Determination of Neutral Sugars by Gas Chromatography of their Alditol Acetates

The neutral monosaccharide composition of cell walls can be determined by first hydrolysing the polysaccharides to their constituent monosaccharides with strong acid and then converting them to alditol acetates by reduction with sodium borohydride to the corresponding alditol, followed by acetylation of the hydroxyls on each alditol. The resultant alditol acetates are volatile and can be identified and quantified by gas chromatography. Thus, a gas chromatograph is essential for this procedure. The amounts of the individual monosaccharides in the cell walls can be determined and then summed to give the total neutral sugar content. While this procedure does not identify the parent polysaccharides, some inferences can be made since the types of polysaccharides in cell walls are well known.

Several procedures have been used to hydrolyze polysaccharides in cell walls and cell wall fractions. For example, the noncellulosic polysaccharides can be hydrolyzed using 1 M sulfuric acid for 2 to 3 hr at 100°C (Selvendran and Ryden, 1990). One of the simplest procedures is that of Albersheim et al. (1967) in which hydrolysis of the noncellulosic polysaccharides is achieved by incubating in 2 M trifluoroacetic acid (TFA) at 121°C for 1 hr. The advantage of the TFA procedure is that it is quick and the acid can be removed by evaporation in a gentle stream of air or nitrogen. However, neither the 1 M sulfuric acid or TFA procedures hydrolyze cellulose. Hydrolysis of cellulose can be achieved by an initial dispersion in 72% (w/w) sulfuric acid (Saeman et al., 1963; Selvendran et al., 1979; Fry, 1988; Harris et al., 1988; Selvendran and Ryden, 1990) followed by hydrolysis in 1 M sulfuric acid.

Determination of the monosaccharide composition by gas chromatography of alditol acetates is commonly used for cell wall analyses because the procedure gives a single peak for each sugar. An alternative is to prepare trimethylsilyl (TMS) derivatives. However, these are easily hydrolyzed by moisture in the atmosphere and therefore should be analyzed in the gas chromatograph immediately, whereas alditol acetates are relatively more stable and could be rerun on the gas chromatograph the following day if required.

This unit provides two protocols (see Basic Protocols 1 and 2) and an alternative procedure (see Alternate Protocol) for estimating neutral sugars. Differences among these protocols are in the method of hydrolysis. The choice of method will depend on the type of information required. Basic Protocol 1 is used when only the amounts of noncellulosic neutral sugars are required. When both the noncellulosic neutral sugars and the cellulose content are required, then Basic Protocol 2 or Alternate Protocol 1 are used.

DETERMINATION OF NONCELLULOSIC NEUTRAL SUGARS BY TRIFLUOROACETIC ACID (TFA) HYDROLYSIS

BASIC PROTOCOL 1

Hydrolysis with TFA is rapid and gives yields of monosaccharides that are equal to those achieved by mineral acids (Albersheim et al., 1967). The great advantage of using TFA is that it can be readily removed by evaporation, i.e., under a gentle stream of clean nitrogen or air.

The preferred method for reduction and acetylation is that of Blakeney et al. (1983). Their innovation was to use DMSO as the solvent for sodium borohydride and to use 1-methylimidazole as the catalyst for the quantitative acetylation of alditols in the presence of borate. Methods commonly used to prepare alditol acetates prior to this had incorporated steps in which the borate was removed by repeated evaporations with methanol, which was slow and tedious.

Contributed by Laurence D. Melton and Bronwen G. Smith
Current Protocols in Food Analytical Chemistry (2001) E3.2.1-E3.2.13

Materials

Dried cell walls or cell-wall fractions (*UNIT E3.1*)
2 M trifluoroacetic acid (TFA; see recipe)
Nitrogen or argon gas
20 mg/ml allose
13-sugar standard (see recipe)
15 M (concentrated) ammonia (analytical grade)
0.5 M sodium borohydride in DMSO (freshly prepared; see recipe)
18 M acetic acid (glacial, analytical grade)
1-methylimidazole
Acetic anhydride
Dichloromethane (DCM; high quality)
Helium (zero grade; carrier gas for GC)

15-ml borosilicate glass tubes and caps with Teflon-lined insert
5-ml glass syringe
Swinney stainless steel 13-mm filter unit (Millipore)
0.22-µm polytetrafluoroethylene (PTFE) filters (Millipore) or glass fiber filters
40°C water bath
Gas chromatograph (e.g., Model HP 6890, Hewlett Packard) fitted with a flame ionization detector and a dedicated cool on-column capillary inlet
BPX-70 open tubular fused silica column (25 m long, 0.33 i.d., and 0.25-µm film thickness; SGE Chromatography Products)
Hewlett Packard ChemStation software

Hydrolyze cell walls or cell-wall fractions with TFA

1. Place 5 mg of dried cell walls or cell-wall fractions (*UNIT E3.1*) into a scrupulously clean (see Critical Parameters) borosilicate glass tube (in duplicate). Record the exact weight of cell walls or fractions.

2. Add 0.5 ml of 2 M TFA to each sample. Set up a tube containing only 0.5 ml of TFA as a control and take this through all the following steps.

   CAUTION: *TFA is a strong acid—wear goggles, gloves, and protective clothing.*

   A clean glass pipet is recommended to deliver the acid, as accidental splashes and fumes from acids can cause corrosion of expensive automatic pipets.

3. Flush tubes well with argon or nitrogen gas (to remove all traces of air) and cap tightly using a screw cap with Teflon-lined insert. Vortex to mix, taking care not to spread the solid material above the level of the liquid.

