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Note

Use of mannitol as an internal standard in the gas–liquid chromatography of methyl glycosides

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A general method for the investigation of carbohydrates in complex glycolipids involves hydrolysis with aqueous acid or with cation-exchange resins in the protonated form, However, under the conditions required for complete hydrolysis of glycosidic bonds, extensive destruction of certain sugars may take place[1–4]. It appears that quantitative recoveries of carbohydrates after hydrolysis by aqueous acids are not routinely achieved[2].

Cleavage of glycosidic bonds can also be catalysed by protons in methanol with the liberation of O-methyl glycosides. This methanolysis is usually brought about by the action of hydrogen chloride in anhydrous methanol. It is claimed that methanolysis is as efficient as hydrolysis for cleaving glycosidic bonds and that this method has certain advantages[4–7], but there have been recent reports[2,3,8] of difficulties encountered in obtaining satisfactory recovery of sugars liberated as their methyl glycosides.

Chambers and Clamp[9] have carried out an assessment of methanolysis and other factors used in the analysis of carbohydrate materials, and one of these factors was the choice of internal standard. Mannitol was chosen as it was claimed to be completely stable under the conditions used by these workers. However, we found that, under certain conditions, there were difficulties in using mannitol as an internal standard for the analysis, by gas–liquid chromatography (GLC), of the methyl glycosides obtained by methanolysis of plant galactolipids.

An internal standard for use in quantitative GLC should: (a) preferably be a solid, (b) be readily available in a highly purified form, (c) be chemically similar to the class of compound under investigation, (d) give a single chromatographic peak that is adequately separated from those of other components, and (e) be stable under all the conditions of the analytical procedure.

Internal standards of two types have been used in the GLC of sugars as their trimethylsilyl (TMS) derivatives, *viz.*, polyhydroxy compounds such as mannitol[9], sorbitol[10], inositol[11], α-D-glucoheptose[12] and phenyl α-D-glucopyranoside[13] (these compounds have the properties (a)–(d) above); and hydrocarbons such as terphenyl, triphenylethylene, chrysene, pyrene[14] and eicosane[15] (these compounds have the properties (a), (b), (d) and (e) above, but are not chemically similar to the sugars).

In the present work both mannitol and octadecane were investigated as to

their suitability as internal standards for the quantitative GLC of the methanolysis products from galactolipids.

EXPERIMENTAL

Octadecane, mannitol, galactose and methyl α-D-galactopyranoside were obtained from Koch-Light Laboratories (Colnbrook, Great Britain).

Pyridine (BDH, Poole, Great Britain) was dried over solid sodium hydroxide and distilled. To prepare 0.7 *M* methanolic hydrogen chloride, freshly distilled acetyl chloride (2.5 ml) was added carefully to methanol (50 ml, AnalaR grade); the reagent was ready for use after 15 min and was freshly prepared each week.

For methanolysis, the sugar (10 mg) was heated under reflux with 0.7 *M* methanolic hydrogen chloride (0.5 ml) for 2 h at 80°, and the resulting solution was evaporated to dryness at 50° in a stream of nitrogen. As soon as the solution was dry, the residue was dissolved in pyridine (1 ml).

The procedure used to prepare the TMS derivatives was essentially that of Sweeley *et al.*[5] and has already been described[7].

Thin-layer chromatography (TLC) was carried out on plates (20 × 20 cm) coated with silica gel (Camag, without binder) layers 0.25 mm thick; GLC was carried out on a Perkin-Elmer (Beaconsfield, Great Britain) PE-800 gas chromatograph with nitrogen (at 5 p.s.i.) as carrier gas and stainless-steel SCOT columns, each 16 m × 0.5 mm, coated with SE-30 (operated at 170°) or XE-60 (operated at 150°). These columns were purchased from Perkin–Elmer.

RESULTS AND DISCUSSION

As an internal standard, octadecane has the advantages that it is a solid obtainable in a high degree of purity, it is soluble in pyridine and it does not react with methanolic hydrogen chloride or with the reagents used for trimethylsilylating the methyl glycosides. It has the disadvantage that it only gives good symmetrical peaks in GLC with hydrocarbon stationary liquids. On the SCOT columns and silicone phases used in this investigation, octadecane gave unsymmetrical peaks with XE-60, and, even on the less polar SE-30, the peaks (although better than on XE-60) were still not symmetrical.

Mannitol has the advantages that it is a solid, obtainable in a high degree of purity, it is a type of compound similar to that being investigated, its TMS derivative gives one symmetrical peak on both XE-60 and SE-30 stationary liquids. One of the disadvantages of mannitol is its low solubility in pyridine. Penick and McCluer[16] found that this was the most troublesome factor and that, unless precautions were taken to ensure that the mannitol was completely dissolved in the pyridine before the silylation, the results were always low and the chromatogram displayed multiple peaks in the area of the fully silylated mannitol. Similar results were found with inositol[14].

