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Note

Interference by phthalic esters in the gas-chromatographic analysis of sugars

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Phthalic esters are used extensively as plasticisers in the formulation of poly(vinyl chloride) and other plastics, and have been reported as widespread contaminants of many environments[1]. In particular, phthalic esters have been found in many samples of biological origin[1–3] and in laboratory solvents and equipment[4–6]. Because of the apparent facility with which these esters are leached from a variety of plastic devices, stringent precautions are required for the gas-chromatographic analysis of phthalates[1] and also for the analysis of materials that closely match the gas-chromatographic behaviour of phthalates[7,8].

Problems associated with contamination by phthalic esters were encountered recently during the analysis of polysaccharides by gas chromatography of the alditol acetate derivatives[9]. Reports in the literature of unidentified peaks in the gas chromatograms of other polysaccharides suggest that the problem has probably occurred elsewhere, but has not been recognised by other investigators[10].

When the extracellular polysaccharide of a *Rhizobium japonicum* strain was analysed as described by Albersheim *et al.*[11], using analytical-grade reagents and solvents as commercially supplied, the chromatogram of the alditol acetate mixture obtained appeared as shown in Fig. 1a. The authentic alditol acetates were identified by their retention times and mass spectra, and the presence of the sugars in the polysaccharide was confirmed by paper chromatography of replicate hydrolysates. The remaining peaks were shown, by mass spectrometry and by gas chromatography of standard samples, to result from phthalic esters. It may be seen (compare Figs. 1b and 1c) that dibutyl phthalate behaves as a tetritol acetate, that di(2-ethylhexyl) phthalate has a retention time very close to that for xylitol acetate, and that butylbenzyl phthalate has a retention time intermediate between those of mannitol and galactitol acetates. Butylbenzyl phthalate was particularly troublesome in the analysis of polysaccharides of certain *Rhizobium* strains that contained 4-*O*-methyl-D-galactose[9], because this phthalate and 4-*O*-methylgalactitol pentaacetate have identical retention times on gas-chromatographic columns (EGS/GE-XF1150 and ECNSS-M) widely used for the separation of alditol acetates[12,13].

