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ANALYSIS OF DERIVATIVES OF CARBOHYDRATES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The behaviour of benzoylated derivatives of alditols, monosaccharides, disaccharides, amino sugars, and methyl glycosides in high-pressure liquid chromatography (h.p.l.c.) has been investigated. A system was devised, using the most basic equipment of a single pump and fixed-wavelength u.v. detector, which gave good separations of the components of mixtures of derivatised methyl glycosides. Fractionation of complex mixtures of many of the other benzoylated carbohydrates was achieved in less than 30 min. The 4-nitrobenzoates were less useful for routine analyses.

INTRODUCTION

G.l.c. has long been used[1,2] to separate volatile derivatives of monosaccharides and to identify anomeric forms. Ion-exchange[3] or gel-filtration[4] techniques have been applied to monosaccharides and oligosaccharides, but may be time-consuming. For maximal sensitivity, both methods use destructive methods of detection, which is a major disadvantage when only small amounts of precious material are available.

H.p.l.c. offers an attractive, alternative approach, since it is relatively rapid and uses non-destructive detectors. The use of refractive-index detectors gave detection limits of ~ 20 μg of sugar[5]. Prior derivatisation with suitable chromophoric reagents allows sensitive u.v.-detectors to be used[6].

A method which would ultimately be applicable for routine analyses should be as simple as possible, involving a single pump and, for example, a fixed-wavelength

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detector. Moreover, the u.v.-absorbing derivatives should (*a*) be easy to prepare in high yield, (*b*) absorb strongly at a convenient wavelength, (*c*) be compatible with preferred solvent systems, (*d*) allow ready regeneration of the carbohydrate precursor, and (*e*) be suitable for n.m.r. spectroscopy and/or mass spectrometry. In this context, we have studied benzoates and 4-nitrobenzoates.

The chromatographic system should also employ commercially available packed-columns or column-packings, and readily available, pure, inexpensive, and stable solvent(s). A solvent mixture is preferred over a single solvent, so that the polarity can be finely adjusted to meet the exact requirements of a particular separation by altering the composition of the mixture. These requirements are met by a Porasil C column and ethyl acetate–hexane (1 : 5).

EXPERIMENTAL

Traces of u.v.-absorbing material were removed from hexane ("fraction from petroleum", b.p. 67–70°; B.D.H.) by stirring with conc. sulphuric acid and nitric acid (1 : 1), then washing with distilled water, 2M sodium hydroxide, and distilled water, drying ($CaCl_2$), passing through a column of basic alumina or silica gel, and distilling from anhydrous calcium chloride. Chromatographic-grade ethyl acetate and dichloromethane (AnalaR) were used directly. Benzoyl chloride (technical grade) was purified[7] and pyridine (AnalaR) further purified[7] and stored over molecular sieves.

^{1}H-N.m.r. spectra were recorded for 10% solutions in $CDCl_3$ (internal Me_4Si) with a Perkin–Elmer R12 spectrophotometer. U.v. spectra were recorded on a Unicam SP800 spectrophotometer. T.l.c. was performed on Kieselgel 60 F_{254} with solutions of benzoates in dichloromethane, the solvent systems given in Table I, and detection with u.v. light.

Preparation of derivatives. — To a solution of a sample (1–100 mg) of sugar in pyridine (1 ml) at 4° was added benzoyl chloride (1 equiv. per OH or NH_2 group + 1 equiv. excess) in 3 portions during 30 min with periodic shaking whilst keeping the temperature at ~4°. The mixture was left at 4° for 17 h followed by 2 h at room temperature. Distilled water (2 mol./mol. of excess benzoyl chloride) at ~0° was added with cooling in an ice-bath. After a further 2 h at room temperature, dichloromethane (1 ml) was added, and the solution was cooled to 4° and washed with M sulphuric acid (2 × 2 ml, at 4°) and saturated, aqueous sodium hydrogen carbonate (2 × 2 ml, at 4°). The organic layer was dried (Na_2SO_4), and the solvent removed. The residue was subjected to h.p.l.c. (Table II).

Small-scale preparations (up to 95 μg) were performed as described above. The reaction was stopped with water (10 μl), and the mixture was washed with 0.5-ml portions, dried, and concentrated in a stream of nitrogen. The residue was dissolved in chloroform (50 μl) prior to h.p.l.c.

