

# Chapter 3

## Oligosaccharide Mass Profiling (OLIMP) of Cell Wall Polysaccharides by MALDI-TOF/MS

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### Abstract

In today's field of plant cell wall research, insights into the structure of wall components are obtained using many different techniques, ranging from spectroscopic and microscopic to chemical and biochemical. In this chapter, we describe one method: oligosaccharide mass profiling (OLIMP). Using OLIMP, we can harness the selective power of a specific wall hydrolase together with the speed and sensitivity of mass spectrometry to provide highly reproducible structural and compositional information about the wall molecule of interest.

**Key words:** Mass spectrometry, Matrix polysaccharides, Oligosaccharides, Glycosylhydrolases, Xyloglucan, Xylan, Pectins

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### 1. Introduction

Oligosaccharide mass profiling (OLIMP) is a useful, rapid, and sensitive tool to reveal structural properties of cell wall polysaccharides (1, 2). The sample preparation and analysis of wall polysaccharides by OLIMP is simple and quick, as shown in Fig. 1. It comprises three steps: (1) the preparation of wall material from plant tissues, (2) enzymatic release of specific oligosaccharides from the wall materials, followed by (3) mass spectrometry on the solubilised oligosaccharides. Based on the observed ions and the known specificity of the enzymes used, specific structures can be assigned to the ions. Hence, OLIMP is capable of giving valuable insights into the diversity and substitution patterns of wall polysaccharides. The analysis of oligosaccharide composition with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) is furthermore a method that does not involve strong acid or base treatments, which allows a

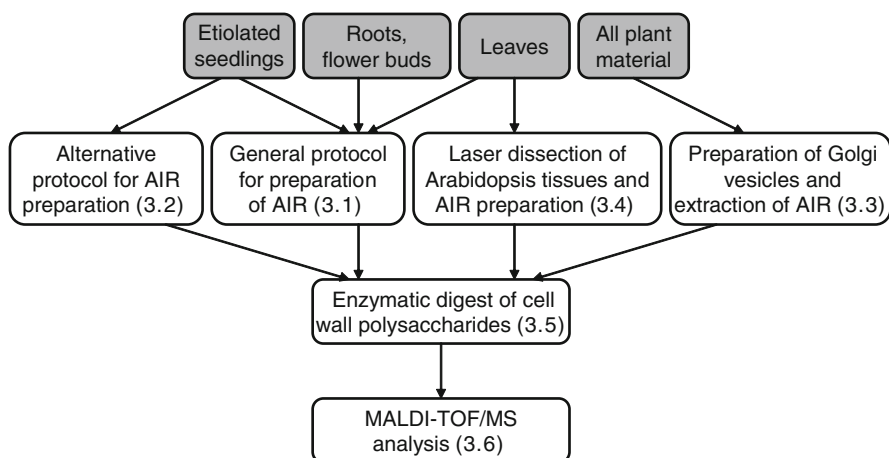


Fig. 1. Flow chart for OLIMP procedure using MALDI-TOF/MS. The first step encompasses preparation of AIR from plant material. AIR can be extracted from a variety of plant tissues, and the preparation method can be modified according to the wall material to be analysed (see [Subheadings 1–4](#)). Oligosaccharides can be released from AIR with enzymes specific for various polysaccharides (see [Subheading 5](#)) and subsequently analysed by MALDI-TOF/MS (see [Subheading 6](#)).

comprehensive analysis of the polysaccharide structure including *O*-acetylation and methylesterification substitution levels. OLIMP is very sensitive, requiring walls of only 5,000 cells for a complete analysis (3). In addition, a rapid analysis of the samples allows high-throughput experiments as shown by Lerouxel *et al.* (1) and Mouille *et al.* (4). However, structural isomers cannot be distinguished with OLIMP unless further structural information is obtained by mass fragmentation methods such as post-source decay (PSD). This procedure will generate several smaller fragments of a single oligosaccharide, which are further analysed by mass spectrometry (5, 6). This information can also be helpful for the analysis of polysaccharides for which limited structural information is available. Another limitation of OLIMP is its non-quantitative nature. Although the relative abundance of the oligosaccharide ions can be delineated from the spectra, the absolute amount present in the sample cannot, because the enzyme might not solubilise the polymer in its entirety from the wall (7), or because of ion suppression of the mass spectrometer at high concentrations or salt contaminants.

