Please cite this article as: C■t■lin and Markus, (2016). Analysis of Monosaccharides in Total Mucilage Extractable from *Arabidopsis* Seeds, *Bio-protocol* 6 (9): e1801. DOI: 10.21769/BioProtoc.1801.

bio-protocol http://www.bio-protocol.org/e1801 Vol 6, Iss 9, May 5, 2016

Analysis of Monosaccharides in Total Mucilage Extractable from *Arabidopsis* Seeds

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[Abstract] The *Arabidopsis thaliana* seed coat epidermis produces copious amounts of mucilage polysaccharides (Haughn and Western, 2012). Characterization of mucilage mutants has identified novel genes required for cell wall biosynthesis and modification (North *et al*., 2014). The biochemical analysis of seed mucilage is essential to evaluate how different mutations affect cell wall structure (Voiniciuc *et al*., 2015c). Here we describe a robust method to screen the monosaccharide composition of *Arabidopsis* seed mucilage using ion chromatography (IC). Mucilage from up to 48 samples can be extracted and prepared for IC analysis within 24 h (only 4 h hands-on). Furthermore, this protocol enables fast separation (31 min per sample), automatic detection and quantification of both neutral and acidic sugars.

Materials and Reagents

1. Sterile Petri dishes
2. Filter paper (MACHEREY-NAGEL GmbH & Co., catalog number: 434009 or similar types)
3. 3M Micropore paper tape (VWR International, catalog number: 115-8172)
4. Square (7 x 7 cm) or round (Ø 5 cm; 35 multi-well insets) plastic pots, and trays
5. Peat-sand-pumice substrate (SoMi 513 Dachstauden) (HAWITA GRUPPE GmbH)
6. ARACON cone and tube (Betatech bvba)
7. Large (sandwich-style) brown paper bags
8. Small white paper bags (Baumann Saatzuchtbedarf, catalog number: 3.065.002, or similar style)
9. 15 ml Falcon tubes (VWR International, catalog number: 734-0452)
10. 2 ml Eppendorf safe-lock tubes (VWR International, catalog number: 211-2165)
11. 2 ml screw-cap tubes (VWR International, catalog number: 211-0093)
12. Chromatography vials with inserts (VWR International, catalog number: 548-0120)
13. Snap cap for chromatography vials (VWR International, catalog number: 548-1151)
14. Manual pipettes tips
15. *Arabidopsis thaliana* seeds
16. Murashige and Skoog (MS) basal salts (Sigma-Aldrich, catalog number: M5519-10L)
17. Agar (Carl Roth GmbH + Co, catalog number: 4807.2)

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18. D-(-)-Ribose (Rib) (Sigma-Aldrich, catalog number: R7500-5 G)
19. L-(+)-Arabinose (Ara) (Sigma-Aldrich, catalog number: A3256-25 G)
20. L-(-)-Fucose (Fuc) (Sigma-Aldrich, catalog number: F2252-5 G)
21. D-(+)-Galactose (Gal) (Sigma-Aldrich, catalog number: G0750-25 G)
22. D-(+)-Galacturonic acid monohydrate (GalA) (Sigma-Aldrich, catalog number: 48280-5G-F)
23. D-(+)-Glucose (Glc) (Sigma-Aldrich, catalog number: G8270-100 G)
24. D-Glucuronic acid (GlcA) (Sigma-Aldrich, catalog number: G5269-10 G)
25. D-(+)-Mannose (Man) (Sigma-Aldrich, catalog number: M8574-25 G)
26. L-Rhamnose monohydrate (Rha) (Sigma-Aldrich, catalog number: R3875-5 G)
27. D-(+)-Xylose (Xyl) (Sigma-Aldrich, catalog number: X3877-25 G)
28. Ultrapure water (18.2 MΩ cm at 25 °C)
29. Sodium hydroxide (NaOH) (VWR International, catalog number: BAKR3727.2500)
30. Trifluoroacetic acid (TFA) (Carl Roth GmbH + Co., catalog number: 6957.1)
31. ½ MS plates (see Recipes)
32. Sugar standard stocks (see Recipes)
33. 9-Sugar mix (see Recipes)
34. 2 M TFA (see Recipes)
35. 10 mM NaOH (see Recipes)
36. 733 mM NaOH (see Recipes)