Characterisation of derivatives. — Benzoates of methyl α- and β-D-glucopyranosides, prepared as described above, were recrystallised twice from ethyl

TABLE I

T.L.C. SEPARATIONS

Solvent system	R_F *values of benzoylated derivatives*					
	Benzoic anhydride	D-*Glucose*	D-*Galactose*		*Methyl* α-D-*glucopyranoside*	*Methyl* β-D-*glucopyranoside*
Methanol–dichloromethane (1:99)	0.75	0.60	0.62	0.52	0.56	0.52
Methanol–chloroform (1:99)	0.75	0.71	0.71	—	0.69	0.60
Dichloromethane–hexane (1:1)	0.41	0.06	0.08	—	0.10	0.06
Ethyl acetate–hexane (1:1)	0.62	0.52	0.55	0.45	0.61	0.56
Ethyl acetate–hexane (1:3)	0.33	0.13	0.15	0.07	0.19	0.17
Ethyl acetate–hexane (1:5)	0.35	0.10	0.12	0.04	0.14	0.10
Carbon tetrachloride	0.06	0	0	0	0	0
Tetrachloroethylene	0.08	0	0	0	0	0
Trichloroethylene	0.20	0	0	0	0	0
Toluene	0.44	0.13	0.14	—	0.12	0.07
Isopropyl ether	0.52	0.38	0.37	0.18	0.40	0.34
Dichloromethane	0.67	0.39	0.41	0.28	0.40	0.35
Butyl ether	0.74	0.47	0.46	0.32	0.43	0.27
Chloroform	0.56	0.48	0.52	0.42	0.54	0.49
Ethyl ether	0.69	0.59	0.57	—	0.60	0.59
Butyl acetate	0.88	0.84	0.79	—	0.86	0.82

TABLE II

ISOMERIC COMPOSITION OF THE PRODUCTS OF BENZOYLATION OF UNEQUILIBRATED CARBOHYDRATES

Carbohydrate	*Isomers*				
	1 T[a] (%)	*2* T (%)	*3* T (%)	*4* T (%)	*5* T (%)
Monosaccharides					
L-Arabinose	0.79 (8)	0.97 (83)	1.12 (9)		
D-Ribose	0.89 (9)	0.94 (65)	1.07 (26)		
D-Xylose	0.86 (100)				
D-Lyxose	0.79 (49)	0.89 (48)	1.01 (3)		
D-Glucose	1.21 (42)	1.33 (58)			
D-Galactose	1.00 (12)	1.08 (43)	1.19 (7)	1.26 (39)	
D-Mannose	1.09 (2)	1.18 (79)	1.60 (20)		
L-Rhamnose[b]	0.76 (23)	0.92 (9)	1.48 (29)	1.55 (12)	2.31 (21)
L-Fucose	0.74 (3)	0.79 (2)	0.86 (81)	1.12 (10)	1.27 (4)
D-Fructose[c]	0.99 (22)	1.04 (6)	1.10 (15)	1.20 (36)	1.44 (12)
Methyl glycosides					
Methyl α-D-glucopyranoside	1.00				
Methyl β-D-glucopyranoside	1.41				
Methyl α-D-galactopyranoside[d]	0.94				
Methyl β-D-galactopyranoside	1.25				
Methyl α-D-mannopyranoside	1.10				
Methyl α-D-xylopyranoside	0.73				
Methyl β-D-xylopyranoside	0.87				
Alditols[e]					
Xylitol	0.98 (98)	1.32 (2)			
D-Glucitol	0.88 (8)	1.28 (92)			
Galactitol	0.89 (2)	1.30 (85)	1.80 (13)		
D-Mannitol	1.30 (94)	2.11 (6)			
Disaccharides					
Lactose	3.30 (100)				
Maltose	2.95 (11)	3.17 (90)			
Sucrose	2.02 (2)	2.21 (97)	2.60 (3)		
Amino sugars					
2-Acetamido-2-deoxy-D-glucose	2.06 (14)	3.04 (86)			
2-Acetamido-2-deoxy-D-galactose	1.24 (36)	1.68 (5)	2.54 (59)		
2-Amino-2-deoxy-D-glucose hydrochloride	1.99 (2)	2.29 (2)	3.55 (89)	3.63 (4)	3.83 (3)
2-Amino-2-deoxy-D-galactose hydrochloride	1.62 (5)	1.86 (13)	2.28 (82)		
Chloroform	0.36				
Benzoic anhydride	0.56				
Methyl benzoate	0.47				

[a]Retention time relative to that of methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside. [b]Smaller peaks at *T* 0.99 (1%), 1.21 (2%), and 1.33 (2%). [c]Smaller peaks at *T* 0.76 (1%), 0.81 (1%), 0.86 (2%), and 1.36 (4%). [d]In two out of three preparations of this derivative, a second peak (*T* 1.34) was observed. These products were separated by column chromatography on silica gel and shown by ^{1}H-n.m.r. spectroscopy to be the tetrabenzoate and a tribenzoate, respectively. [e]Major peaks arise from the fully-benzoylated compound; faster-running, minor peaks are impurities, and slower-running, minor peaks are incompletely-benzoylated compounds.

