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SEPARATION OF SOME PERBENZOYLATED CARBOHYDRATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A number of hydroxy and polyhydroxy compounds, monosaccharides and disaccharides as their perbenzoylated derivatives were separated by high-performance liquid chromatography (Corasil II). The preparation of these derivatives and several parameters affecting their chromatographic resolution are discussed.

INTRODUCTION

During an investigation on substitution patterns of sugars with benzoyl chloride, it became necessary to establish chromatographic methods for separating and quantitating these derivatives. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were selected because of their speed, selectivity and sensitivity. Even though the benzoate esters are generally crystalline and easily detectable with ultraviolet (UV), literature references on TLC analysis[1,2] were few and none were found on HPLC analysis. Consequently, the chromatographic results obtained with perbenzoylated carbohydrates are now reported. The data should assist in identifying carbohydrates and related alcohols found in some complex mixtures.

The benzoate derivatives offer several advantages: (1) They can be detected easily by nondestructive means, *i.e.*, UV (Fig. 1) for TLC and HPLC or by a spray of water for TLC[3]. (2) Isolation is facilitated by virtue of their crystallinity and marked increase in molecular weight. (3) Because of their low volatility, losses due to evaporation are minimized. This is especially important when analyzing mixtures covering a broad range of molecular weights. Gas–liquid chromatographic (GLC) analysis of such carbohydrate mixtures can give widely variable results due to differential losses of the volatile trimethylsilyl (TMS) derivatives. The acetates, however, are useful alternatives for GLC analysis[4]. (4) The derivatives are easily and quickly prepared from common reagents.

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

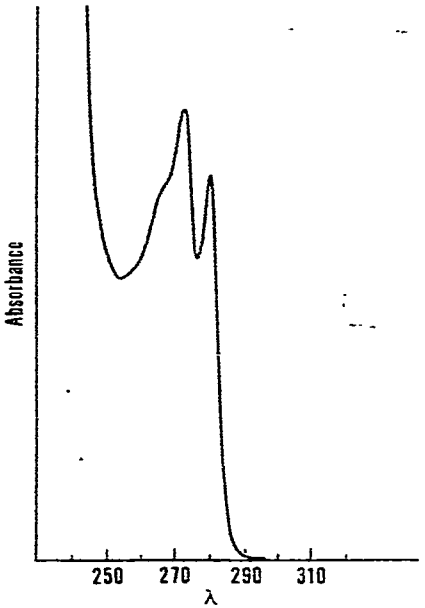

Fig. 1. UV scan of methyl 2,3,4,6-tetra-O-benzoyl α-D-glucopyranoside in hexane.

Because benzoates absorb in the UV range, the widely available and more sensitive UV detectors can be used for HPLC. Gains in sensitivity of up to 1000-fold over that possible with a differential refractometer can be obtained[5]. In addition, solvent gradients can be used with minimal baseline drift.

EQUIPMENT AND METHODS

Sample preparation

A 1- to 10-mg sample of sugar was dissolved in 2 ml pyridine in a 1-dram vial, and the anomers were equilibrated by heating at 62° for 1 h. After cooling to room temperature, benzoyl chloride (0.2 ml) was added and the solution re-heated at 62° for 45 min. The solution was cooled, methanol (0.5 ml) was added to decompose the residual benzoyl chloride and then evaporation by a dry stream of nitrogen produced an amorphous solid. The residue was redissolved in 2.5 ml chloroform, extracted once with 1 ml 1.0 *N* hydrochloric acid (the aqueous layer was removed with a Pasteur capillary pipette), neutralized with solid sodium bicarbonate and dried with anhydrous sodium sulfate. The solution was filtered and the residue was twice washed with 1 ml chloroform. The filtrate and the washings were combined and evaporated to approximately 1 ml. Sample size for HPLC and TLC varied from 0.5–3 μl.

Apparatus

HPLC was performed on a Nester and Faust Model 1210 liquid chromatograph equipped with a UV detector and fitted with a Corasil II 2 m × 2 mm I.D. stainless-steel column. Low void fittings and capillary tubing were used throughout. Samples were applied to the column via a septum injection port. A Hewlett-Packard Model

5751B gas chromatograph fitted with a 5-ft. 3% OV-17 column was used for GLC analysis.

