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ANALYSIS OF PHENYLDIMETHYLSILYL DERIVATIVES OF MONOSACCHARIDES AND THEIR ROLE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBOHYDRATES

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SUMMARY

The preparation and characterisation of phenyldimethylsilyl derivatives of alditols, monosaccharides and disaccharides are described. The use of such derivatives in high-performance liquid chromatography of carbohydrates has been investigated and a simple isocratic system for the separation and quantitation in sub microgram amounts of complex mixtures of derivatised methyl glycosides and derivatised monosaccharides in under 20 min is described.

INTRODUCTION

Gas–liquid chromatography (GLC) 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 are normally time-consuming methods. For maximal sensitivity, such methods use destructive methods of detection, which is a major disadvantage when only small amounts of precious material are available.

High-performance liquid chromatography (HPLC) offers an attractive, alternative approach, since it is more rapid and normally uses non-destructive detectors. The use of refractive-index detection has been made for the separation of monosaccharides[5,6] and oligosaccharides[7] but the method is only sensitive to *ca.* 20 μg. The use of the mass detector, a destructive detection system has resulted in an increase in sensitivity, by a factor of ten[8], combined with improved stability and the ability to run gradient elution. Non-specific detection, via ultraviolet (UV) absorption at,

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or below, 200 nm has also been used[9], but requires high-purity (high-cost) solvents to run even limited gradient systems.

The use of pre-column derivatisation of carbohydrates, with a suitable chromophoric reagent allows the use of UV detectors at higher wavelength, with the advantage of cheaper solvents and cheaper detectors (fixed wavelength). The UV-absorbing derivative 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 nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry. A number of derivatives have been proposed, including acetates[10], benzoates[11], 4-nitrobenzoates[12] and benzyloxime-perbenzoates[13], but the major drawback to their use is their time-consuming preparation.

We now report on the development of a substituted silyl derivative which can be prepared easily and chromatographed using a simple isocratic system based on a commercially available column packing (Partisil 5) and readily available, pure, stable and inexpensive solvents (ethyl acetate and hexane).

EXPERIMENTAL

Materials

Phenyldimethylsilyl chloride obtained from Fluorochem or Cambrian Chemicals and imidazole (GPR) obtained from BDH Chemicals were used directly. N,N-Dimethylformamide (AnalaR) was purified[14] and stored over molecular sieves. Ethyl acetate (spectroscopy grade, BDH) and hexane (HPLC grade, Rathburn Chemicals) were used directly.

Large-scale preparation of derivatives

The method of preparation is based on that of Dodd *et al.*[15]. To a solution of monosaccharides (100 mg) in dry N,N-dimethylformamide (2.5 ml) was added imidazole (2.5 equiv. per OH group) and the mixture heated at 100°C for 1 h. After cooling in ice, phenyldimethylsilyl chloride (1.2 equiv. per OH group) was added and allowed to react for 6 h at ambient temperature. The product was extracted with hexane (2 × 3 ml) and the combined extracts washed with water (3 × 3 ml), dried over anhydrous sodium sulphate and evaporated with a nitrogen stream. The resulting oil was stored at −20°C.

For analytical studies the oil was diluted with hexane and purified by a modification of the flash chromatographic method described by Still *et al.*[16], using silica gel 60 with ethyl acetate–hexane (1:9) as eluent.

Small-scale preparation of derivatives

For routine application of the method to the HPLC analysis of carbohydrates, the following modified method was employed. To a solution of carbohydrate (0–10 mg) in N,N-dimethylformamide (150 μl) contained in a screw cap spectrum vial was added imidazole solution (0.33 g/ml N,N-dimethylformamide, 200 μl). After heating at 100°C for 1 h and subsequent cooling in ice phenyldimethylsilyl chloride (70 μl) was added and the reaction allowed to proceed for 6 h at ambient temperature. (For convenience the reaction can be allowed to continue for 18 h or heated at 100°C for 1 h without detriment.) The product was extracted with hexane (2 × 200 μl) and

used directly for analysis or stored at −20°C. For increased sensitivity the hexane extract can be concentrated using a stream of nitrogen.

Elemental analysis

Purified derivatives of D-glucose, D-galactose, methyl α- and β-D-glucopyranosides were found to have the following analyses: D-glucose derivative (Found: C, 61.5; H, 6.8%. Calc. for $C_{46}H_{62}O_6Si_5$: C, 64.9; H, 7.34%); D-galactose derivative (Found: C, 65.4; H, 7.7%); methyl α-D-glucopyranoside derivative (Found: C, 65.2; H, 8.2%. Calc. for $C_{39}H_{54}O_6Si_4$: C, 64.1; H, 7.44%); and methyl β-D-glucopyranoside derivative (Found: C, 65.0; H, 8.0%).