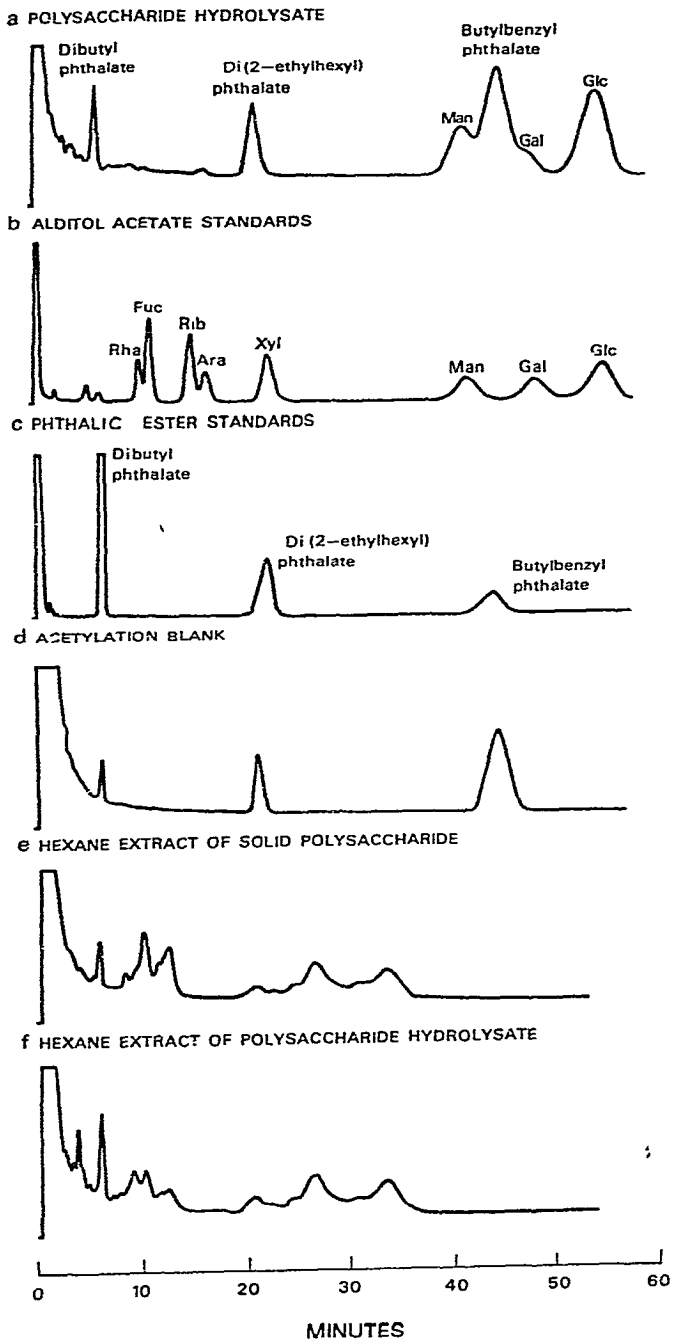

Fig. 1. Gas-chromatographic analysis of (*a*) components of hydrolysed *Rhizobium japonicum* polysaccharide examined as alditol acetates, (*b*) alditol acetate standards, (*c*) phthalic ester standards, (*d*) alditol acetate reaction-blank, using solvents and reagents as commercially supplied, omitting only the polysaccharide hydrolysate, (*e*) hexane-extractable substances in purified solid polysaccharide of *R. japonicum* before hydrolysis, and (*f*) hexane-extractable substances in acid hydrolysate of the polysaccharide. Examined on 1.5% EGS+GE-XF1150 columns at 195°.

The possibility that phthalic esters are present among alditol acetate preparations from polysaccharides creates obvious problems in the identification and determination of sugars by gas chromatography. The number of possible phthalic esters that could be present is large and unpredictable, and the esters may give rise to peaks having a wide range of retention times. It would be desirable, therefore, to be able to recognise the presence of phthalates in alditol acetate preparations, preferably by a gas-chromatographic method that could separate phthalates as a recognisable class. A number of columns representing a wide range of polarities was examined for this purpose, but none provided the desired separation of phthalates and alditol acetates.

The problem of phthalate contamination may be minimised by distilling all solvents used in the various analytical steps. As an alternative to distillation, it is possible to decrease the level of phthalate contamination by use of analytical procedures that involve the use of smaller volumes of potentially contaminated solvents. Whichever course of action is adopted, it is necessary first to be aware of the existence of the problem of phthalic ester contamination. If solvents and reagents are not distilled before use, the presence of phthalates may be found by examining a "reaction blank" (Fig. 1d) in which all steps are performed, omitting only the polysaccharide.

However, even purification of reagents is not necessarily sufficient to eliminate the problem posed by the phthalic esters because the polysaccharide samples themselves may be contaminated. Extraction of one of the *R. japonicum* polysaccharides with distilled hexane revealed the presence of volatile, extractable contaminants which, when analysed by gas chromatography, yielded peaks suggestive of phthalic esters (Fig. 1e). The identity of the contaminants was not investigated further, and it is possible that hexane-soluble contaminants other than phthalates were responsible. Largely similar peaks were obtained from hexane extracts of hydrolysates of the polysaccharide after it had been treated with 0.5M sulphuric acid for 24 h at 100°, indicating that the compounds responsible for the peaks, whatever their nature, were not degraded and eliminated by the acid hydrolysis (Fig. 1f). Treatment of a mixture of phthalic ester standards, in the presence of pyrene as internal standard[14], under the same acid conditions showed that di(2-ethylhexyl) and butylbenzyl phthalates are resistant to hydrolysis under these conditions commonly used for the hydrolysis of polysaccharides.