4. Incubate for 60 min at 121°C. Allow to cool.

   A heating block may be used for the incubations.

5. Add 25 µl of 20 mg/ml allose (internal standard). Vortex to mix.

6. Filter hydrolysate using glass syringe fitted with a Swinney stainless steel 13-mm filter unit and a 0.22-µm PTFE filter into a clean borosilicate glass tube. After each sample has been filtered, discard the PTFE filter and wash the syringe and filter unit 6 times in Milli-Q-purified water to ensure there is no carryover from one sample to another.

   Alternatively, glass-fiber filters can be used, but they should be heated to 500°C in an oven for 1 hr to remove all traces of contaminating carbohydrates prior to use.

7. Evaporate filtrate to dryness in a gentle stream of air or nitrogen gas.

   Use new, washed Pasteur pipets (or needles) to ensure there are no traces of debris, especially from cardboard boxes in which the pipets are transported.

Reduce monosaccharides to corresponding alditols

8. Take dried hydrolysates and add 100 µl Milli-Q-purified water to each tube.
9. Set up two clean tubes as controls. Add 100 µl Milli-Q water (water control) to one tube and 100 µl of the 13-sugar standard to the other tube.

   Allow the standard to thaw and mix well before use.

10. Add 20 µl of 15 M ammonia to each tube.

    Do this step in a fume hood.

11. Add 1 ml of 0.5 M sodium borohydride in DMSO to each tube. Cap the tubes and vortex to mix.
12. Incubate for 90 min at 40°C.

    Use either a heating block or a water bath for incubation.

13. Add 100 µl of 18 M acetic acid to each tube. Vortex to mix.

    Do this step in a fume hood. The mixture should effervesce as the sodium borohydride is destroyed.

Acetylate the alditols

14. Add 200 µl of 1-methylimidazole.

    The 1-methylimidazole is stored over silica gel at 4°C. Allow it to come to room temperature before opening the bottle. Decant a small volume into a vial and use this rather than putting pipet into bottle.

15. Add 2 ml acetic anhydride to each tube and vortex to mix.
16. Incubate for 10 min at room temperature.
17. Add 5 ml Milli-Q-purified water to each tube to destroy the excess acetic anhydride. Vortex to mix.
18. Incubate for 10 min at room temperature or until cool.
19. Add 1 ml dichloromethane (DCM) to extract the alditol acetates. Vortex to mix (do not cap the tubes, as the glue holding Teflon liners may be soluble in DCM). Allow the phases to separate and transfer the lower DCM phase to a clean borosilicate glass tube using a Pasteur pipet.

    Do this by first expelling the air from the Pasteur pipet and then lowering it through the aqueous phase and into the DCM phase. Take care to ensure that very little or preferably none of the aqueous phase enters the pipet. The DCM is volatile and tends to squirt out of the pipet; hold both tubes together in one hand so that it is possible to transfer the DCM quickly without losses.

20. Add another 1 ml DCM to the original solution (aqueous phase) and repeat the extraction process.

    If necessary, centrifuge at slow speed for ~2 min to separate the phases. Remove the lower DCM phase and combine the DCM extracts.

21. Add 4 ml Milli-Q-purified water to the combined DCM extracts and vortex to mix. Remove upper aqueous phase and discard. Add 4 ml water and repeat the wash procedure an additional three times.

    If necessary, centrifuge at slow speed for ~2 min to separate the phases. After the last wash, centrifuge as above and remove the lower DCM phase to a clean glass vial (caps should be Teflon-lined).

22. Gently evaporate the DCM completely in a stream of instrument-grade air or nitrogen gas, and add 2 ml of DCM. Proceed to gas chromatography of the fully acetylated alditols.

    Take care when evaporating the DCM, as some alditol acetates are particularly volatile—see Troubleshooting.

Run gas chromatography

23. Separate and quantify alditol acetates using a gas chromatograph (e.g., Model HP 6890, Hewlett Packard), fitted with a flame ionization detector and a dedicated cool on-column capillary inlet (set on "oven track mode") on a BPX-70, open tubular fused silica column (25 m long, 0.33 i.d. and 0.25-µm film thickness).

    For an alternative column and gas chromatography system see Carrington et al. (1993).

    a. Set the initial oven temperature at 38°C and maintain it for 30 sec, increase to 170°C at 50°C/min, and then increase to 230°C at 2°C/min and hold at 230°C for 5 min.

    The detector temperature is held at 250°C. Helium (zero grade) is used as the carrier gas at a column head pressure of 40 kPa. The flow rate of the other gases are: hydrogen, 40 ml/min; air, 450 ml/min; and nitrogen, 45 ml/min. Total run time is 38 min. The results are integrated using Hewlett Packard ChemStation software.

    b. Inject 0.5 µl of samples and controls.

    Reference alditol acetates elute from the BPX-70 column in the following order: erythritol triacetate, 2-deoxyribitol tetraacetate, rhamnitol pentaacetate, fucitol pentaacetate, ribitol pentaacetate, arabinitol pentaacetate, xylitol pentaacetate, 2-deoxyglucitol hexaacetate, allitol hexaacetate, mannitol hexaacetate, galactitol hexaacetate, glucitol hexaacetate, and myo-inositol hexaacetate. Their identification can be verified by using individual standards.