Thin-layer chromatographic (TLC) analysis

TLC was performed on Kieselgel 60 F_{254} on aluminium foil with solutions of phenyldimethylsilyl derivatives in hexane, using the solvent systems given in Table I. Detection was by UV light or 1-naphthol reagent[17] (see Fig. 1). Fluorescence quenching densitometry was carried out, using a Perkin-Elmer MPF 4 fluorimeter equipped with a scanning attachment, on the products obtained from a large-scale preparation prior to purification, in order to assess the amount of product and impurities obtained (see Table II).

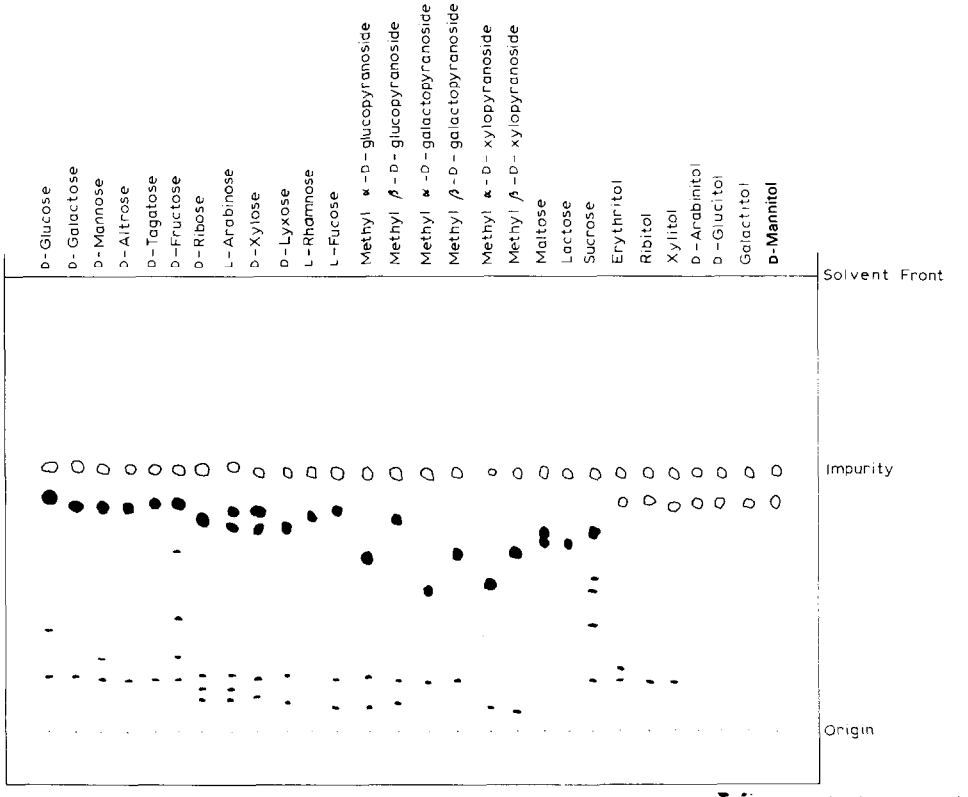


Fig. 1. TLC separation of the products obtained by the small-scale derivitisation method (eluent, ethyl acetate–hexane, 1:9). All spots were detected with UV light, but the unshaded spots did not react with the 1-naphthol reagent.

TABLE I

TLC SEPARATIONS

Solvent system	*R_F values of phenyldimethylsilyl derivatives*				
	Impurity band	D-*Glucose*	D-*Galactose*	*Methyl* α-D-*glucopyranoside*	*Methyl* β-D-*glucopyranoside*
Methanol–dichloromethane (1:99)	0.96	0.96	0.96	0.89	0.96
Methanol–chloroform (1:99)	0.83	0.83	0.83	0.83	0.83
Dichloromethane–hexane (1:1)	0.74	0.52	0.53	0.11	0.25
Ethyl acetate–hexane (1:9)	0.69	0.44	0.42	0.32	0.42
Ethyl acetate–hexane (1:3)	0.70	0.63	0.62	0.61	0.65
Diethyl ether–hexane (1:3)	0.56	0.50	0.45	0.41	0.47
1,1-Dichloroethane–hexane (1:9)	0.72	0.63	0.61	0.21	0.43
1,1-Dichloroethane–hexane (1:1)	0.76	0.76	0.76	0.38	0.58
Toluene–hexane (3:1)	0.77	0.50	0.44	0.13	0.29
Chloroform	0.44	0.06	0.05	0.03	0.03
Carbon tetrachloride	0.70	0.70	0.70	0.67	0.70
1,1-Dichloroethane	0.91	0.91	0.91	0.91	0.91