HPLC

Traces of UV absorbing material were present in the diethyl ether, hexane and light petroleum (b.p. 30–60°) used as solvents. They were readily removed by passing the solvent through an activated silica gel column.

A linear gradient of diethyl ether in hexane (0–99% in 110 min) gave the best resolution with the mixtures studied. The flow-rate was maintained at 1 ml/min.

GLC

The simple benzoate esters were separated by GLC on a 5-ft. 3% OV-17 column at 150°. Retention times of the esters were as follows: methyl benzoate (0.41 min), ethyl benzoate (0.62 min), 1-propyl benzoate (1.04 min) and 1-butyl benzoate (1.74 min). The helium flow-rate was 30 ml/min.

The TMS-mono- and disaccharides were separated on the same 5-ft. 3% OV-17 column by holding the column at 150° for 3 min and then temperature programming to 215° at a rate of 4°/min. The retention times of the TMS ethers were: glucofuranose (5.6 min), α-D-glucopyranose (9.7 min), β-D-glucopyranose (14.1 min), α-maltose (22.5 min), and β-maltose (24.1 min).

TLC

For TLC analysis, Brinkman silica gel F_{254} plates were used. Sample spots on developed plates were located by spraying with 5% sulfuric acid in ethanol–water (7:3) and then heating at 130° for 30 min. Alternatively, if spots were to be extracted, they were detected by spraying the plate with water or by exposure to short-wave UV light.

RESULTS AND DISCUSSION

Reducing sugars, because of changes in ring size and anomeric configuration, afford a unique opportunity for identification. A multiplicity of spots or peaks on chromatograms and different relative intensities permit a better identification —a kind of fingerprint for each sugar. To utilize this characteristic, the forms that the sugars assume must be frozen at some equilibrium point. Benzoylation fixes the ring size and anomer configuration.

To compare samples from varied sources and physical states, a standard set of conditions was established. The carbohydrates were heated in dry pyridine at 62° for 1 h. To determine if equilibrium was reached under these conditions, solutions of D-glucose, L-rhamnose, maltose, D-mannose and D-galactose were treated as described. No statistical differences were found between samples taken at 1 and 3 h. D-Glucose was studied over a period of 27 h to determine the rate of anomerization and to detect possible isomerization or degradation (Fig. 2). Samples were periodically removed, trimethylsilylated and analyzed by GLC. No significant differences were found between the 1-h and 27-h samples.

Results were similar when glucose in pyridine was treated with benzoyl chloride. Benzoylation of the anomeric hydroxyl and consequent locking of the conforma-

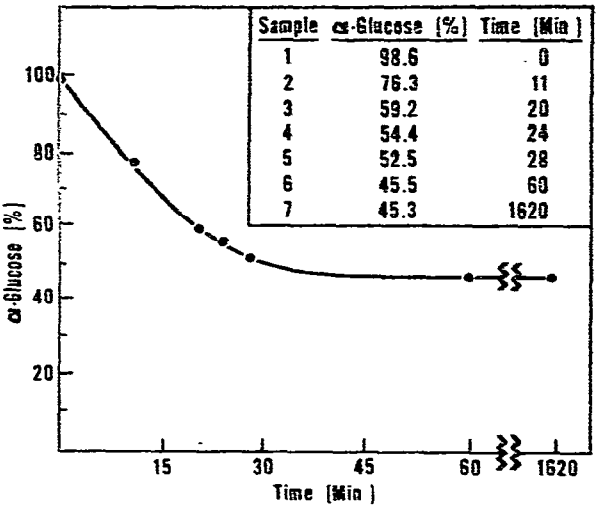

Fig. 2. Rate of anomerization of α-D-glucose in dry pyridine at 62°.

tion occur early in the reaction because no change in anomeric ratio of the perbenzoylated products was detected. For example, when crystalline α-D-glucopyranose was treated with benzoyl chloride shortly after dissolution in pyridine, it formed essentially α-D-glucopyranose pentabenzoate. Prior equilibration in pyridine at 62° produces a mixture of α- and β-D-glucopyranose pentabenzoates. A sample of the mixture was spotted on a TLC plate, developed with a diethyl ether–hexane (1:1) mixture and quantitated as previously described[6]. The same samples were run on Corasil II by HPLC. Agreement between HPLC, GLC and TLC was within 6%. Results were similar with L-rhamnose, D-mannose, and D-fructose.