24. Identify the alditol acetates by the retention times relative to the standards in the 13-sugar standard.

    The amount of each neutral monosaccharide in the samples can be calculated relative to the internal standard, allose, using response factors. The relative response of the detector for the individual alditol acetates can be calculated from the areas under the peaks for each alditol acetate, relative to the area under the peak for allitol acetate. There should be no peaks in the chromatogram of the water control and only one peak, corresponding to allitol hexaacetate from the internal standard allose, in the chromatogram of the TFA control.

BASIC PROTOCOL 2

DETERMINATION OF NONCELLULOSIC NEUTRAL SUGARS AND CELLULOSE CONTENT BY TRIFLUOROACETIC ACID HYDROLYSIS FOLLOWED BY SULFURIC ACID HYDROLYSIS

In this procedure, the cell walls are first treated with TFA to determine the neutral monosaccharide composition of the noncellulosic polysaccharides. The TFA-insoluble residue is then hydrolyzed using a two-stage sulfuric acid procedure to determine cellulose content.

Materials

Dried cell walls or final residue from cell wall fractionation (see *UNIT E3.1*)
Methanol
Nitrogen gas
72% (w/w) sulfuric acid (see recipe)
Milli-Q-purified water
15 M ammonia (analytical grade)
20 mg/ml allose
13 sugar standard (erythritol, 2-deoxyribose, rhamnose, fucose, ribose, arabinose, xylose, 2-deoxyglucose, allose, mannose, galactose, glucose, *myo*-inositol)

15-ml borosilicate glass tubes and screw-caps with Teflon-lined inserts
100°C and 30°C heating block or bath
5-ml glass syringe
Swinney stainless steel 13-mm filter unit (Millipore)
0.22-µm polytetrafluoroethylene (PTFE) filters or glass fiber filters
Gas chromatograph fitted with a flame ionization detector and a dedicated cool-on-column capillary inlet

Additional reagents and equipment for hydrolysis with TFA (see Basic Protocol 1)

Hydrolyze cell walls or final residue fraction using TFA

1. Perform TFA hydrolysis (see Basic Protocol 1, steps 1 to 5).

2. Add 2 ml methanol and centrifuge at 1000 × *g*, room temperature. Remove the supernatant using a Pasteur pipet and place in a clean glass tube.

   Use new, washed Pasteur pipets to ensure there are no traces of debris, especially from cardboard boxes that the pipets are transported in.

3. Add 2 ml methanol to pellet, vortex to mix, and centrifuge as in step 2. Remove the supernatant and combine with the supernatant from step 2. Evaporate the combined supernatants to dryness in a gentle stream of air or nitrogen gas. Proceed with reduction in step 5.

4. Retain the pellet, i.e., TFA-insoluble material, and proceed with two-stage sulfuric acid hydrolysis in step 6.

Reduce TFA-soluble material

5. Reduce TFA-soluble material in the combined supernatants from step 3 (see Basic Protocol 1, steps 8 to 13), then proceed with acetylation (see Basic Protocol 1, steps 14 to 22).

Hydrolyze TFA-insoluble residue with a two-stage sulfuric acid hydrolysis

6. Add 125 µl of 72% sulfuric acid to the TFA-insoluble residue pellet from step 4 using a glass pipet. Set up a control tube containing 125 µl of 72% sulfuric acid and take this through all the following steps.

   CAUTION: *Sulfuric acid is very corrosive. Wear goggles, gloves, and protective clothing.*

7. Flush tubes well with argon or nitrogen gas to remove all traces of air and cap using a screw-cap with Teflon-lined insert. Vortex to mix (take care not to splatter the solid material above the level of the liquid).

8. Incubate for 3 hr at 30°C. Vortex to mix at frequent intervals to aid dissolution (take care not to splatter the solid material above the level of the liquid). Allow to cool.

9. Add 1.375 ml Milli-Q-purified water, then mix thoroughly and quickly. Flush with argon or nitrogen gas. Cap tightly. Incubate for 3 hr 100°C. Allow to cool.
10. Add 300 µl of 15 M ammonia to neutralize the solution. Vortex to mix.

    Do this step in a fume hood.

    IMPORTANT NOTE: *Sugars are unstable when left in alkaline solutions, so proceed with next steps quickly.*
11. Add 50 µl of 20 mg/ml allose (internal standard). Vortex to mix.
12. Filter the hydrolysate using glass syringe fitted with a Swinney stainless steel 13-mm filter unit and a 0.22-µm PTFE filter into a clean borosilicate glass tube.

    After a sample has been filtered, discard the PTFE filter and wash the syringe and stainless steel filter unit six times in Milli-Q-purified water to ensure there is no carryover from one sample to another.

    Alternatively glass fiber filters can be used, but they should be heated to 500°C in an oven prior to use to remove all traces of contaminating carbohydrates.
13. Add 200 µl of the filtered hydrolysate to a scrupulously clean borosilicate glass tube (see Critical Parameters).

Prepare controls

14. Prepare a water control by adding 200 µl Milli-Q-purified water to a clean borosilicate glass. Add 20 µl of 15 M ammonia and vortex to mix.
15. Prepare a 13-sugar standard control by adding 100 µl of the 13-sugar standard to a clean borosilicate glass tube (allow the standard to thaw and mix well before use), then add 100 µl of Milli-Q-purified water (total volume 200 µl) and 20 µl of 15 M ammonia. Vortex to mix.

Reduce and acetylate sugars from TFA-insoluble material

16. Reduce the monosaccharides in the filtered hydrolysates and controls to the corresponding alditols (see Basic Protocol 1, steps 11 to 13). Acetylate the alditols (see Basic Protocol 1, steps 14 to 22).