During this investigation, analytical-grade solvents and reagents from a number of commercial sources were examined and many were found to be contaminated by phthalates, the most common contaminants being butylbenzyl phthalate in methanol (up to 1.8 μg/ml) and in chloroform (15 μg/ml), and di(2-ethylhexyl) phthalate in acetic anhydride. Distilled water that had been allowed to flow through 3 m of plastic tubing was found to accumulate up to 15 μg of phthalic esters per litre. For acetylation procedures involving the addition of 5 × 5-ml portions of methanol and extraction into 10 ml of chloroform, in the worst case a total of 195 μg of butylbenzyl phthalate would be added to each sample from these two solvents alone. Quantities of this

magnitude may be relatively large in the analysis of the unavoidably small samples of polysaccharides and glycoproteins encountered in some biological investigations.

The presence of butylbenzyl phthalate as a contaminant may also cause problems in the gas-chromatographic analysis of sugars as their trimethylsilyl (Me_3Si) derivatives. On columns of OV-17 and OV-101, this phthalic ester matched the retention times of Me_3Si-cellobiitol and Me_3Si-D-*glycero*-D-*gulo*-heptitol, respectively.

The widespread occurrence of phthalic esters, the close similarity of their retention times to those of sugar derivatives on gas-chromatographic columns usually used for these derivatives, and the absence at present of any chromatographic procedure for distinguishing between peaks arising from phthalates and sugar derivatives, all combine to make it essential that the identification of sugar components in polysaccharides should not depend on gas–chromatographic methods alone. Whenever possible, the identity of sugars suggested by peaks on gas chromatograms should be confirmed by independent procedures, such as paper chromatography, that are not subject to interference by phthalic esters. Mass spectrometry is a particularly useful adjunct to gas chromatography in this context, because phthalates and sugar derivatives have quite discrete mass spectra. Phthalic esters as a class may be readily recognized from their electron-impact mass spectra because, except for the dimethyl ester, they all give a very characteristic ion at *m/e* 149 (ref. 15).

EXPERIMENTAL

Hydrolysis and analysis of polysaccharide. — A *Rhizobium japonicum* extracellular polysaccharide (10 mg) was hydrolysed in 0.5M sulphuric acid (10 ml) for 24 h at 100°, and a 1-ml sample taken for analysis by gas chromatography. The sample was neutralised with barium carbonate and centrifuged, and the sugars in the supernatant liquid were reduced by treatment with sodium borohydride (10 mg) overnight. After addition of excess glacial acetic acid, the sample was evaporated to dryness and then 5-ml amounts of methanol were evaporated five times from it. The dry residue was treated with acetic anhydride (1 ml) for 3 h at 120°. After addition of water (10 ml), the mixture was extracted with chloroform. The chloroform layer was washed successively with 5% sodium hydrogen carbonate and water, and dried with anhydrous sodium sulphate. The residue was dissolved in approximately 0.1 ml of chloroform. A reaction blank, omitting only the polysaccharide, was also prepared.

Gas-chromatographic analyses were performed with a Pye Series 104 Model 64 dual-column instrument fitted with flame-ionization detectors. Stainless-steel columns [2 m × 2 mm (i.d.)] were used throughout. The samples were analysed on columns of (*a*) 1.5% ethylene glycol succinate (EGS) + 1.5% silicone GE-XF1150 on acid-washed Chromosorb W (80–100 mesh), and (*b*) 3% ECNSS-M on Gas Chrom Q (80–100 mesh), using a nitrogen carrier-gas at a flow rate of 30 ml/min and isothermal conditions (195°) (injection port 250°; detector oven 250°).

Samples of the individual components were isolated by preparative gas-

chromatography by collecting them in chilled capillaries, and the mass spectra were determined with an AEI MS-902 instrument using a cooled insertion probe.

The presence of sugars in the hydrolysate was confirmed by paper chromatography of another neutralised sample, using 10:3:3 (v/v) butanol–pyridine–water as solvent and detection by the alkaline silver nitrate method.

