhamnose/UDP-galactose transporter family of Arabidopsis. *Proc Natl Acad Sci U S A, 111*(31), 11563-11568. doi:10.1073/pnas.1406073111

Ruta, J., Rudaz, S., McCalley, D. V., Veuthey, J. L., & Guillarme, D. (2010). A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography. *J Chromatogr A, 1217*(52), 8230-8240. doi:10.1016/j.chroma.2010.10.106

Ryll, T., & Wagner, R. (1991). Improved ion-pair high-performance liquid chromatographic method for the quantification of a wide variety of nucleotides and sugar-nucleotides in animal cells. *J Chromatogr, 570*(1), 77-88.

Selvendran, R. R., & Ryden, P. (1990). Isolation and analysis of plant cell walls. In P. M. Dey (Ed.), *Methods in Plant Biochemistry* (Vol. 2, pp. 549-579): Academic Press.

Soo, E. C., Aubry, A. J., Logan, S. M., Guerry, P., Kelly, J. F., Young, N. M., & Thibault, P. (2004). Selective detection and identification of sugar nucleotides by CE-electrospray-MS and its application to bacterial metabolomics. *Anal Chem, 76*(3), 619-626. doi:10.1021/ac034875i

Sweeley, C. C., Bentley, R., Makita, M., & Wells, W. W. (1963). Gas-Liquid Chromatography of Trimethylsilyl Derivatives of Sugars and Related Substances. *Journal of the American Chemical Society, 85*(16), 2497-&. doi:Doi 10.1021/Ja00899a032

Author Manuscript

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Taylor, R. L., & Conrad, H. E. (1972). Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. *Biochemistry, 11*(8), 1383-1388.

Turner, S. R., & Somerville, C. R. (1997). Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. *Plant Cell, 9*(5), 689-701. doi:10.1105/tpc.9.5.689

Turnock, D. C., & Ferguson, M. A. (2007). Sugar nucleotide pools of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. *Eukaryot Cell, 6*(8), 1450-1463. doi:10.1128/EC.00175-07

van den Hoogen, B. M., van Weeren, P. R., Lopes-Cardozo, M., van Golde, L. M., Barneveld, A., & van de Lest, C. H. (1998). A microtiter plate assay for the determination of uronic acids. *Anal Biochem, 257*(2), 107-111. doi:10.1006/abio.1997.2538

Zhao, X., Liu, N., Shang, N., Zeng, W., Ebert, B., Rautengarten, C., Zeng, Q. Y., Li, H., Chen, X., Beahan, C., Bacic, A., Heazlewood, J. L., & Wu, A. M. (2018). Three UDP-xylose transporters participate in xylan biosynthesis by conveying cytosolic UDP-xylose into the Golgi lumen in Arabidopsis. *J Exp Bot, 69*(5), 1125-1134. doi:10.1093/jxb/erx448

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FIGURE LEGENDS

Figure 1. Representative analysis of nucleotide sugars by LC-MS/MS from *Arabidopsis thaliana*.

(A) Extracted ion chromatogram of MRM using a standard mix containing 14 nucleotide sugars (20 pmol each) including UDP-Gal (5) and UDP-Glc (6).

(B) Extracted ion chromatogram of MRM using a standard mix containing 14 nucleotide sugars (20 pmol each) with UDP-Gal and UDP-Glc removed.

(C) Representative extracted ion chromatogram of MRM of nucleotide sugars extracted from Arabidopsis developing leaves.

(D) Representative extracted ion chromatogram of MRM of nucleotide sugars extracted from Arabidopsis developing leaves with UDP-Gal and UDP-Glc removed to enable visualization of all nucleotide sugars in the sample.

1, UDP-GalA; 2, UDP-GlcA; 3, UDP-Arap; 4, UDP-Rha; 5, UDP-Gal; 6, UDP-Glc; 7, UDP-Xyl; 8, UDP-GlcNAc/GalNAc; 9, UDP-Araf; 10, GDP-Man; 11, GDP-L-Gal; 12, GDP-Glc; 13, GDP-Fuc; 14, UDP-Api.