OLIMP can be used on a wide range of wall materials; it has been successfully used for the analysis of various *Arabidopsis* tissues such as leaves, roots, and flower buds. Because only very small samples are required for OLIMP analysis, it is possible to analyse specific cell types, cell compartments, or single etiolated seedlings (3).

OLIMP can be used to analyse a variety of wall polymers, limited only by the type of enzymes available (Fig. 2). A wide variety of commercially available hydrolases are suitable for use in OLIMP.

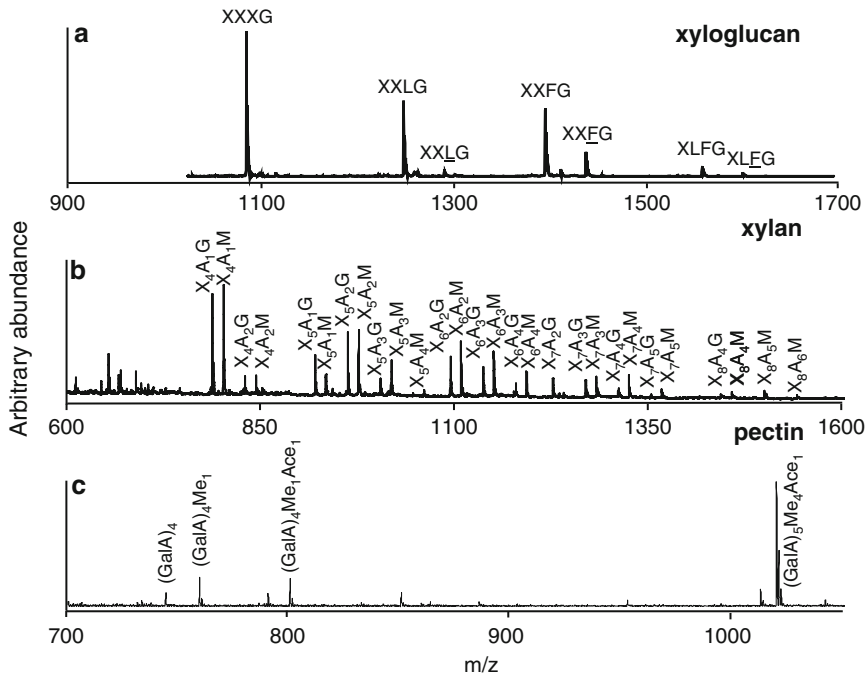


Fig. 2. OLIMP spectra of solubilised oligosaccharides (see Note 10). (a) Xyloglucan OLIMP spectrum from an *Arabidopsis* hypocotyl using a xyloglucan-specific endoglucanase. Nomenclature of oligosaccharide structures is according to Fry *et al.* (26), whereby the single letter indicates the reducing end sugar of the side-chain: e.g. *G* non-substituted glucosyl-residue; *X* xylosyl; *L* galactosyl; *F* fucosyl-residue. Underlined structures indicate galactosyl *O*-acetylation. (b) Xylan OLIMP spectrum derived from rice leaves using a xylanase M6 (Megazyme). Nomenclature for putative structures from Lee *et al.* (14).  $X_n$  number of pentoses, *G* glucuronic acid, and *M* methylated glucuronic acid. (c) Pectin OLIMP spectrum from *Arabidopsis* leaf walls using *endo*PG and pectin methylesterase.  $(GalA)_n$  number of galacturonic acid;  $Me_n$  number of methylester;  $Ac_n$  number of *O*-acetyl substituents.

For example, for xyloglucan OLIMP, *endo*- $\beta$ -(1–4)-glucanases are commonly used (8–12); these enzymes can be specific for xyloglucan (10–12). Similarly, for the analysis of xylan and pectin, *endo*- $\beta$ -(1–4)-xylanases (13, 14) and *endo*-polygalacturonases (*endo*PG) (3, 15) are utilised, respectively. For the analysis of the pectic polysaccharides, a pectin methylesterase (PME) is added to the digestion to increase the susceptibility of pectin to hydrolysis by *endo*PG (3, 15). A large number of other enzymes have been recently cloned and are available to the public (16) with a potential to expand the wall components that can be assessed by OLIMP. For instance, digestion of cell wall material with lichenase leads to the release of mixed-linkage glucan-derived oligosaccharides that can be analysed with MALDI-TOF/MS (17, 18). Furthermore, with slightly altered experimental set-ups, glycosyl-transferase and glycoside hydrolase activities can be assayed (19–23). For this purpose, purified enzymes or plant extracts are incubated with suitable substrates, and the product released is analysed by mass spectrometry.