Attempted group separation of phthalic esters and alditol acetates. — In addition to the columns already referred to, the following columns were used, under the conditions shown in parentheses: 5% squalane on Chromosorb W (80–100 mesh) (100° and 150°, isothermal; 40 ml nitrogen/min), 3% OV-101 on Chromosorb W (80–100 mesh) (temperature-programmed at 5°/min from 150 to 250°), 1.5% OV-17 on Gas Chrom Q (80–100 mesh) (temperature-programmed at 5°/min from 150 to 250°), 5% OV-210 on Gas Chrom Z (80–100 mesh) (isothermal at 220°; 20 ml/min), 3% OV-225 on Gas Chrom Q (80–100 mesh) (temperature-programmed at 5°/min from 150 to 250°), 3% 1,2,3-tris(2-cyanoethoxy)propane on Gas Chrom Q (80–100 mesh) (temperature-programmed at 5°/min from 100 to 150°, and also isothermally at 150°), Porapak Q (250°), and Carbopak A (250°).

Samples of (*a*) a chloroform solution of equimolar amounts of diethyl, di-(2-ethylhexyl) and butylbenzyl phthalates, and (*b*) a chloroform solution of the alditol acetates shown in Fig. 1b, were injected into the columns listed above. In no case was a clear separation of the two classes of compounds obtained.

Resistance of phthalic esters to hydrolysis. — A mixture of butylbenzyl phthalate (15.7 mg), di(2-ethylhexyl) phthalate (5.7 mg), and pyrene (7.2 mg) was dissolved in benzene (10 ml), and 0.05-ml aliquots were transferred to ampoules. The solvent was removed by evaporation, 1 ml of 0.5M sulphuric acid was added to each, and the ampoules were sealed. The samples were heated for 24 h at 100°, cooled, and the ampoules opened. The contents were transferred to a separatory funnel, and the ampoule and the aqueous solutions extracted with redistilled benzene (10 ml). The extracts were evaporated to dryness at room temperature in a stream of nitrogen, and the residues were dissolved in chloroform and analysed by gas chromatography on the EGS + GE-XF1150 columns isothermally at 195°. The phthalic esters were determined by comparison with the pyrene standard. The recoveries of butylbenzyl and di-(2-ethylhexyl) phthalates after the acid treatment were 40 and 96% of the initial values, respectively.

Interference by butylbenzyl phthalate with Me_3Si derivatives. — On columns of 1.5% OV-17, temperature-programmed at 2°/min from 200° and 40 ml of nitrogen, min, the following retention times were recorded: Me_3Si-sucrose 1.00 (13.0 min), Me_3Si-lactitol 1.14, Me_3Si-cellobiitol 1.21, Me_3Si-maltitol 1.32, and butylbenzyl phthalate 1.20. On columns of 3% OV-101 used under the same conditions as the OV-17 columns already described, butylbenzyl phthalate and Me_3Si-D-*glycero*-D-*gulo*-heptitol were found to have identical retention-times (10.2 min).

Determination of phthalate concentrations in solvents. — Experiments with mixtures of pyrene and phthalic ester standards established that, for equal weights of

compound, the relative detector-response was: pyrene 1.00, dibutyl phthalate 0.66, di(2-ethylhexyl) phthalate 0.62, and butylbenzyl phthalate 0.41.

The phthalate content of analytical grades of methanol was determined by evaporating 25-ml quantities under diminished pressure to remove volatile material, and then adding pyrene (0.35 mg) as a solution in hexane. The mixtures were analysed by gas chromatography on columns of EGS+GE-XF1150 isothermally at 195°. One brand of methanol was contaminated with two phthalates, one unidentified and the other the butylbenzyl ester, which constituted 82% of the 1.8 μg of total phthalate per ml of methanol. However, three other brands of methanol were free from detectable amounts of phthalates. Similarly, 50-ml volumes of analytical grade chloroform were analysed; butylbenzyl phthalate, present in amounts equal to 14.8 μg/ml chloroform, was the only contaminant detected.

The level of phthalate contamination in distilled water that passed through plastic tubing was determined by extraction of 1 litre with distilled hexane (100 ml), evaporation of the hexane, addition of the pyrene standard, and analysis as described. Dibutyl, di(2-ethylhexyl) and butylbenzyl phthalates, and two other contaminants, all in approximately equal amounts, were detected. Their total peak-areas were equivalent to a phthalate content of 15 μg/litre of water.

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