Run gas chromatography

17. Separate and quantify alditol acetates for both the TFA-soluble (step 5) and insoluble material (step 16) using a gas chromatograph (see Basic Protocol 1, steps 23 to 24).

ALTERNATE PROTOCOL

DETERMINATION OF NONCELLULOSIC NEUTRAL SUGARS AND CELLULOSE CONTENT BY SULFURIC ACID HYDROLYSIS

This protocol is an alternative to Basic Protocol 2 and can be used to fully hydrolyze cellulose as well as other polysaccharides simultaneously. In this protocol a two-stage sulfuric acid procedure is used (Harris et al., 1988). This procedure is simpler than that described in Basic Protocol 2; however, it has the disadvantage of some noncellulosic neutral sugars being degraded during cellulose hydrolysis. Moreover, one cannot tell the source of glucose (e.g., whether it comes from cellulose or xyloglucan).

For materials see Basic Protocol 2.

Hydrolyze by two-stage sulfuric acid hydrolysis

1. Place 10 mg of dried cell walls or final residue fraction (*UNIT E3.1*) into a scrupulously clean (see Critical Parameters) borosilicate glass tube (in duplicate). Record the exact weight.

   This procedure can be done using 5 mg of cell walls or fraction and half volumes of reagents to step 6.

2. Add 250 µl of 72% sulfuric acid using a glass pipet. Set up a tube containing 250 µl of 72% sulfuric acid as a control and take this through all the following steps.

   A clean glass pipet is recommended to deliver the acid, as accidental splashes and fumes from acids can cause corrosion of expensive automatic pipets.

   CAUTION: *Sulfuric acid is very corrosive—wear goggles, gloves, and protective clothing.*

3. Flush tube well with argon or nitrogen gas to remove all traces of air and cap using a screw-cap with Teflon-lined insert. Vortex to mix (take care not to splatter the solid material above the level of the liquid).

4. Incubate for 3 hr at 30°C. Vortex to mix at frequent intervals to aid dissolution (take care not to splatter the solid material above the level of the liquid). Allow to cool.

5. Add 2.75 ml Milli-Q-purified water mix thoroughly and quickly. Flush with argon or nitrogen gas, cap tightly, and incubate for 3 hr at 100°C. Allow to cool.

6. Working in a fume hood, add 600 µl of 15 M ammonia to neutralize the solution. Vortex to mix.

   IMPORTANT NOTE: *Sugars are unstable when left in alkaline solutions so proceed quickly.*

7. Add 50 µl of 20 mg/ml allose (internal standard). Vortex to mix.

8. Filter the hydrolysate using a glass syringe fitted with a Swinney stainless steel 13-mm filter unit and a 0.22-µm PTFE filter into a clean borosilicate glass tube.

   After a sample has been filtered, discard the PTFE filter and wash the syringe and filter unit six times with Milli-Q-purified water to ensure there is no carryover from one sample to another.

   Alternatively, glass-fiber filters can be used, but they should be heated to 500°C in an oven prior to use to remove all traces of contaminating carbohydrates.

9. Add 200 µl of the filtered hydrolysate to a scrupulously clean borosilicate glass tube (see Critical Parameters).

Prepare controls

10. Prepare a water control by adding 200 µl Milli-Q-purified water to a clean borosilicate glass. Add 20 µl of 15 M ammonia and vortex to mix.

11. Prepare a 13-sugar standard control by adding 100 µl of the 13-sugar standard to a clean borosilicate glass tube (allow the standard to thaw and mix well before use), then add 100 µl Milli-Q-purified water (total volume 200 µl) and 20 µl of 15 M ammonia. Vortex to mix.

Reduce and acetylate sugars

12. Reduce monosaccharides to corresponding alditols (see Basic Protocol 1, steps 11 to 13).

13. Acetylate the alditols (see Basic Protocol 1, steps 14 to 22).

Run gas chromatography

14. Separate and quantify alditol acetates using a gas chromatograph as described in Basic Protocol 1, steps 23 to 24.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Sodium borohydride in DMSO, 0.5 M

Weigh out 0.5 g sodium borohydride into a glass bottle with a Teflon-lined lid. Add 25 ml of DMSO (use the highest quality only, if necessary dry over molecular sieves) loosely cap and place in oven for 1 hr at 100°C, with occasional gentle swirling. Remove from oven, tighten cap, cool, and store in dark (or wrap in aluminum foil). Prepare fresh on the day of use.

Take care when handling, wear gloves, goggles, and mask—proceed quickly to keep moisture out of the stock chemical.

13-sugar standard

Thoroughly dry 30 mg of each of the following monosaccharides for several days over an effective desiccant (e.g., phosphorus pentoxide): erythritol (i-erythritol, meso-erythritol), 2-deoxy-D-ribose, L(+)-rhamnose, α-D(+)-fucose (6-deoxy-D-galactose), D(−)-ribose, L(+)-arabinose, D(+)-xylose, 2-deoxy-D-glucose, β-D-allose, D(+)-mannose, D(+)-galactose, α-D (+)-glucose, *myo*-inositol. Weigh exactly 20 mg of the dried sugars into individual vials and add 1 ml Milli-Q-purified water to each. Mix together equal volumes (e.g., 100 µl) of each of the 13 monosaccharide solutions. Store the vials of 13 sugar standard at −20°C.

Sulfuric acid, 72% (w/w)

Place 20 g of ice-cold water in a beaker and add ice-cold analytical grade sulfuric acid with stirring to bring the weight to 93.5 g. Add more ice-cold water, with stirring, to bring the final weight to 100 g. Store in a glass bottle with a Teflon-lined cap.