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## 2. Materials

### **2.1. Preparation of Alcohol-Insoluble Residue (AIR) from Arabidopsis Plant Materials**

1. 2 mL Microcentrifuge tubes (Eppendorf, Hamburg, Germany) (see Note 1).
2. 70% (v/v) Aqueous ethanol.
3. 1:1 (v/v) Chloroform:methanol.
4. Acetone, pure.

### **2.2. Alternative Protocol for Preparation of AIR from Dark-Grown Seedlings**

1. 2 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany) (see Note 1).
2. Methanol, pure.
3. 1:1 (v/v) Chloroform:methanol.

### **2.3. Preparation of Golgi Vesicles and Extraction of AIR**

1. Sucrose buffers: 0.1 M  $\text{KH}_2\text{PO}_4$  buffer (pH 6.65), 10 mM  $\text{MgCl}_2$ , 8, 16, 33, 36, and 38% (w/v) sucrose.
2. STM buffer: 0.25 M sucrose, 1 mM  $\text{MgCl}_2$ , 10 mM Tris-HCl (pH 8.0).
3. Ethanol, pure.
4. 80% (v/v) Aqueous ethanol.
5. 1:1 (v/v) Chloroform:methanol.

### **2.4. Laser Dissection and AIR Extraction from Arabidopsis Leaf Tissue**

1. Ethanol dilution series for dehydration of leaf tissue: 20, 30, 40, 50, 60, 70, 80, 90% (v/v) aqueous ethanol, and pure ethanol.
2. Pure xylene and 1:1 ethanol/xylene (v/v) solution.
3. Paraffin solutions for embedding: 30, 50, and 80% paraffin in xylene (v/v) and pure paraffin.

### **2.5. Enzymatic Digest of Cell Wall Polysaccharides**

1. Enzymes: Xyloglucan-specific *endo*-glucanase (XEG) (EC 3.2.1.151, (24)), PME (Novozymes, Bagsvaerd, Denmark) *endo*-polygalacturonase M2 (*endo*PG), and xylanase M6 (Megazyme, Bray, Ireland).
2. 1 M ammonium formate stock solution (pH 4.5).
3. 4 M sodium hydroxide.
4. 1 M hydrochloric acid.
5. Float-A-Lyzer G2 dialysis devices (MWCO 8–10 kDa; Spectrum Labs, Greensboro, NC, USA).
6. 1 M sodium acetate stock solution (pH 6.0).

### **2.6. MALDI-TOF MS Analysis**

1. 2,5-dihydroxybenzoic acid 10 mg/mL in water (see Note 2).
2. Bio-Rad MSZ-501 (D) cation exchange resin beads (see Note 3).

### 3. Methods

#### **3.1. General Procedure for Preparation of Alcohol-Insoluble Residue (AIR) from Arabidopsis Plant Material**

1. 0.1–10 mg plant material is placed in a 2 mL microcentrifuge tube and snap frozen in liquid nitrogen (see Note 4).
2. The plant tissue is ground using a Retsch ball mixer mill MM301 for 1 min at 25 Hz with a single steel ball (3 mm).
3. After the tissue has been ground, the steel ball is removed from the tube via a magnet.
4. 1 mL 70% Ethanol is added to the ground material; the microcentrifuge tube is vortexed briefly and centrifuged for 10 min at  $20,000\times g$  in a tabletop centrifuge.
5. The supernatant is removed and the pellet is washed with 1 mL chloroform:methanol and vortexed briefly; the plant tissue is spun down for 10 min at  $20,000\times g$  in a tabletop centrifuge.
6. The supernatant is discarded and the pellet dried under a stream of air. The dried tissue can be directly used for enzyme digestion (see [Subheading 3.5](#); Note 5).