Equipment

1. Laminar flow clean bench
2. Growth chamber (Johnson Controls)
3. Autoclave
4. Water purification system (Milli-Q or similar style)
5. Manual pipettes (Eppendorf AG, Research plus and Repeater Plus style)
6. Analytical balance (Mettler-Toledo, model: XSE205DU)
7. Ball mill (Retsch GmbH, catalog number: MM400)
8. Two 24 TissueLyser adapters for ball mill (QIAGEN, catalog number: 69982)
9. Safety glasses
10. Lab coat
11. Chemical resistant gloves (Honeywell, Dermatril, catalog number: 740, or similar style)
12. Fume hood
13. Sample concentrator (Bibby Scientific Limited, Techne, catalog number: FSC400D), equipped with
    a. 127 mm needles (Bibby Scientific Limited, Techne, catalog number: F7210)
    b. A Dri-block heater (Bibby Scientific Limited, Techne, catalog number: DB200/3)
    c. Three aluminium blocks (Bibby Scientific Limited, Techne, catalog number: F3505)

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   d. Connected to a central air supply, in a fume hood
14. Ice machine
15. Ion chromatography (IC): Dionex DX-600 system equipped with
   a. AS50 autosampler
   b. GP50 gradient Pump
   c. ED50 electrochemical detector
   d. CarboPac PA20 guard column (Thermo Fisher Scientific, Dionex Softron, catalog number: 060144)
   e. CarboPac PA20 analytical column (Thermo Fisher Scientific, Dionex Softron, catalog number: 60142)
16. Vortex mixer (Scientific Industries Inc., model: Vortex-Genie 2, or similar style)
17. Benchtop centrifuge (compatible with 2 ml tubes)
18. Racks with lids for 2 ml tubes (VWR International, catalog number: 211-0215)
19. Boxes with lids for vials (neoLab, catalog number: 2-2580)
20. Fine-tipped forceps (VWR International; catalog number: 232-0107, or similar types)
21. Serological pipettes (Thermo Fisher Scientific, Nunc-type or similar style)
22. 2 L volumetric flask
23. Helium gas tank

Software

1. Chromeleon 6.8 chromatography data system software (Thermo Fisher Scientific, Dionex)
2. Microsoft Excel with the Real Statistics Resource Pack (http://www.real-statistics.com/)

Procedure

A. Plant growth, seed harvest and storage

The manner in which seeds are produced, harvested and stored can impact the content and composition of mucilage (see recent review, Voiniciuc *et al*., 2015c). To facilitate comparisons between different genotypes, it is necessary to always grow plants under the same conditions. We recommend the following procedures, which yield high quality seeds and consistent mucilage chemotypes (Voiniciuc *et al*., 2015a; Voiniciuc *et al*., 2015b; Voiniciuc *et al*., 2015c).

1. Using filter paper, sprinkle seeds on ½ MS plates, and seal plates with 3M Micropore Paper Tape. Germinate seeds under continuous light.
2. After five to seven days (when the cotyledons fully open), transfer seedlings using forceps to individual pots filled with wet peat-sand-pumice substrate. Cover with plastic dome for one week.

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3. Grow plants under constant light (~170 µE m^{-2} s^{-1}), temperature (20 °C) and relative humidity (60%), watering every two to three days as needed.
4. Before flowering, cover each plant with an ARACON cone and tube to prevent cross-pollination and seed dispersal. Remove any branches that cannot be contained within the ARACON tubes.
5. When most of the siliques turn yellow (almost 60 days after step A1), cut plants from their base and harvest mature seeds by shaking each plant into large brown paper bags for 10 sec.

Note: To preserve the biological variation, use one bag per plant and do not pool seeds.

6. Empty the contents of each brown bag onto a clean piece of filter paper and carefully remove the vegetative material using forceps. Transfer only seeds to small white paper bags.

B. Total mucilage extraction

1. Prepare a spreadsheet for each experiment, with the relevant information (*e.g.,* Sample #; Seed Weight; Seed Bag; Genotype). Up to 48 unknown samples can be processed at once. To simplify labeling and later processing, samples should be assigned a letter (*e.g.,* experiment A) and a two-digit number, making sure that single digit numbers are preceded by a zero (*e.g.,* A01 to A48).
2. Pre-label the sides of 2 ml Safe Lock tubes with the sample numbers. Use an analytical balance to add 4-6 mg seeds to each tube and record precise weight in the spreadsheet.
3. Prepare a dilution series of 9-Sugar mix standards by adding the volumes listed in Table 1 to 2 ml Screw-Cap Tubes.