TABLE III

SEPARATION OF MIXTURES

Mixture	T[a]	*Benzoate (isomer number)*
L-Arabinose, D-Lyxose, D-Ribose, D-Xylose	0.79	D-Lyxose (1), L-Arabinose (1)
	0.87	D-Xylose (1)
	0.89	D-Lyxose (2), D-Ribose (1)
	0.91	D-Ribose (2)
	0.95	L-Arabinose (2)
	1.05	D-Ribose (3)
	1.13	L-Arabinose (3)
D-Galactose, D-Glucose, D-Mannose	0.98	D-Galactose (1)
	1.09	D-Galactose (2), D-Mannose (1)
	1.12	D-Mannose (2), D-Galactose (3)
	1.19	D-Glucose (1)
	1.26	D-Galactose (4)
	1.32	D-Glucose (2)
	1.60	D-Mannose (3)
L-Fucose, D-Fructose, L-Rhamnose	0.75	L-Rha (1), L-Fuc (1), L-Fuc (2)
	0.86	L-Fuc (3)
	0.90	
	0.95	L-Rha (2)
	0.98	D-Fru (1)
	1.04	D-Fru (2)
	1.12	D-Fru (3), L-Fuc (4)
	1.20	D-Fru (4)
	1.27	L-Fuc (5)
	1.43	D-Fru (5)
	1.50	L-Rha (3)
	1.60	L-Rha (4)
	2.39	L-Rha (5)
L-Fucose, D-Fructose, D-Galactose, D-Glucose, D-Mannose, L-Rhamnose	0.76	L-Rha (1), L-Fuc (1), L-Fuc (2)
	0.86	L-Fuc (3)
	0.91	
	0.95	L-Rha (2)
	0.98	D-Fru (1), D-Gal (1)
	1.08	D-Gal (2), D-Man (1)
	1.12	D-Man (2), D-Gal (3), L-Fuc (4), D-Fru (3)
	1.19	D-Fru (4), D-Glc (1)
	1.27	D-Gal (4), L-Fuc (5)
	1.34	D-Glc (2)
	1.43	D-Fru (5)
	1.51	L-Rha (3)
	1.61	L-Rha (4), D-Man (3)
	2.39	L-Rha (5)

[a]Retention time relative to that of methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside.

acetate, by addition of hexane to a hot, concentrated solution (after filtration) until the mixture became turbid. After precipitation had occurred (10–15 min), the products were collected by filtration, washed with hexane, and dried. Characterisation of the products gave the following data.

Methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside: m.p. 102–103.5° (lit.[8] m.p. 104°), λ_{max}^{EtOH} 231 nm (ε 3.9 × 10^4). N.m.r. data: δ 3.49 (s, MeO), 4.5 (m, CH_2 and H-5), 5.3 (m, H-1,2), 5.71 (t, *J* 10.4 Hz, H-3 or H-4), 6.22 (t, *J* 10.4 Hz, H-3 or H-4), 7.4 and 8.0 (Ar*H*) (Found: C, 68.7; H, 5.3. Calc. for $C_{35}H_{30}O_{10}$: C, 68.8; H, 5.0%). This substance gave a single peak in h.p.l.c., and was used as a standard (*T* 1.00).

Methyl 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranoside: m.p. 159–160° (lit.[9] m.p. 159–160°), λ_{max}^{EtOH} 231 nm (ε 3.8 × 10^4). N.m.r. data: δ 3.54 (s, MeO), 4.1 (m, H-5), 4.7 (m, CH_2 and H-1), 5.7 (m, H-2,3,4), 7.4 and 8.0 (Ar*H*) (Found: C, 68.5; H, 5.3%). This substance gave a single peak in h.p.l.c. (*T* 1.41).

H.p.l.c. — A Waters Associates Model 204 liquid chromatograph was used, equipped with U6K septumless injector, 6000A solvent-delivery system, and a 400 single-channel absorbance detector (254 nm). A μ Porasil column (30 cm × 3.9 mm i.d.) was eluted with ethyl acetate–hexane (1 : 5) at 1 ml/min.