Extreme care is necessary, wear goggles, gloves, and protective clothing. Make up the acid in a fume hood.

Trifluoroacetic acid, 2 M

Use high-purity TFA. Preparation will depend on specifications of the product purchased. For example: for TFA density 1.480: assay 99.5%; mol. wt. 114.02, 1 liter of liquid contains ~1472.6 g TFA; thus, 1 ml contains 1.4726 g TFA (or 0.679 ml/g) and 2 M TFA contains 28.04 g/liter.

To prepare 100 ml of 2 M TFA, carefully add 15.5 ml of the concentrated TFA to a 100-ml volumetric flask containing water, then adjust the volume to 100 ml with water. Store in a brown glass bottle, flush with argon or nitrogen gas, and cap tightly with a Teflon-lined lid.

CAUTION: *Extreme care is necessary; wear goggles, gloves, and protective clothing. Make up the acid in a fume hood.*

COMMENTARY

Background Information

Plant cell wall polysaccharides are composed of varying proportions of the neutral monosaccharides rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose, and the acidic monosaccharides galacturonic acid, glucuronic acid, and 4-*O*-methylglucuronic acid (see Fig. E3.2.1). Other monosaccharides have been identified but are present only in trace amounts. The monosaccharides are linked by a variety of glycosidic linkages with differing susceptibility to acid hydrolysis (Aspinall, 1982). Moreover, the monosaccharides released show variable susceptibility to degradation in acid. While procedures may be designed to achieve maximum hydrolysis, they are often a compromise to minimize destruction of the monosaccharides (Aspinall, 1982). An alterna-

tive to acid hydrolysis is to use commercially available hydrolytic enzymes. These are often highly specific for a particular glycosidic linkage and are especially suitable for detailed analysis of polysaccharide composition.

Traditionally, polysaccharides were hydrolyzed with a strong acid, such as 1 M or 2 M hydrochloric acid for 1 hr in a boiling water bath (Adams, 1965). However, some polysaccharides are completely hydrolyzed at lower acid concentrations or shorter times, and others require more strenuous conditions. Concomitantly, the monosaccharides formed by acid hydrolysis may be degraded by the acid (Aspinall, 1982). Consequently, for each polysaccharide, the investigator should vary the conditions to find the parameters that give the maximum level of hydrolysis with the minimum degradation. With a mixture of polysaccharides, finding the optimal conditions is more of a challenge and a compromise is the inevitable outcome.

Albersheim et al. (1967) made a major breakthrough when they substituted trifluoroacetic acid (TFA) for HCl or H_2SO_4. TFA is easily removed by evaporation, whereas HCl and H_2SO_4 have to be neutralized on completion of the hydrolysis. Soluble salts formed had to be removed by ion-exchange resins, with