Table 1. 9-Sugar mix (10 mg/ml) dilution series for monosaccharide quantification

Label	S000	S001	S002	S005	S010	S025	S050	S075	S100	S125
µl	0	1	2	5	10	25	50	75	100	125
µg	0	1	2	5	10	25	50	75	100	125

4. Prepare enough 30 µg/ml Rib (Internal Standard) to add 1 ml to all samples and sugar standards in one experiment. For a typical experiment with 48 samples and 10 standards, add 180 µl of the 10 mg/ml Rib and fill up to 60 ml with ultrapure water.
5. Using a repeater pipette, add 1 ml of Internal Standard to all samples and standards.
6. Perform the total mucilage extraction by shaking the seed-containing tubes for 15 min at 30 Hz in a ball mill using two 24 Tissue Lyser Adapters, at room temperature (~24 °C).
7. Rotate block 180° and shake for an additional 15 min at 30 Hz to complete the total mucilage extraction. This detaches all mucilage from wild-type seeds (Figure 1).

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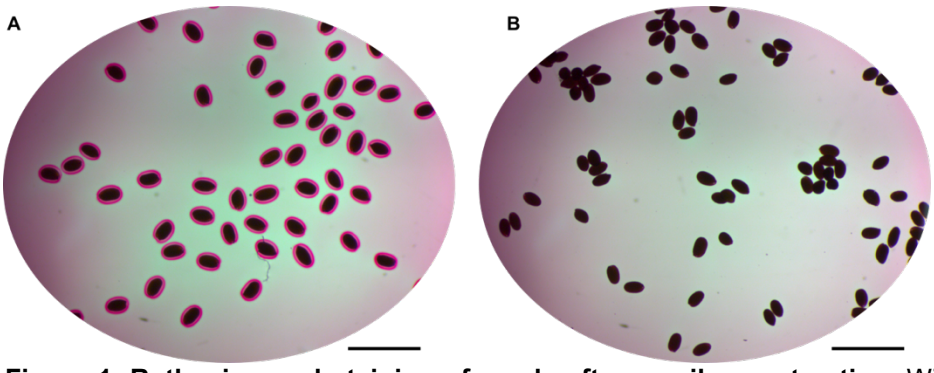

Figure 1. Ruthenium red staining of seeds after mucilage extraction. Wild-type (Col-0) seeds stained in a 24-well plate as previously described (Voiniciuc *et al.*, 2015b), after gentle shaking in water (A) or the total mucilage extraction (B). Bars = 2 mm.

8. Let seeds settle for at least 30 sec. For each sample, transfer 800 µl of the supernatant to a 2 ml Screw-Cap tube, pre-labeled on its side.
   Note: Do not transfer any seeds, and do not cap the tubes at this point.
9. Dry samples and standards under air flow at 45 °C using the sample concentrator. Process all tubes identically from this point onwards.

C. Hydrolysis of matrix polysaccharides
   1. Using a repeater pipette, add 300 µl of 2 M TFA to all tubes.
      Note: Perform TFA work in a fume hood, with the appropriate personal protective equipment.
   2. Cap each tube tightly, and vortex for 3 sec.
   3. Transfer tubes to the Techne Dri-block (preheated to 120 °C) and incubate for 60 min.
   4. Cool heating blocks and tubes on ice. Centrifuge tubes for 30 sec at maximum speed.
   5. Uncap tubes, and evaporate TFA under air flow at 45 °C using sample concentrator.
      Note: Keep caps on a clean paper towel, in the correct order.

D. Final elution of samples
   1. Add 600 µl of autoclaved, ultrapure water to all tubes using a repeater pipette. Vortex mix for 3 sec.
      Note: If using medium shaking intensity and exercising caution, the tubes do not need to be capped for this step because the solution will not spill over.
   2. Transfer 150 µl from each tube to pre-labeled IC vials with inserts. Seal vials with caps.
   3. Place tubes in the autosampler of a Dionex DX-600 system in ascending order, but randomize (*e.g.,* using Microsoft Excel) the injection order of the sample and standards.

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E. Separation and quantification of monosaccharides

1. Separate and quantify monosaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using CarboPac PA20 Guard and Analytical columns, at 40 °C and a constant flow rate of 0.4 ml/min.
2. Ensure that sufficient volumes of the three IC eluents (ultrapure water, 10 mM NaOH, and 733 mM NaOH; Figure 2) are available to run all samples and standards in an experimental batch.