A solution of benzoate in ethyl acetate was diluted with hexane (5 vol.) immediately prior to injection. Precipitation of the derivative can occur on storage of the solution. Alternatively, a solution of benzoate in chloroform was injected (solvent peak, *T* 0.36).

The elution times of benzoates (relative to that of methyl 2,3,4,6-tetra-*O*-

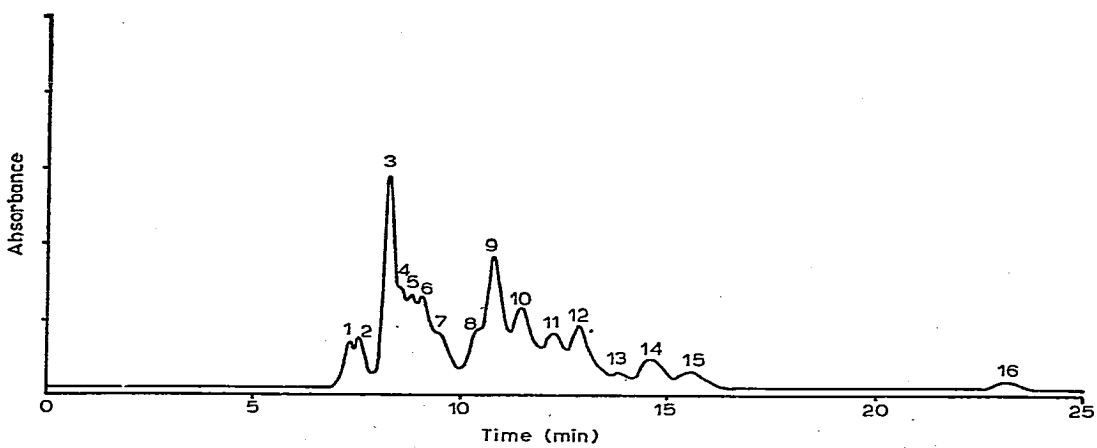

Fig. 1. H.p.l.c. of a benzoylated mixture of L-arabinose, L-fucose, D-fructose, D-galactose, D-glucose, D-lyxose, D-mannose, L-rhamnose, D-ribose, and D-xylose. Composition of peaks (benzoate isomer indicated in brackets): 1 (*T* 0.75) Fuc (1), Fuc (2), Rha (1); 2 (*T* 0.78) Ara (1), Lyx (1); 3 (*T* 0.86) Fuc (3), Xyl (1); 4 (*T* 0.88) Lyx (2), Rib (1); 5 (*T* 0.91) Rib (2); 6 (*T* 0.94) Ara (2), Rha (2); 7 (*T* 0.98) Fru (1), Gal (1); 8 (*T* 1.07) Gal (2), Man (2), Rib (3); 9 (*T* 1.12) Ara (3), Fuc (4), Fru (3), Gal (3), Man (2); 10 (*T* 1.19) Fru (4), Glc (1); 11 (*T* 1.26) Fuc (5), Gal (4); 12 (*T* 1.33) Glc (2); 13 (*T* 1.42) Fru (5); 14 (*T* 1.51) Rha (3); 15 (*T* 1.61) Man (3), Rha (4); 16 (*T* 2.39) Rha (5).

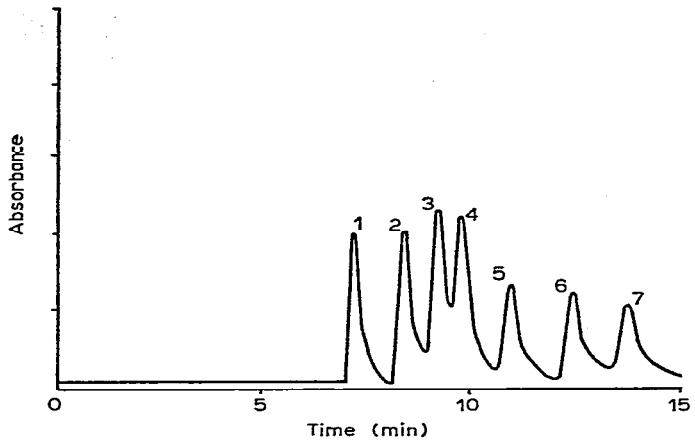

Fig. 2. H.p.l.c. of a mixture of the fully benzoylated methyl glycopyranosides of D-galactose, D-glucose, D-mannose, and D-xylose; 1 (*T* 0.73) Me-α-D-Xyl; 2 (*T* 0.87) Me-β-D-Xyl; 3 (*T* 0.95) Me-α-D-Gal; 4 (*T* 1.00) Me-α-D-Glc; 5 (*T* 1.13) Me-α-D-Man; 6 (*T* 1.28) Me-β-D-Gal; 7 (*T* 1.41) Me-β-D-Glc.