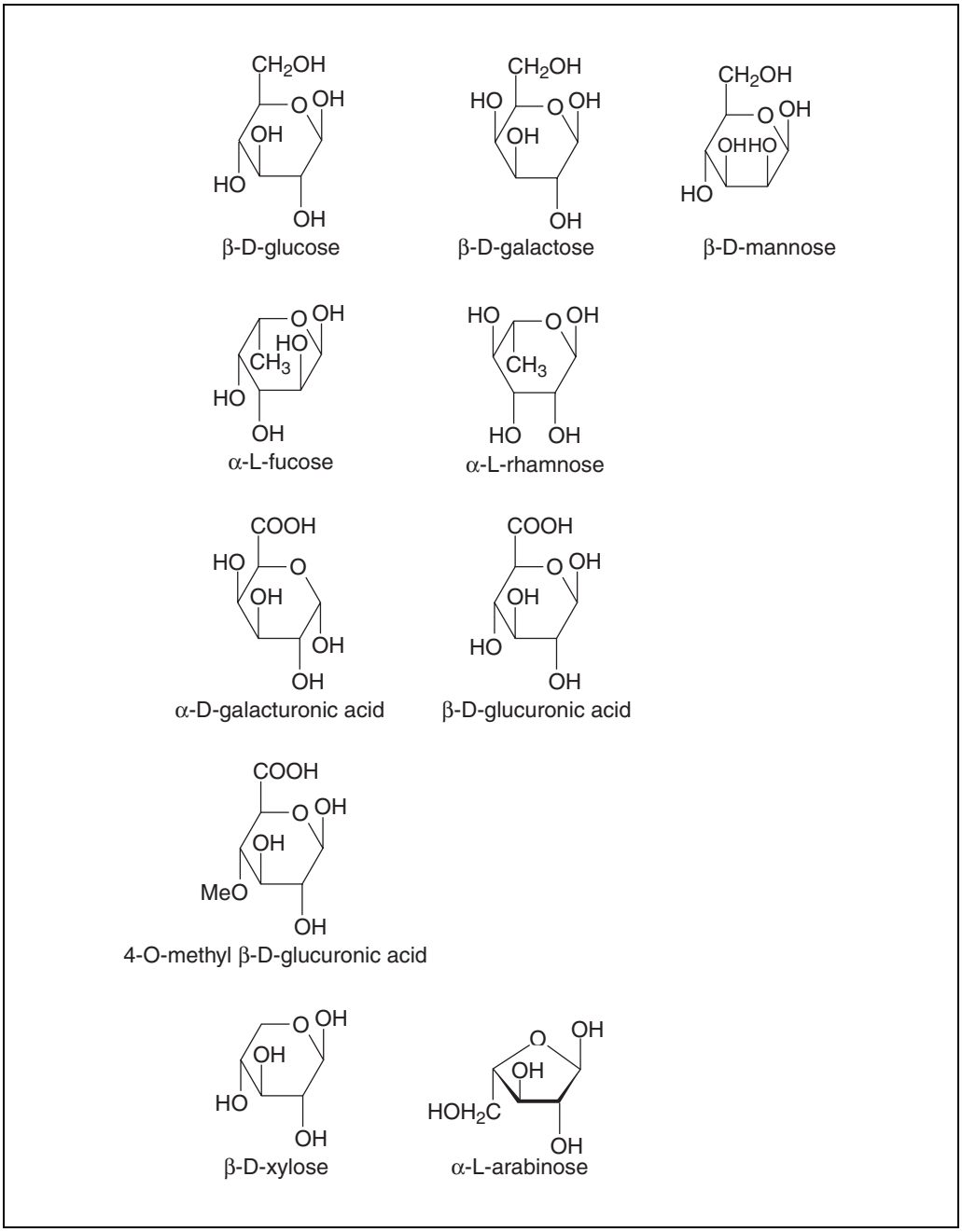

Figure E3.2.1 Structures of monosaccharides from plant cell walls.

attendant handling losses of monosaccharides. It was common to neutralize H_2SO_4 by forming the insoluble Ba_2SO_4 salt, but this adsorbs sugars, resulting in poor recoveries. However, polysaccharides containing uronic acids are resistant to acid hydrolysis, and the TFA hydrolysis procedure (Albersheim et al., 1967; Fry, 1988) gives reduced yields of the monosaccharide. Furthermore neutral sugars, such as rhamnose, are incompletely hydrolyzed if they have a uronic acid residue glycosidically linked to them. Thus, protocols described in this unit will give an estimate of the neutral monosaccharides, including the neutral side chains of the pectic polysaccharide rhamnogalacturonan, but the yield of rhamnose may be slightly underestimated. For estimation of uronic acid content see *UNIT E3.3.*

A second important breakthrough was in the acetylation step. With 1-methylimidazole as the catalyst, complete acetylation can be achieved without removing the borate formed from sodium borohydride in the prior reduction step (Blakeney et al., 1983; Harris et al., 1988). Borate complexes with *cis*-diol groups in the monosaccharides. In earlier methods (Albersheim et al., 1967; Selvendran et al., 1979), the borate had to be removed or acetylation would not go to completion (Wolfram and Thompson, 1963). The reduction and acetylation reactions are summarized in Figure E3.2.2.

Table E3.2.1 Response Factors of Alditol Acetates

Order of elution	Alditol acetate	Retention time (min)	Peak area	Response factor relative to allitol hexaacetate
1	Erythritol triacetate	14.321	1108.54	0.880
2	2-Deoxyribitol tetraaceate	18.279	1095.77	0.870
3	Rhamnitol pentaacetate	18.937	1015.50	0.806
4	Fucitol pentaacetate	19.593	1186.82	0.942
5	Ribitol pentaacetate	22.743	1191.66	0.946
6	Arabinitol pentaacetate	23.520	1195.11	0.949
7	Xylitol pentaacetate	27.075	1180.65	0.937
8	2-Deoxyglucitol hexaacetate	28.265	1214.87	0.965
9	Allitol hexaacetate	30.493	1259.49	1.000
10	Mannitol hexaacetate	31.887	1259.48	1.000
11	Galactitol hexaacetate	33.218	1254.58	0.996
12	Glucitol hexaacetate	34.828	1224.28	0.972
13	*Myo*-inositol hexaacetate	36.923	1135.60	0.902

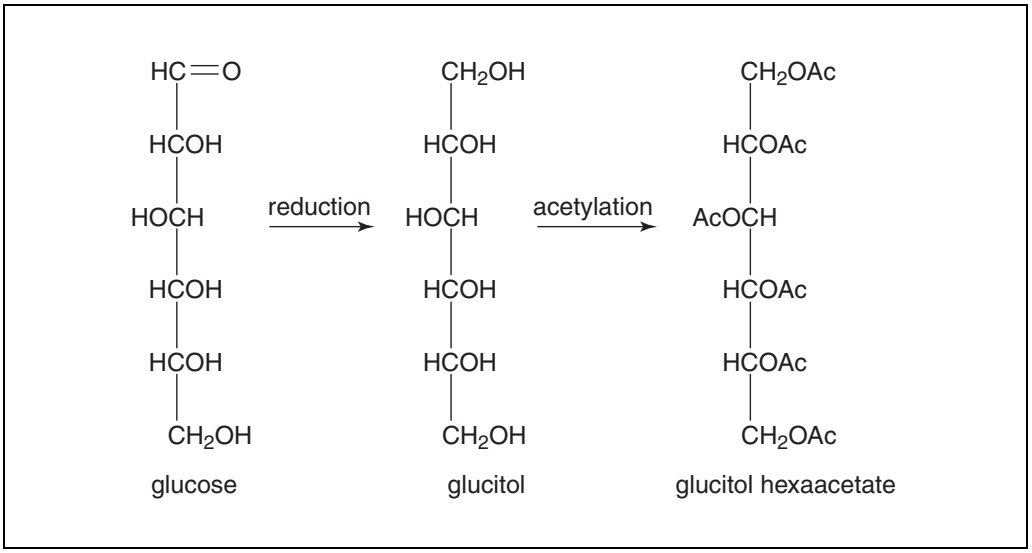

Figure E3.2.2 Reaction scheme for formation of alditol acetates.