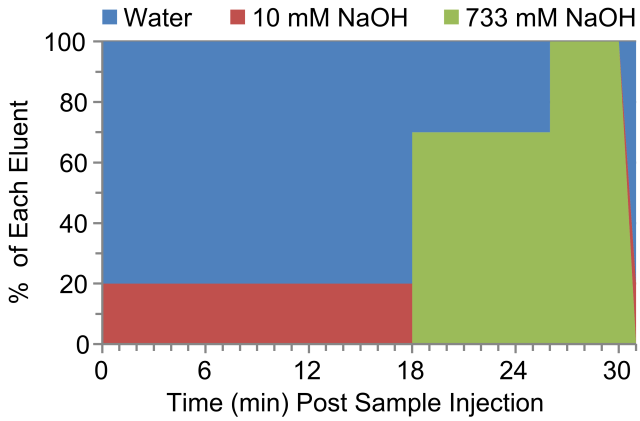

Figure 2. The proportion of eluents pumped after sample injection

3. Equilibrate the columns with 2 mM NaOH (80% water, 20% 10 mM NaOH) for 10 min.
4. Inject 10 µl of each sample.
5. Separate neutral sugars with 2 mM NaOH over the course of 18 min (Figure 2).
6. Afterwards, pump 513 mM NaOH for 7.5 min to separate uronic acids (Figure 2).
7. Finally, rinse the column with 733 mM NaOH for 4 min (Figure 2).

   Note: Repeat the methods in steps E3-7 for each sample and standard (Figure 2).
8. Automatically annotate the monosaccharide peaks by configuring the Peak Table (Table 2) and the Detection Parameters (Table 3) in the Chromeleon Chromatography Data System software. These parameters work correctly for most standards and samples (Figure 3), and require only minor adjustments if the retention times shifts slightly between experiments.

   Note: Depending on your individual set-up and column age, you might have to adjust the flow rate or the NaOH concentration during the neutral sugar elution. Do not decrease NaOH concentration too much as this has an effect on sensitivity.

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Table 2. Chromeleon 6.8 Peak Table to identify and calibrate monosaccharides

Peak name	Ret. time (min)	Window	Standard	Int. type	Cal. type	Peak type
Fuc	4.50	0.270 AN	Internal rib	Area	Quad	B-M-B
Rha	8.00	0.400 AN	Internal rib	Area	Quad	B-M
Ara	8.70	0.400 AN	Internal rib	Area	Quad	M-B
Gal	11.00	0.800 AF	Internal rib	Area	Quad	B-M-B
Glc	12.25	0.800 AN	Internal rib	Area	Quad	B-M-B
Xyl	14.40	1.000 AF	Internal rib	Area	Quad	B-M
Man	15.00	1.000 AG	Internal rib	Area	Quad	M-B
Rib	18.70	1.300 AN	ISTD internal	Area	Lin	B-M-B
GalA	26.26	0.800 AG	Internal rib	Area	Lin	B-M-B
GlcA	28.40	0.800 AG	Internal rib	Area	Lin	B-M-B

Table 3. Chromeleon 6.8 Detection Parameters to annotate monosaccharide peaks

Time	Param. name	Param. value	Channel
0.00	Inhibit integration	On	All channels
3.60	Inhibit integration	Off	All channels
4.10	Minimum area	0.5	All channels
4.75	Minimum area	1	All channels
5.00	Inhibit integration	On	All channels
6.60	Inhibit integration	Off	All channels
6.60	Lock baseline	On	All channels
7.25	Minimum area	0.5	All channels
9.80	Lock baseline	Off	All channels
9.80	Inhibit integration	On	All channels
10.20	Inhibit integration	Off	All channels
10.40	Lock baseline	On	All channels
12.70	Inhibit integration	On	All channels
12.80	Lock baseline	Off	All channels
13.10	Inhibit integration	Off	All channels
14.00	Minimum area	0.25	All channels
14.00	Lock baseline	On	All channels
16.00	Lock baseline	Off	All channels
18.00	Minimum area	10	All channels
20.50	Inhibit integration	On	All channels
25.50	Inhibit integration	Off	All channels
25.60	Minimum area	0.5	All channels
27.00	Inhibit integration	On	All channels
28.20	Inhibit integration	Off	All channels
28.20	Minimum area	0.5	All channels
29.00	Inhibit integration	On	All channels

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9. Inspect chromatograms in Chromeleon to ensure that all peaks are correctly labeled. Manually annotate the peaks of Ara and Man if necessary, which overlap with more abundant sugars.
10. By defining the standards and their concentrations in Chromeleon (Tables 1 and 2), calibration plots are automatically generated for all monosaccharides, normalized to Rib (Internal Standard).