TABLE IV

EFFECT OF REACTION CONDITIONS ON THE ISOMERIC COMPOSITION OF THE PRODUCTS OF BENZOYLATION OF D-RIBOSE

Reaction conditions[a]	*Abundance of isomers (%)*		
	1	*2*	*3*
1 Standard	8	67	25
	8	64	28
2 Pre-heating D-ribose in pyridine (100°/30 min)	13	69	18
	13	68	19
3 Pre-heating D-ribose in pyridine (60°/1 h)	12	67	21
4 Reaction temperature (100°/4 h)	9	66	25
5 Reaction time (20°/4 h)	4	70	26
6 100 mg of D-ribose in 20 ml of pyridine-	3	64	33
	4	60	36
7 Dilution with dichloromethane (2.5 ml) prior to benzoylation	3	70	26
8 Pre-mixed reagents added to solid D-ribose	12	66	22

[a]D-Ribose (250 mg) in pyridine (2 ml) was used (see Experimental), except for 3 (50 mg, 0.5 ml) and 6.

benzoyl-α-D-glucopyranoside) and compositions (based on the combined areas of peaks) are recorded in Table II. Data for the analysis of mixtures are given in Table III and Figs. 1 and 2.

The effect on isomeric composition of varying the benzoylation conditions of D-ribose is shown in Table IV. The effect[10] of heating the carbohydrate in pyridine at 60° for 1 h before derivatisation is shown in Table V.

TABLE V

ISOMERIC COMPOSITION OF THE PRODUCTS OF BENZOYLATION OF EQUILIBRATED CARBOHYDRATES

Carbohydrate	*Isomers*				
	1 T[a] (%)	*2* T (%)	*3* T (%)	*4* T (%)	*5* T (%)
L-Arabinose	0.77 (14)	0.93 (46)	0.99 (7)	1.10 (32)	
D-Ribose	0.85 (12)	0.90 (67)	1.03 (21)		
D-Lyxose	0.75 (78)	0.86 (22)			
D-Xylose	0.85 (100)				
D-Glucose	1.20 (63)	1.35 (37)			
D-Galactose	1.01 (5)	1.11 (69)	1.21 (5)	1.27 (21)	
D-Mannose	1.09 (3)	1.17 (74)	1.63 (20)	1.99 (3)	
L-Rhamnose[b]	0.77 (55)	0.92 (24)	1.48 (11)	1.55 (1)	2.31 (7)
L-Fucose	0.77 (8)	0.88 (37)	1.12 (55)		
D-Fructose[c]	1.01 (32)	1.08 (13)	1.24 (18)	1.31 (7)	1.50 (7)

[a]Retention time relative to that of methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside. [b]Smaller peaks at *T* 0.58 (1%), 1.04 (1%), and 1.28 (0.3%). [c]Smaller peaks at *T* 0.96 (3%), 1.13 (4%), 1.42 (2%), and 1.59 (2%).

TABLE VI

REPRODUCIBILITY OF H.P.L.C. SEPARATION OF BENZOYLATED D-RIBOSE

Separation	*Isomers*		
	1 T[a] (%)	*2* T (%)	*3* T (%)
1	0.89 (9)	0.94 (65)	1.07 (26)
2	0.83 (8)	0.86 (67)	0.99 (25)
3	0.84 (8)	0.88 (64)	1.01 (28)
4	0.81 (5)	0.85 (73)	0.98 (21)
5	0.82 (7)	0.86 (69)	0.99 (24)

[a]Retention time relative to that of methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside.

Reproducibility of separations. — The same column has been used over several months, giving excellent reproducibility with various samples. Reproducibility was checked with D-ribose (Table VI).

