Composition Analysis by Alditol Acetates

MATERIALS

- 4 N Trifluoroacetic Acid (TFA)
- Acetic Anhydride (Sigma, 539996)
- Pyridine (Sigma, 270970)
- $NaBH_4$ (Sigma, 213462) or $NaBD_4$ (Sigma, 205591)
- 1 M NH_4OH
- Myo-Inositol (Sigma, 57569)
- 50% Iso-propyl alcohol (IPA)
- 1 mg/mL solution of standard sugars used for alditol acetates (AA): Rhamnose, Fucose, Ribose, Arabinose, Xylose, Mannose, Galactose, Glucose, N-acetyl Glucosamine, N-acetyl Galactosamine, N-acetyl Mannosamine.

Standard Preparation:

- To prepare an alditol acetate standard place 2 µg of neutral and amino sugars, into a sample tube along with 1 µg of myo-inositol as internal standard

Sample Preparation:

- 50-100 µg of sample are taken in a screw cap tube and 1 µg of myo-inositol is added to it as internal standard. The samples can also be in known volume of water.

PROCEDURE

1. Add an equal volume of 4 N TFA to the standard and samples to make a final concentration of 2 N TFA and hydrolyze at 100 ^{o}C for 4 hrs

2. Cool samples to room temperature and centrifuge at 2000 rpm for 2 min to bring the condensation down to the bottom of the tube.

3. Remove the TFA using dry nitrogen flush on an evaporation heat block. Use slight heat ~37 ^{o}C to fasten the evaporation.

4. Add 100 µL of 50% IPA and re-evaporate the sample. Repeat the IPA wash once more to fully remove TFA. Repeating this step twice will remove the residual acid completely, however to ensure complete removal of the acid check the pH with a pH paper.

5. Prepare the reducing agent by dissolving 10 mg of $NaBH_4$ in 1 mL of 1M ammonium hydroxide. Add ~100-150 µL of reducing agent to each sample and mix by vortexing.

6. Incubate samples at room temperature for 16h or overnight. Make sure to loosen the cap as this reaction forms hydrogen gas.

7. Check the pH of the reduced sample with pH paper, it should be alkaline (pH>12). Cool down the sample on ice-water bath for 1 min then add ice-cold 30% aqueous acetic acid drop wise to neutralize the sample. Vortex thoroughly after addition of each drop of acid and return the sample to the ice-water bath frequently throughout this process. Complete neutralization occurs when there is no effervescence after acid is added. To ensure neutralization use pH paper to check the pH.

8. Dry the neutralized samples by dry nitrogen flush, slight heat (<37 ^{o}C) can be applied to fasten the evaporation however don't use heat when handling permethylated sample). The dried sample may look like syrupy or like a gel. At this stage add 100 µL of Methanol, vortex thoroughly to completely dissolve the sample and again evaporate the sample by dry nitrogen flush, repeat for a total of three times.

   Note: Boric acid formed by neutralization is removed as volatile methyl borate, this step is necessary as excess boric acid interferes with acetylation of sugars.

9. Next add 100 µL of 9:1 methanol:glacial acetic acid to the dried sample and vortex to dissolve completely. Re-evaporate using dry nitrogen flush. Repeat this step at least three times followed by evaporating the samples using absolute methanol (3 times, 100 µL each time). The sample should form a white crust of sodium acetate around the wall of the glass tube.

10. Place the dried samples in a vacuum desiccator over P_2O_5 for 2-3 hours.

11. To acetylate the sample add 50 µL of pyridine and 50 µL of acetic anhydride. Vortex to mix the reagents and sonicate (30-40 sec) to break the solid sticking on the wall of the tube. Heat the reaction mixture at 100 ^{o}C for 1 hour with vortexing at 20 min intervals.

12. Cool the samples to room temperature and then centrifuge at 2000 rpm for 2 min to bring the condensation down to the bottom of the tube. Remove pyridine and acetic anhydride using dry nitrogen flush on the evaporator (DO NOT APPLY HEAT at this stage as the samples are highly volatile). After the samples are dried down completely add 100 µL of toluene, vortex and evaporate toluene using dry nitrogen flush.

    Note: Toluene is added to remove trace of pyridine and acetic anhydride which prevents peak trailing in the GC-MS spectrum.

13. Dissolve the dried samples in 1 mL of dicholormethane and sonicate to break down the solid crust. Vortex the reaction mixture and centrifuged at 2000 rpm for 2 min at 15 ^{o}C. The crust should be settled down at the bottom,

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14. Filter the supernatant through a Pasteur pipette filled with glass wool. Pre-wash the glass wool with a small amount of dichloromethane.

15. Collect the filtrate and remove the dichloromethane by dry nitrogen flush.

16. Reconstitute the samples in 100-150 µL of dichloromethane and transfer into a sample vial for analysis by GC-MS.

GC-MS SETTINGS FOR ALDITOL ACETATES:

Carrier Gas: Helium
Inlet Conditions:

- Temperature: 220 ^{o}C
- Pressure: 11.649 psi
- Flow: 22.24 mL/min

GC-MS Transfer Line Temp: 280 ^{o}C
MS Source: 230 ^{o}C
MS Quad: 150 ^{o}C
Column: DB-5 or Equivalent, 30 m x 0.25 mm x 0.25 µm
Column Flow: 1.1971 mL/min
Injection Volume: 1 µL
Run Time 48 min

Conditions	^{o}C/min	^{o}C	Hold Time (min)	Run Time (min)
Initial		80	2	2
Ramp1	10	180	2	14
Ramp2	2	220	5	39
Ramp3	5	240	5	48

GC-MS SETTINGS FOR PARTIALLY METHYLATED ALDITOL ACETATES:

Carrier Gas: Helium
Inlet Conditions:

- Temperature: 220 ^{o}C
- Pressure: 9.855 psi
- Flow: 22.24 mL/min

GC-MS Transfer Line Temp: 280 ^{o}C
MS Source: 230 ^{o}C
MS Quad: 150 ^{o}C
Column: DB-5 or Equivalent, 30 m x 0.25 mm x 0.25 µm

Column Flow: 10396 mL/min
Injection Volume: 1 µL
Run Time 49.667 min

Conditions	°C/min	°C	Hold Time (min)	Run Time (min)
Initial		80	0	0
Ramp1	5	120	1	9
Ramp2	3	230	4	49.667

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