    Note: Inspect the calibration plots and disable significant outliers if necessary.
11. The amounts of all monosaccharides (expressed in µg) in a sample are automatically calculated based on the calibration plots and can be exported from Chromeleon for further calculations.

    Note: Fuc is detected in trace amounts in total mucilage extracts from Arabidopsis seeds (Figure 3; Voiniciuc et al., 2015c), while GlcA is generally not detected in these samples.

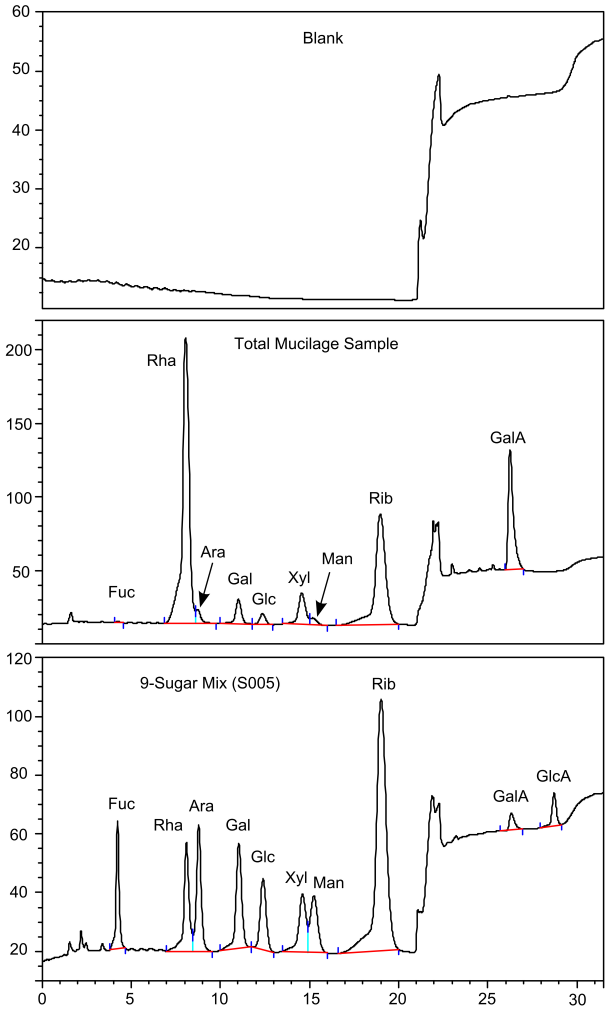

Figure 3. Example chromatograms exported from the Chromeleon software. The vertical axes represent detected signals (nC). The horizontal axes show time post-injection (min).

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F. Final calculations and statistical analysis

Further calculations are performed in Microsoft Excel. The monosaccharide composition of total mucilage extracts can be expressed as absolute (Figure 4A), or relative amounts (Figure 4B).

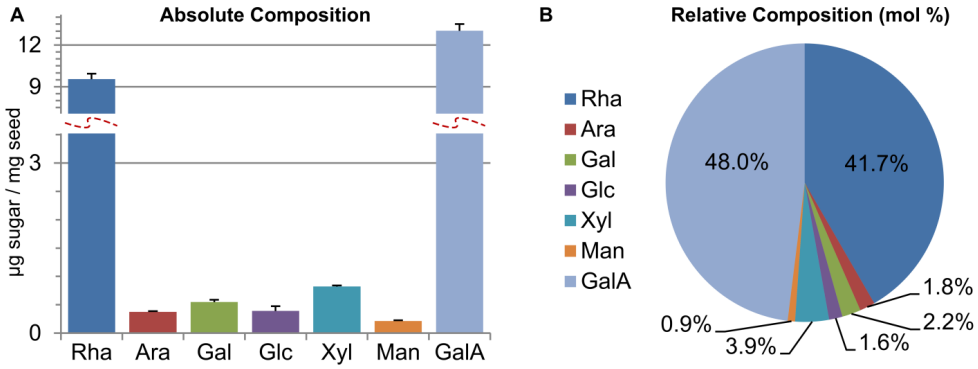

Figure 4. Composition of total mucilage extracts from the Col-0 wild type. The data show means of three biological replicates. The error bars in (A) represent standard deviations.