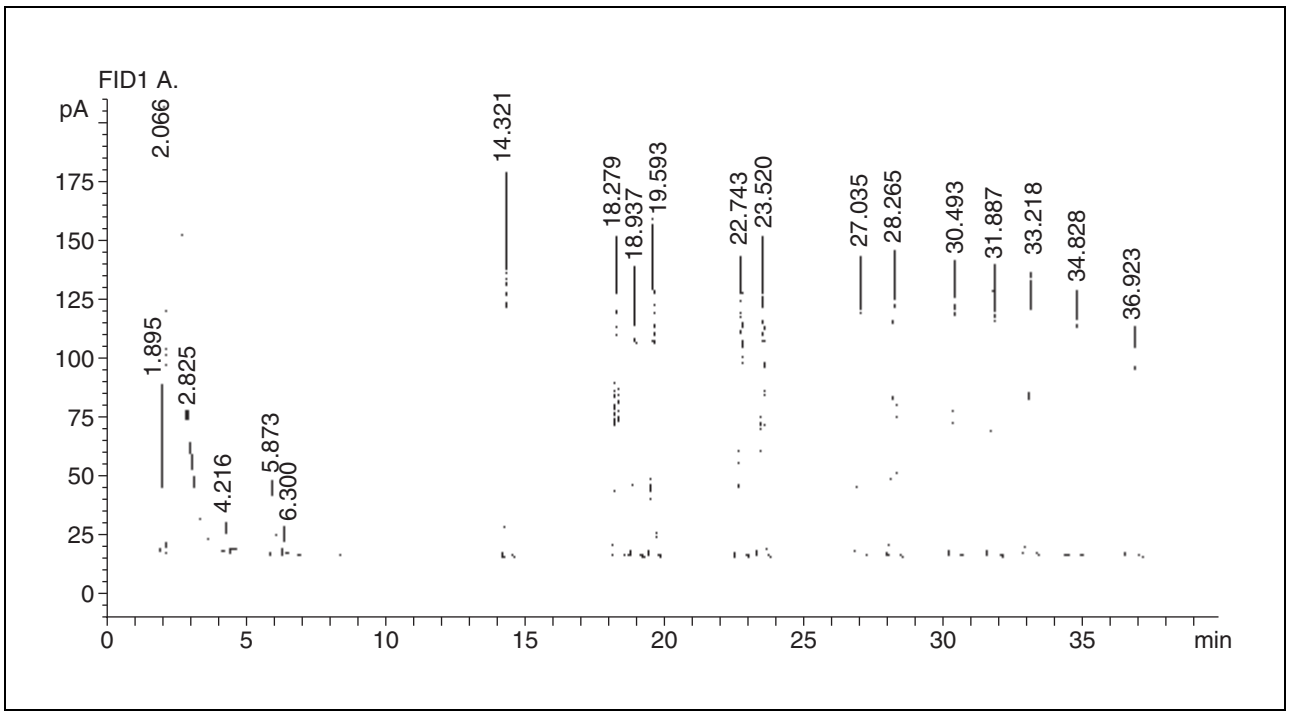

Figure E3.2.3 Chromatogram of a 13-sugar standard.

Critical Parameters

Scrupulously cleaned glassware is essential. Glassware that has previously been used for alditol acetates should first be washed in dichloromethane (DCM) to remove any traces of alditol acetates. Tubes should then be washed using high-quality detergent, rinsed at least 6 times in hot tap water, and then at least two times in distilled water, and allowed to dry.

When performing these protocols, the highest-quality analytical reagents should be used.

The syringe used to inject samples into the gas chromatograph should be washed at least ten times in fresh DCM, making sure that the outside of the needle is equally as clean as the inside. Cleaning in methanol may also assist.

Use only caps and lids that have Teflon-lined inserts.

A chromatogram from a 13-sugar standard is shown in Figure E3.2.3. The retention times, peak areas, and the response factors (i.e., allose = 1.000) are also shown. The response factors for the other monosaccharides are usually just above or below 1.000 (Table E3.2.1). The authors have found the response factor for rhamnose to be consistently lower than the other monosaccharides.

It is useful at the start of each session on the gas chromatograph to inject 0.5 µl DCM to ensure the baseline is flat and that there are no extraneous compounds eluting from the column. It is also useful to do this when one has inadvertently injected a sample that is too concentrated. Once it has been established that there are no compounds eluting, then dilute the sample further in DCM and rerun.

Troubleshooting

Some of the alditol acetates formed from deoxy sugars, pentoses, and tetroses are particularly volatile, and care should be taken when evaporating to dryness (see Basic Protocol 1, step 21). It is best to stop the evaporation as soon as the sample is dry.

Make sure exact dry weights of samples are recorded, to avoid errors associated with weighing. Special care is needed in humid environments, as cell walls and fractions readily absorb water.

Check volume of solutions in 121°C incubation at 30 min to see if tubes are leaking.

All efforts need to be made to reduce the likelihood of plasticizers (Henry et al., 1983) and other contaminants getting into one's samples. Avoid DCM making contact with the glue that attaches Teflon liners to caps, as this glue can give rise to peaks in the chromatogram.