#### **3.2. Alternative Protocol for Preparation of AIR from Dark-Grown Arabidopsis Seedlings**

1. Approximately 10 seedlings (3–7 days old) are placed in a 2 mL microcentrifuge tube and 1 mL of methanol is added (see Note 4).
2. The tissue is homogenised using a Retsch ball mixer mill MM301 for 1 min at 25 Hz with a 5-mm stainless steel ball.
3. After the tissue has been homogenised, the metal ball is removed from the tube using a magnet.
4. The macerated material is centrifuged for 15 min at  $20,000\times g$  and the supernatant is discarded.
5. The pellet is resuspended in 1 mL chloroform:methanol and centrifuged for 15 min at  $20,000\times g$ .
6. The supernatant is discarded and the pellet air dried. Alternatively, the material can be dried under vacuum.
7. The dried tissue is ready for enzyme digestion (see [Subheading 3.5](#)).

#### **3.3. Preparation of Golgi Vesicles and Extraction of AIR**

The method described here is based on Muñoz *et al.* (25). Ultracentrifugation was performed using a Beckman Coulter SW 32 Ti rotor and 38.5 mL thin-wall rotor tubes. To prevent degradation, all preparation steps are performed in the cold room or on ice.

1. 5–10 mg of fresh plant material is transferred into a cold Petri dish and the material is finely chopped (1–2 mm sections) with a razor blade.

2. The chopped material is added to a cold mortar and 8 mL of cold 16% sucrose buffer is added.
3. The material is homogenised for 3 min with a pestle by rotation, applying only light pressure.
4. The suspension is filtered through a nylon mesh (diameter 30  $\mu\text{m}$ ) into a 50 mL Falcon tube, and the filtrate is centrifuged for 10 min at  $2,000\times g$  and  $4^{\circ}\text{C}$ .
5. The ultracentrifugation tube is prepared by adding 8 mL of cold 38% sucrose buffer.
6. Add the supernatant of step 4 on top of the sucrose layer, trying not to disturb the sucrose solution. This can be accomplished by transferring the supernatant with a Pasteur pipette onto the side of the centrifuge tube walls. The sample is centrifuged for 100 min at  $100,000\times g$ . The microsomal fraction will form as a milky layer on top of the 38% sucrose cushion with the microsome free cell extract layer above.
7. The microsome free cell extract (top layer) is removed with a Pasteur pipette.
8. 8 mL of cold 36% sucrose buffer is carefully added on top of the microsomal layer. Be careful not to disturb the layer. Then, 8 mL of cold 33% sucrose buffer is added on top of this layer, with care not to disturb the layer.
9. The tube is filled up to 3.5 mL with cold 8% sucrose buffer and centrifuged for 90 min at  $100,000\times g$ . During this centrifugation step, the microsomal fraction (on top of the 36% sucrose layer) will separate and float on top, resulting in an enriched ER and Golgi fraction.
10. The fraction above the 33% cushion is the Golgi-enriched fraction. This fraction is transferred with a Pasteur pipette into a new ultracentrifuge tube and the fraction is diluted 1:2 with water.
11. The diluted fraction is centrifuged for 60 min at  $100,000\times g$ .
12. The supernatant is discarded, and the pellet is washed twice with STM buffer to remove inorganic phosphate.
13. The pellet is resuspended in 1 mL STM buffer and stored at  $-80^{\circ}\text{C}$  until needed.
14. To prepare AIR for OLIMP using MALDI-TOF/MS analysis, a portion of the solution containing 100  $\mu\text{g}$  protein equivalent is used. The protein content can be determined (e.g. by Bradford assay).
15. Pure ethanol is added to the sample to reach a final concentration of 80% (v/v), and then the sample is filled up with 80% ethanol to a final volume of 1 mL.

16. The sample is centrifuged for 10 min at  $20,000\times g$  and the supernatant is discarded.
17. 1 mL of chloroform:methanol is added to the pellet and the pellet is vortexed gently.
18. The sample is centrifuged for 10 min at  $20,000\times g$ , the supernatant is discarded and the sample is air dried for 30 min.
19. The sample is now ready for enzyme digestion (see [Subheading 3.5](#)).