1. Sugar amounts (expressed in µg) can be divided by the amount of seeds (expressed in mg) used for each mucilage extraction, to calculate the absolute composition (µg sugar/mg seed).

   Note: The total amount of mucilage per mg seed is the sum of all sugar values in a sample.

2. Absolute monosaccharide levels can also be expressed as the number of molecules normalized to the amount of seeds used. To calculate the nmol sugar per mg seed, divide the value obtained in step F1 by the molecular mass of the respective sugar (Table 4), and multiply the result by 1,000.

Table 4. Molar mass of the monosaccharides for final calculations

Sugar	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA
Mass	164.16	164.16	150.13	180.16	180.16	150.13	180.16	194.14	194.14

3. Relative monosaccharide composition in mucilage can be expressed as a molar percentage (mol%), and equals the amount of each monomer divided by the sum of all sugars (both from step F2).
4. Statistical analyses to compare the mucilage composition of wild-type and mutants can be performed in Excel. Significant changes in a particular monosaccharide (expressed either in absolute or relative amounts) between two genotypes with multiple biological replicates can be identified using the built-in T.TEST function. When many mutants (and their various sugars components) are compared to wild-type, conditional

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formatting (*e.g.,* highlight cells with *P*-value < 0.05) can be used to quickly reveal which components are significantly altered. Two-Factor Analysis of Variance (ANOVA) can also be performed in Excel with the Real Statistics Resource Pack (http://www.real-statistics.com/) to evaluate how mucilage composition is affected by two independent mutations (via the analysis of wild-type, single and double mutant samples).

Recipes

1. ½ MS plates
   For 500 ml solution (yields around 25 plates), mix 1.08 g MS basal salts, 3.5 g agar, and water.
   Autoclave, and pour media (while still warm) into sterile Petri dishes on a clean bench.
   When kept in a sterile bag, plates can be stored at 4 °C for at least 6 months.
2. Sugar standard stocks (10 mg/ml)
   For each monosaccharide except GalA and Rha, prepare individual stocks by dissolving 100 mg sugar in 10 ml of autoclaved, ultrapure water in a sterile 15 ml Falcon tube.
   Since they are sold in monohydrate forms, use 109 mg of GalA and 111 mg of Rha rather than the standard 100 mg.
   Aliquot the Rib stock into 2 ml Safe Lock tubes since it is frequently used.
   Store all stocks at -20 °C.
3. 9-Sugar mix (1 mg/ml)
   For a 10 ml solution, mix 1 ml of water with 1 ml of Fuc, Rha, Ara, Gal, Glc, Xyl, Man, GalA, and GlcA (all 10 mg/ml stocks).
   Aliquot the 9-Sugar mix into 2 ml Safe Lock tubes and stored at -20 °C.
4. 2 M Trifluoroacetic acid (TFA)
   Prepare 500 ml of a 2 M TFA solution by slowly adding 77 ml of TFA (12.98 M) to 423 ml of ultrapure water
   Note: Perform TFA work in a fume hood, with the appropriate personal protective equipment.
5. 10 mM NaOH (IC eluent)
   Use a volumetric flask to transfer 2 L of ultrapure water to the appropriate eluent container
   Add 1,040 µl of 50% (w/v) NaOH using a 1 ml serological pipette
   Degas eluent using helium for 5 min
6. 733 mM NaOH (IC eluent)
   Use a volumetric flask to transfer 2 L of ultrapure water to the appropriate eluent container
   Add a total of 80 ml of 50% (w/v) NaOH using a 50 ml serological pipette

Degas eluent using helium for 5 min

Acknowledgments

The methods described here were derived from (Voiniciuc *et al*., 2015c), and were employed by (Voiniciuc *et al*., 2015a; Voiniciuc *et al*., 2015b). We thank Björn Usadel for helpful comments on the protocol. This work was supported by the Natural Sciences and Engineering Research Council of Canada (PGS-D3 grant to C. V.). Author Contributions: C. V. and M. G. developed the method. C. V. wrote the protocol.

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