Complex mixtures of hydroxyl-containing compounds can be easily and rapidly analyzed by conversion to the benzoate ester. For example, a mixture containing compounds with a wide range of molecular weights —such as methanol, 1-butanol, ethylene glycol, glycerol, D-xylose, D-glucose, D-mannose, D-galactose, sucrose, maltose, lactose, and maltotriose— can readily be separated by conversion to their benzoate esters and subsequent analysis by HPLC (Fig. 3).

The simple alcohol benzoates, *i.e.*, methyl, ethyl, propyl and butyl, can all be resolved at a low flow-rate. Unfortunately, the detector used was so sensitive to changes in flow-rate that attempts to flow program in order to resolve all the simple esters, along with the rest of the compounds, resulted in marked baseline shifts. The newer UV detectors do not present this problem.

Several unidentified impurities were noticed. The small peak at 6 min coincides with benzoyl chloride. The shoulder on the ethylene glycol peak probably represents an impurity in the starting material. The broad peak at 72 min is associated with lactose and may represent partially benzoylated products or another oligosaccharide present in commercial lactose.

Analysis time can be cut to 40 min by starting development with 1% diethyl ether–hexane and programming the gradient over 60 min rather than 110 min. Under these conditions peaks 7 and 8 almost coalesce. Nevertheless, mannose and glucose

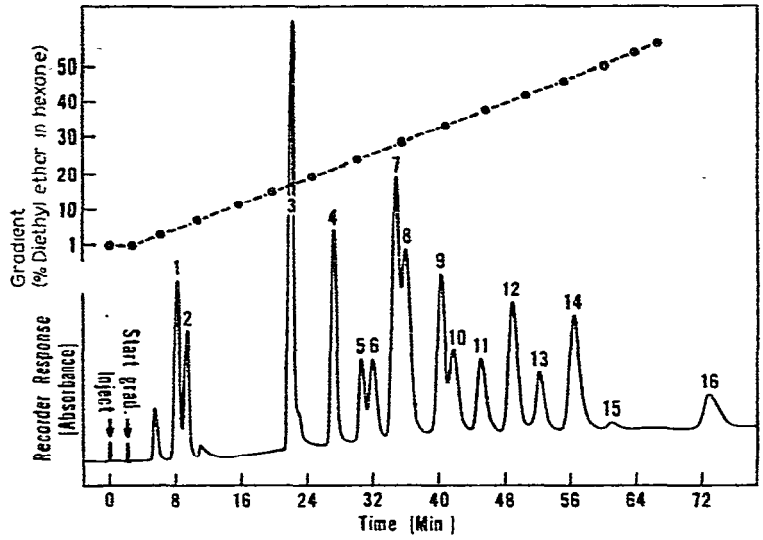

Fig. 3. Separation of 16 perbenzoylated hydroxy compounds on Corasil II (37–50 μm; Waters Assoc.) by HPLC. Temperature, ambient. Benzoates of: 1, 1-butanol; 2, methanol; 3, ethylene glycol; 4, glycerol; 5, α-D-xylose; 6, β-D-xylose; 7,α-D-mannose; 8, α-D-glucose, α-D-galactose; 9, β-D-glucose, D-galactose; 10, D-galactose; 11, β-D-mannose; 12, sucrose; 13, α- and β-maltose; 14, lactose; 15, maltotriose; 16, lactose oligosaccharides impurities.