#### **3.4. Laser Dissection of Arabidopsis Tissues and Their AIR Preparation**

1. A leaf from a 5-week-old plant is harvested, directly transferred to a 2 mL microcentrifuge tube containing 1 mL 20% ethanol, and incubated at room temperature.
2. After 6 h, the 20% ethanol is removed and the procedure is repeated with increasing ethanol concentrations in 10% increments, each 6 h long, until incubation with pure ethanol is achieved. Incubation with pure ethanol is carried out twice. The pure ethanol is removed and ethanol:xylene (1:1, v/v) is added. The ethanol:xylene incubation is followed by three incubations with pure xylene. All incubations are carried out for at least 6 h with 1 mL of solvent.
3. After removal of pure xylene, the leaf tissue is incubated in 1 mL 30% paraffin xylene solution for 8 h at  $42^{\circ}\text{C}$ , followed by sequential incubation in 50 and 80% paraffin for 8 h at 52 and  $58^{\circ}\text{C}$ , respectively. Then, the tissue is transferred to pure paraffin and incubated for 7 h at  $64^{\circ}\text{C}$ . This step is repeated once.
4. The tissue is then placed in aluminium tray (3 mL) containing melted paraffin. After the paraffin solidifies at room temperature, the leaf is excised and mounted on a cutting block.
5. Sections of 20–40  $\mu\text{m}$  are made and placed on glass microscope slides. The paraffin is removed by adding xylene dropwise onto the tissue, and soaking up the solubilised paraffin with a paper tissue from the side.
6. Tissues of interest are dissected using a laser dissector, according to the guidelines of the manufacturer. To analyse oligosaccharide composition, the equivalent of approximately 5,000 cells must be collected.
7. The collected cells (fragments) are transferred to a 0.5 mL microcentrifuge tube, washed with 200  $\mu\text{L}$  xylene, and spun down at  $20,000\times g$  in a tabletop centrifuge. After decanting the supernatant, 200  $\mu\text{L}$  methanol:chloroform is added and the sample is briefly vortexed and centrifuged at  $20,000\times g$  in a tabletop centrifuge.
8. Discard the supernatant and air dry the pellet. The dried cell fragments can be directly used for enzyme digestion (see [Subheading 3.5](#); Note 5).

### 3.5. Enzymatic Digest of Cell Wall Polysaccharides

1. Depending on the polysaccharide to be investigated, the following digests can be carried out.
  - (a) *Xyloglucan-specific endoglucanase (XEG) digest for analysis of the cross-linking glycan xyloglucan*: 50  $\mu$ L of 50 mM ammonium formate (made with 2.5  $\mu$ L ammonium formate stock solution) containing 0.2 U XEG (1 U of XEG releases 1  $\mu$ mol xyloglucan oligosaccharides per min) are added to previously prepared AIR from plant material (see [Subheadings 3.1–3.4](#)) and incubated overnight (16 h) at 37°C and shaking at 120 rpm.
  - (b) *Pectin digest with endo-polygalacturonase M2 (endoPG)*: 50  $\mu$ L of 100 mM ammonium formate (prepared with 5  $\mu$ L ammonium formate stock solution) containing 0.15 U *endoPG* (1 U of *endoPG* releases 1  $\mu$ mol polygalacturonic acid oligosaccharides per min) and 0.08 U PME (1 U of PME releases 1  $\mu$ mol methanol per min) are added to previously prepared AIR from plant material and incubated overnight (16 h) at 37°C, shaking at 120 rpm.
  - (c) *Xylan digest*: 200  $\mu$ L of 4 M sodium hydroxide is added to the previously prepared AIR and incubated for 1 h at 37°C under shaking (500 rpm). The samples are neutralised by adding 800  $\mu$ L of 1 M hydrochloric acid and spun at 20,000 $\times g$  for 10 min in a tabletop centrifuge. The supernatant is transferred into a Float-A-Lyzer G2 dialysis device (MWCO 8–10 kDa), and dialysis is performed against ultrapure water with at least one change of water. After dialysis, the sample is transferred into a microcentrifuge tube and dried down in a speed vac. The dried material is digested overnight (16 h) in 200  $\mu$ L 50 mM sodium acetate (prepared with 10  $\mu$ L sodium acetate stock solution) containing 8 U xylanase M6 (1 U of xylanase releases 1  $\mu$ mol arabinoxylan oligosaccharides per min at 40°C and 120 rpm).
2. The digest is spun down for 10 min at 20,000 $\times g$  in a tabletop centrifuge, and the supernatant containing the released oligosaccharide fragments is transferred to a new microcentrifuge tube (see Note 6).