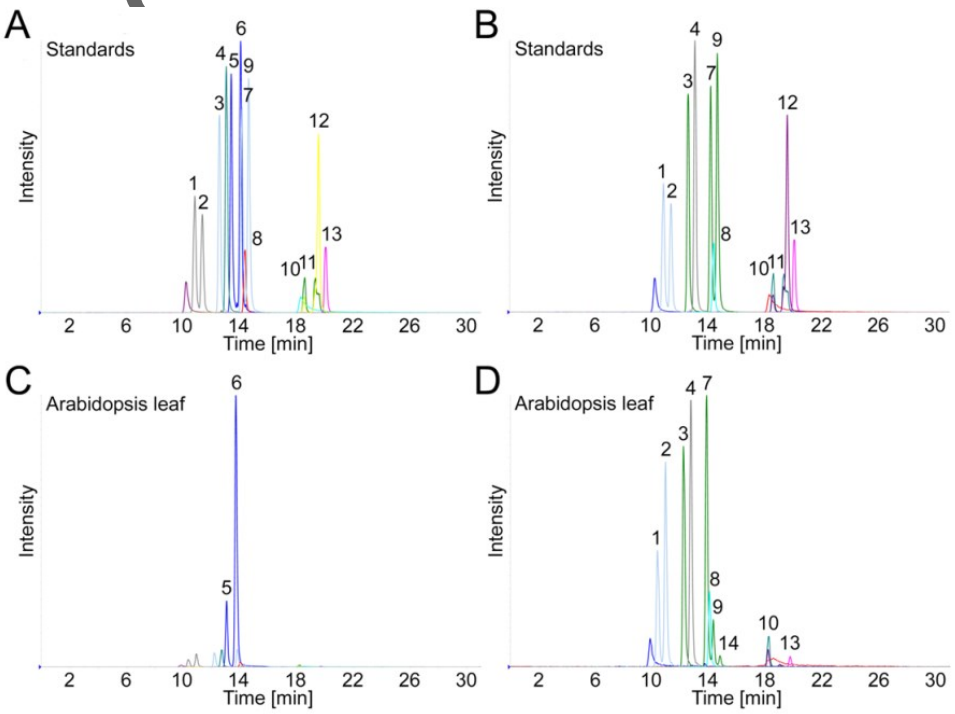


Figure 1

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Figure 2. Representative analysis of monosaccharides by HPAEC-PAD from *Arabidopsis thaliana*.

(A) Separation of monosaccharide standards by anion exchange with a CarboPac PA20. GalA, galacturonic acid; GlcA, glucuronic acid.

(B) Separation of monosaccharides from a processed sample from *Arabidopsis thaliana* using the standard protocol.

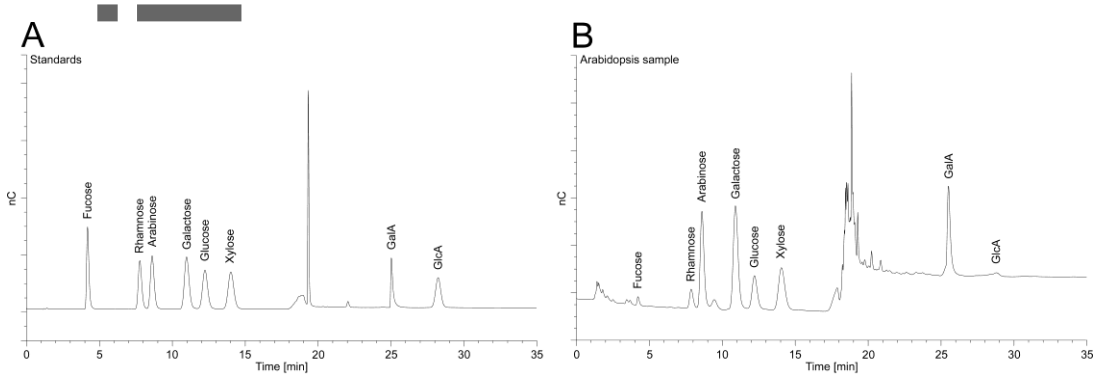


Figure 2

TABLES

Table 1. Buffer gradient conditions used for nucleotide sugar separation (Buffer A: 0.3 % formic acid pH 9.0; Buffer B: acetonitrile).

Total Time (min)	Flow Rate (µL / min)	A %	B %
1	50	95	5
1	50	95	5
20	50	75	25
22	50	20	80
24	50	20	80
26	50	95	5
32	50	95	5

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Table 2. Compound-dependent mass spectrometry parameters.

Q1 mass (Da)	Q3 mass (Da)	Compound	CE (volts)
565.0	323.0	UDP-Glc/Gal	-30
606.0	385.0	UDP-GlcNAc/GalNAc	-33
604.0	424.0	GDP-Man/Gal	-36
579.0	403.0	UDP-GalA/GlcA	-30
535.0	323.0	UDP-Arap/Xyl/Araf/Api	-30
588.0	442.0	GDP-Fuc	-30
549.0	323.0	UDP-Rha	-33
604.0	362.0	GDP-Glc	-36
588.0	346.0	ADP-Glc	-33

Table 3. Gradient employed for monosaccharide analysis (Buffer A: 0.01 N NaOH; Buffer B: 0.45 N NaOH).

Total Time (min)	Flow Rate (mL / min)	A %	B %
-10	0.4	100	0
0	0.4	100	0
15	0.4	100	0
15.1	0.4	0	100
35	0.4	0	100

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