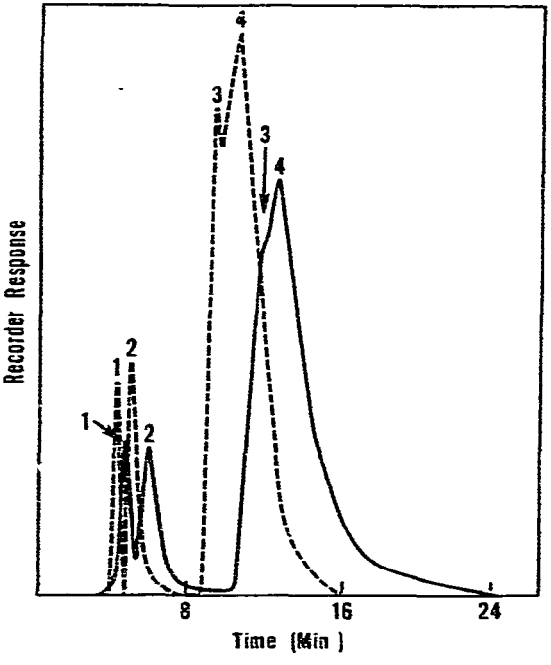

Fig. 4. Effect of temperature on the resolution of a mixture containing perbenzoylated α- and β-D-glucose and α- and β-maltose. 1 = α- D-Glucose; 2 = β- D-glucose; 3 = α-maltose; 4 = β-maltose. ———, Room temperature; — — —, 40°.

can still be differentiated by noting the presence or absence of peak 11. When an intermediate gradient time is used (80 min), peaks 7 and 8 once again separate and peak 15 occurs at 44 min.

An increase in flow-rate also decreased retention time. For example, at a flow-rate of 2 ml/min and a gradient elution time of 110 min, peak 15 occurs at 46.5 min rather than the 61 min shown in Fig. 3; however, the last two peaks (15 and 16) are excessively broadened.

Sometimes excessively broad peaks are sharpened by running the chromatogram at a raised temperature. Resolution may or may not suffer. For example, glucose and maltose perbenzoates were run at room temperature and at 40° (Fig. 4). At the higher temperature, maltose tailing was reduced by 20%, and the width of the peak at half height by 25%. Whereas the separation factor between α- and β-D-glucose was reduced by 16%, the resolution of maltose improved. A barely perceptible shoulder (α- and β-maltose) at room temperature became a doublet at 40°.

Use of the gradient did not cause any problems due to a shifting baseline. Although the percentage of diethyl ether in hexane varied from 0.5 to 99, the baseline

TABLE I

COMPARISON OF RETENTION TIMES OF A STANDARD BENZOATE MIXTURE WITH CHANGES IN GRADIENT

*Peak**	*Retention time (min)*				
	*1 ml/min, 110 min***	*1 ml/min, 60 min***	*2 ml/min, 110 min***	*1 ml/min, 70 min****	*1 ml/min, 80 min****
1	8.2	—	—	—	5.8
2	9.5	—	—	—	6.3
3	22.2	16.5	15.5	12.0	12.0
4	27.2	20.3	18.9	16	16.6
5	30.6	22.4	20.96	17.9	19.5
6	32	23.5	21.9	18.9	21.0
7	34.8	25.3§	24.0	21.0	23.4
8	36.1	26.1	24.9	21.0	24.2
9	40.2	28.7	28.3	24.3	27.7
10	41.7	29.7	30.0	24.3	28.8
11	45.2	32.0	32.4	27.2	31.5
12	48.9	33.7	35.3	29.8	34.5
13	52.3	35.4	38.4	32	37.1
14	56.3	37.4	—	34.4	40.2
15	60.9	40.3	46.5	36.5	43.1
16	71.4	—	—		54.2
Sorbitol	39.1	27.6			
L-Rhamnose		20.3, 25.3			
D-Ribose	33.8, 36.1				
Methyl α-D-glucopyranoside		24.5			
Methyl β-D-glucopyranoside		27.8			

* For the explanation of peaks, see the legend to Fig. 3.
** Starting concentration 1% diethyl ether in hexane.
*** Starting concentration 5% diethyl ether in hexane.
§ Shoulder.

did not deviate more than 2%. However, if impure solvents were used, a baseline shift of more than 40% was noted, and some spurious peaks appeared.

HPLC results are summarized in Table I.

CONCLUSIONS

HPLC of the benzoate esters offers an alternate and efficient means for identifying and isolating carbohydrates in complex mixtures. In addition, alcohols and glycols of low molecular weight, which might be missed by other techniques because of volatilization, can be detected and quantitated. The system described could be used as part of an overall scheme for analyzing a complex multicomponent system. The initial section of the chromatogram is open, and compounds of low polarity would show up in this region. Compounds of high polarity and molecular weight could probably be detected beyond peak 16 after switching to a more polar eluting solvent, such as 2-propanol–diethyl ether.

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