$NaBH_4$ is degraded by reaction with moisture in the air. Therefore it should be kept in a desiccator. To check that $NaBH_4$ is reactive, take a drop of $NaBH_4$ solution and add one small drop of glacial acetic acid, which should

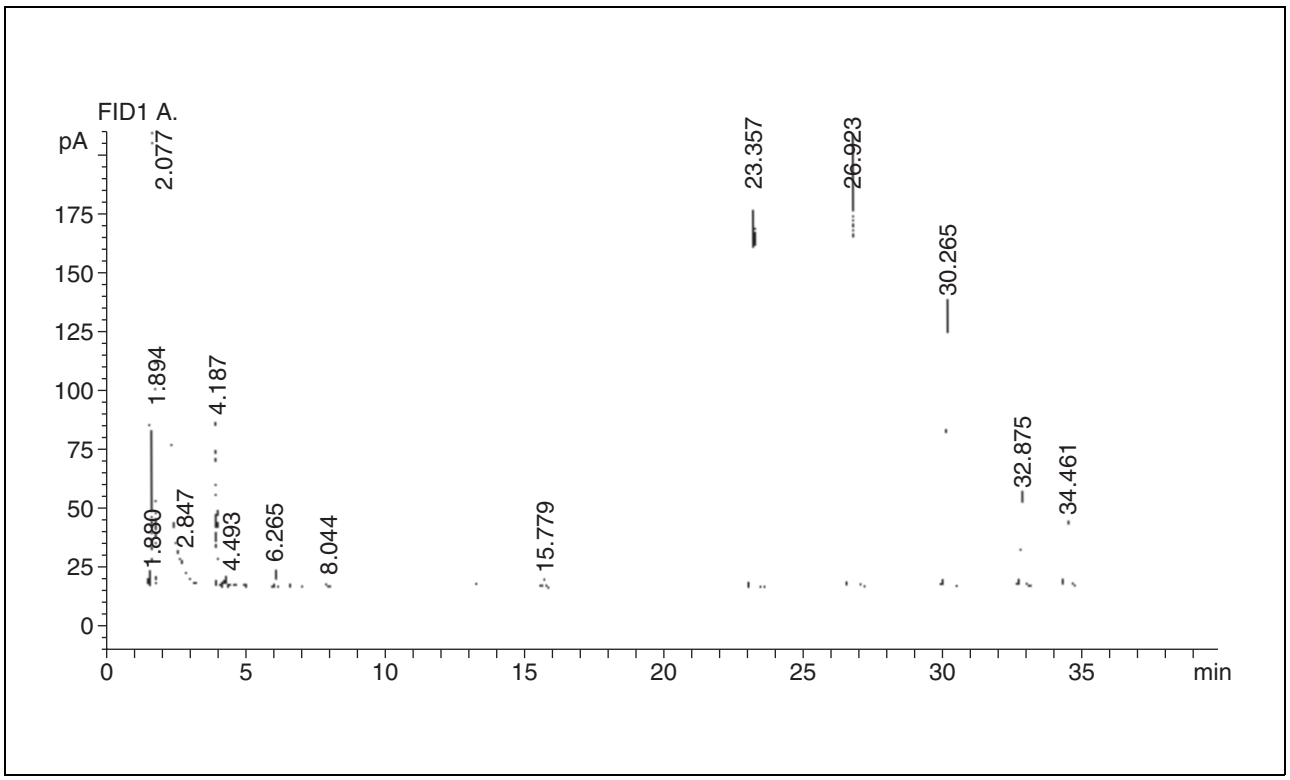

Figure E3.2.4 Chromatogram of a sample.

result in copious effervescence. Check again at the end of the reduction that there is still some unreacted $NaBH_4$ present.

Anticipated Results

Reproducibility between duplicate samples should be very high.

A sample chromatogram from a TFA hydrolysate of a cereal grain cell wall preparation is shown in Figure E3.2.4. The major monosaccharides in this sample are xylose and arabinose, with smaller amounts of galactose and glucose. The monosaccharide composition, together with the method of hydrolysis, indicate that the major polysaccharide is likely to be arabinoxylans. The glucose could arise from several different polysaccharides, including xyloglucans and (1→3,1→4)-β-D-glucans since this sample was from a cereal grain. The galactose is possibly pectic in origin. It is important when one is speculating on the types of polysaccharides in one's samples to take into account the uronic acid composition (see *UNIT E3.3*). More definitive identification requires analysis of the linkages.

When identifying the alditol acetates in a sample by comparison with the retention times of the 13-sugar standards, the times should be within 0.05 min. However, during the course of the day the retention times tend to change a little, and so it is important to rerun the 13-sugar standard after every fourth or fifth sample. Over a longer time, one will also observe slight differences in retention times, such as shown in Figures E3.2.3 and E3.2.4 for a sample and standard run 2 months apart.

Time Considerations

Do not try to process too many samples in a batch. It is suggested that initially two samples should be tried in duplicate, with an acid control, water control, and 13-sugar standard. Handling too many tubes may lead to delays during the evaporation process. Evaporation of TFA can be assisted by warming the samples to 30°C.

For Basic Protocol 1 allow 3 to 4 hr to complete steps 1 to 7. Allow a further 5 hr for steps 8 to 22. For gas chromatography allow ~50 min per sample. For Basic Protocol 2 add several more hours to that required for Basic Protocol 1, to allow for the extra hydrolysis step. The Alternate Protocol can be completed in ~8 hr.

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Key References

Albersheim et al., 1967. See above.

Describes the basic procedure for TFA hydrolysis and how the resulting monosaccharides are degraded at different rates.

Blakeney et al., 1983. See above.

Describes the procedure for reduction of monosaccharides using $NaBH_4$ in DMSO solution followed by acetylation using 1-methylimidazole as the catalyst.

Harris et al., 1988. See above.

Describes the procedure and its application to neutral detergent fiber preparations from a wide range of plants.

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