DISCUSSION

None of the common derivatives of carbohydrates (acetates, methyl ethers, and trimethylsilyl ethers) are suitable for h.p.l.c. using a u.v. detector. Compared with acetates, benzoates absorb strongly at 254 nm (the fixed wavelength of the standard

detector) and are generally easier to crystallise and more stable, and were therefore investigated. Some h.p.l.c. data on methyl glycopyranoside tribenzoates have been reported[11], and more recently a thorough study of perbenzoylated carbohydrates appeared[10] (gradient elution from Corasil II with ether–hexane). Our system (single solvent, faster analysis time) is as efficient as, and more convenient than, that of Lehrfeld[10].

When benzoylation mixtures are poured into a large excess of water[12,13], benzoic anhydride is formed and is difficult to separate from the product[14]. Use of a calculated amount of methanol to destroy the excess of benzoylating agent[10] results in contamination of the product with methyl benzoate as well as benzoic anhydride. Both benzoic anhydride (T 0.56) and methyl benzoate (T 0.47) can be separated from the derivatised carbohydrate by h.p.l.c. Recrystallisation of the products from aqueous ethanol[14] may lead to an ethyl glycoside. Recrystallisation from ethyl acetate–hexane removed benzoic anhydride but not materials with retention times in t.l.c. lower than that of the product, whereas silica gel filtration with ethyl acetate–hexane (1:3) removed these materials but not completely the benzoic anhydride.

The standard method finally adopted was a modification of Fletcher's method[14] whereby free sugars gave a mixture of fully benzoylated α- and β-anomeric forms of pyranoid and furanoid structures, possibly accompanied by small proportions of under-benzoylated products, and methyl glycosides gave a single product (with the exception of methyl α-D-galactopyranoside which was contaminated with some tribenzoate). Characterisation of the products of methyl glucosides, using combustion analysis and spectroscopic methods, showed that fully benzoylated products were formed in all cases.

From the t.l.c. data in Table I, ethyl acetate–hexane (1:5) was selected for h.p.l.c., and the results are shown in Table II and Fig. 1. Reproducibility of retention times relative to that of methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside was good (Table IV). The sensitivity of the method was such that the product of benzoylation of 20 ng of methyl α-D-glucopyranoside gave a 10% scale-deflection at maximum sensitivity. The limit of detectability could be increased 10-fold if the detecting wavelength was 230 nm, since this corresponds to the λ_{max} for benzoates.

Good separations of anomeric pairs of methyl glycosides were obtained (Fig. 2). Fractionation of simple mixtures of sugars is possible, but overlap of some peaks occurs with more complex mixtures, thereby complicating interpretation (*cf.* Table III and Fig. 1). The fact that more than one peak per carbohydrate is obtained can be useful when analysing materials of biological origin, since more-certain identification is possible. Hexitol hexabenzoates had similar retention times, although separation from pentitol pentabenzoates should be possible (Table II). The benzoates of amino sugars and disaccharides were well separated from the other groups.

The effect of the reaction conditions on the ratio of isomeric products is important (Table IV), and the use of standardised conditions is essential. Lehrfeld[10] ensured that equilibrium between isomers had been reached by heating a pyridine solution of the sugar at 62° for 1 h. The mixture of isomeric benzoates obtained after

pre-equilibration will not correspond to the equilibrium if isomerisation is more rapid than benzoylation and the various isomers are not benzoylated at the same rate. For D-ribose, which has a propensity to adopt furanoid forms (*e.g.*, 24% of furanoid anomers in D_2O at 35°)[15], extensive isomerisation occurred under all conditions (Table IV). In most cases, equilibration before derivatisation showed (*cf.* Tables II and V) that the isomeric compositions were markedly different, with a tendency for an increase in isomer 1. Benzoylation of some sugars in hot pyridine is reported[12,13] to lead to furanose derivatives. When the concentration of D-ribose was decreased 10-fold, there was an increase in the amount of isomer 3 at the expense of isomers 1 and 2. The use of a 4-h reaction time at either room temperature or 100°, compared to 17 h at 4° or 2 h at room temperature, had no effect on the isomeric composition of the product mixture.

4-Nitrobenzoates have[16] a λ_{max} (260 nm) close to that (254 nm) of the standard u.v. detectors for h.p.l.c., and they provide an ~10-fold increase in sensitivity compared to benzoates. When 4-nitrobenzoyl chloride was used in the standard esterification procedure, a mixture of fully and partially derivatised products was formed, which were less soluble in ethyl acetate–hexane and more polar than benzoates and therefore less useful for h.p.l.c., particularly if 230- or 235-nm detectors are used. Hexane–chloroform–acetonitrile–water mixtures have been reported[17] for the h.p.l.c. of 4-nitrobenzoates.

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