In our work, dilute solutions (1 mg per ml) of mannitol in pyridine were used; these solutions, even on standing for several weeks, gave reproducible detector responses when silylated and when GLC was carried out with octadecane as standard.

The following experiments were carried out to determine if silylated mannitol was stable under the conditions of the methanolysis and the GLC .

(a) A mixture of mannitol and octadecane in pyridine was trimethylsilylated; the detector response of the TMS-mannitol was 153 relative to octadecane.

(b) A similar mixture was heated for 2 h at 80° with 0.7 *M* methanolic hydrogen chloride (0.5 ml) and the solvent and acid were removed in a stream of nitrogen at 50°; the relative detector response to TMS-mannitol was 152. Thus, under these conditions, there was no loss of mannitol. Chambers and Clamp[9] found that mannitol was completely stable in both 1 *M* and 2 *M* methanolic hydrogen chloride, but that destruction of the mannitol occurred under more extreme conditions, 38% being lost in 4 *M* acid and 48% in 6 *M* acid at 100°.

(c) A mixture of mannitol, octadecane and galactose was treated as in (b); the relative detector response of TMS-mannitol was 114 (25% loss of mannitol). When this procedure was repeated with 2.5 ml of the methanolic hydrogen chloride, the relative detector response was 87 (45% loss of mannitol). These findings are not in agreement with those of Chambers and Clamp[9], who found that the response factors of monosaccharides were remarkably constant whether mannitol was added before or after the methanolysis step. Their response factors would remain constant only if there was no loss of mannitol, or if there was an equal amount of degradation of both the mannitol and the monosaccharide.

(d) A mixture of mannitol and galactose was heated with methanolic hydrogen chloride as in (b), and, after removal of the methanol and acid, the residue was dissolved in a small amount of methanol. This solution was examined by TLC on silica gel. The resulting chromatogram indicated that there were present in the solution (i) various components with R_F values lower than those of mannitol, galactose or methyl galactosides (perhaps due to polymeric carbohydrates) and (ii) two compounds with R_F values higher than those of mannitol, galactose or methyl galactosides; these two spots were shown to be due to methyl galactofuranosides[7].

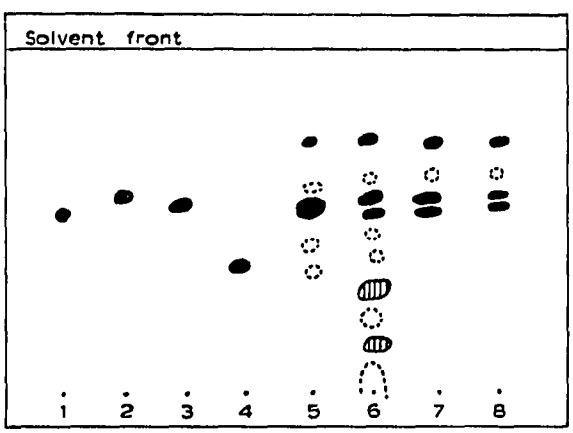

Fig. 1. TLC of products obtained by the action of methanolic hydrogen chloride on mixtures of mannitol and galactose: silica gel (unlined tank); solvent, propanol–ethyl acetate–water (7:2:1). 1, Mannitol; 2, galactose; 3, methyl α-D-galactopyranoside; 4, lactose; 5, galactose heated under reflux with 0.7 *M* methanolic hydrogen chloride, solvent and acid removed in a stream of nitrogen at 50°; 6, galactose + mannitol treated as for 5; 7, galactose + mannitol heated under reflux with 0.7 *M* methanolic hydrogen chloride, acid removed with solid silver carbonate; 8, solution from 7 evaporated in a stream of nitrogen at 50°.

(e) A mixture of mannitol and galactose was heated with methanolic hydrogen chloride as in (b), but the acid was removed by treatment with solid silver carbonate. The filtrate was evaporated in a stream of nitrogen and the residue was dissolved in methanol. Its thin-layer chromatogram did not indicate any components with lower R_F values, but spots due to galactofuranosides were present. Diagrams of the chromatograms from solutions (d) and (e) are shown in Fig. 1. The detector response for TMS-mannitol prepared from the above solution was 87. This means that, although no polymeric products were indicated by TLC, there was still considerable loss of mannitol.

The results of these experiments indicate that mannitol should not be added as an internal standard before the methanolysis step. In the procedure finally adopted for the plant galactolipid investigation, mannitol was added after the methanolysis step but before trimethylsilylation of the methyl glycosides.

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