### 3.6. MALDI-TOF/MS Analysis

The analysis can be performed on a Kratos AXIMA CFR MALDI-TOF/MS instrument using a stainless steel MALDI target type DE1580TA (Kratos).

1. Prepare the MALDI-TOF/MS sample target by adding a layer of matrix. Use 2  $\mu$ L of 2,5-dihydroxybenzoic acid per sample, spot and dry the matrix under vacuum (see Note 7).
2. Transfer 10  $\mu$ L digest into a fresh 1.5 mL or 0.5 mL microcentrifuge tube.



3. Add approximately 5–10 cation exchange beads to each sample and incubate at room temperature for 15 min. (see Notes 3 and 8).
4. Transfer the desalted remaining liquid into a new microcentrifuge tube.
5. Spot as many samples as possible within a 3 min time frame (2  $\mu$ L of the desalted oligosaccharide sample solutions) onto the target plate mesas containing the dried matrix spot (step 1). This time limit ensures that the first spot is not fully air-dried. Wait another 2 min and dry the target under vacuum. Repeat these steps until all your samples are spotted (see Note 9).
6. The mono- and oligosaccharides are detected by the MS as their sodium  $[M+Na]^+$  and to a lesser degree potassium  $[M+K]^+$  adducts. Therefore, the molecular mass detected will be 23 or 39 m/z larger than that of your analyte.

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## 4. Notes

1. Testing different microcentrifuge brands and sizes showed that the Eppendorf 2 mL tubes are best suited to withstand milling with the ball mixer at very low temperatures. Of course, once in a while plastic pieces break or crack.
2. You can prepare a stock solution of the matrix chemical and store aliquots at  $-20^{\circ}\text{C}$  for as long as 6 months. Thaw the aliquot 30 min before use and vortex vigorously several times. Make sure that the matrix is completely dissolved. Other solvents than water can be used, such as acetone, acetonitrile, methanol, or chloroform. However, in our experience water is the most effective solvent to achieve good spectra of oligosaccharides.
3. The MSZ501 (D) resin comes as a mixture of anion and cation resin beads, and the two forms can be distinguished visually. We use only the cation exchange resin beads. The anion exchange beads are lighter than the cation beads and have a blue indicator dye irreversibly bound, whereas the cation exchange beads are of a golden brown colour. To prepare the cation exchange resin you have to separate the brown and blue beads. For this purpose, transfer several spoonfuls of Bio-Rad MSZ-501 (D) resin beads into a 50 mL Falcon tube and fill with water. Swirl the tube a few times and decant the lighter, blue cation beads into a second 50 mL Falcon tube. Repeat until you have reached separation. The beads can be stored at room temperature for 6 months in water. However, before use, wash the beads several times with fresh water.

4. In our experience, a wide range of plant material and tissues can be used, but there are some limitations. Tissues that work very well are 4-day-old dark-grown seedlings and flower buds, and leaves of 2–3-week-old plants. In many cases, weighing of material may not be necessary, and we have found that the following amounts work well: 1–20 four-day-old dark-grown seedlings, flower buds of 1 inflorescence, 1 leaf of a 2–3-week-old plant.
5. To dry samples more quickly, an additional washing step with 1 mL acetone is recommended.
6. For samples that contain very small amounts of material, it is advisable to dry the supernatant containing the solubilised oligosaccharides using a vacuum centrifuge followed by re-dissolving the pellet in 10  $\mu$ L water to increase the oligosaccharide concentration.
7. Prepare about 20 sample spots at a time with the matrix. Preparation of too many sample spots at a time can lead to longer drying times under vacuum, which can lead to poor quality of the matrix crystals. Prepare more sample spots than you have samples to provide some leeway in case some samples mix and you have to re-spot.
8. To add the ion exchange beads, use a small spoon-shaped spatula to hold a small amount of beads. With a second tapered spatula, move a small quantity of beads over the edge of the spoon. The beads will stick to the tapered spatula. Tap the tapered spatula on the rim of the microcentrifuge tube to transfer the beads into the tube.
9. As a quality control and to simplify troubleshooting, it is advisable to include a sample that is known to produce good spectra on each target that you prepare. If possible, use a previous sample derived from plant material rather than a pure standard.
10. Ionised oligosaccharides will be analysed as their sodium or potassium adducts; therefore, you should not expect to find masses that exactly match the predicted oligosaccharide masses, but with an additional +23 or +39 m/z.

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