TABLE II

FLUORESCENCE QUENCHING DENSITOMETRIC SCAN OF TLC SEPARATION OF PHENYLDIMETHYLSILYL DERIVATIVES USING ETHYL ACETATE–HEXANE (1:9)

Derivative	R_F	$\frac{Peak\ area}{Total\ area}$ (%)
D-Glucose	0.52*	11.1
	0.46 }	80.9
	0.42 }	
	0.11**	8.0
D-Galactose	0.55*	10.3
	0.45 }	82.9
	0.39 }	
	0.09**	6.8
Methyl α-D-glucopyranoside	0.54*	11.8
	0.34	72.1
	0.99** }	16.1
	0.04 }	
Methyl β-D-glucopyranoside	0.53*	8.0
	0.44	80.1
	0.19** }	11.9
	0.10 }	

* Impurity.

** Partially derivatised material arising from incomplete derivatisation or partial degradation during the washing process of the large-scale preparation. This material does not occur when the small-scale preparation method is used.

Infrared (IR) analysis

IR spectra of the purified derivatives were recorded as thin films using a Pye Unicam SP1050 calibrated at 907 and 1602 cm^{-1} (see Fig. 2a–d).

Ultraviolet analysis

UV spectra were recorded on a Hilger and Watts Ultrascan equipped with 10-mm path length cells using hexane solutions of the purified derivatives. Series of peaks in the range 240–280 nm were obtained (see Table III).

^1H NMR analysis

^{1}H NMR spectra (100 MHz) were recorded for solutions of the purified de- showed characteristic multiplets (δ = 0.2 ppm) for the Si-CH_3 protons, arising from the diastereotopic nature of these methyl protons, and (δ = 7.10 ppm) for the aromatic protons. Other characteristic resonances observed included:

For *methyl α-D-glucopyranoside derivative*, a doublet (δ = 4.18 ppm, J = 4 Hz) for the anomeric proton and a singlet (δ = 3.00 ppm) for the glycosidic methyl group.

For *methyl β-D-glucopyranoside derivative*, a doublet (δ = 3.90 ppm, J = 7 Hz) for the anomeric proton and a singlet (δ = 2.78 ppm) for the glycosidic methyl group.

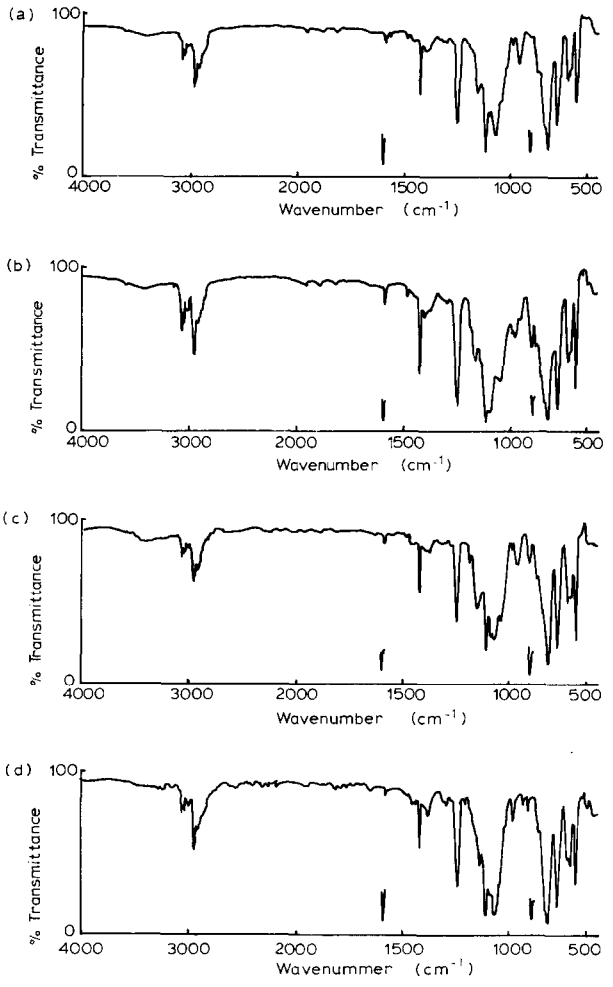


Fig. 2. Infra-red spectra of the fully phenyldimethylsilylated derivatives of D-glucose (a), D-galactose (b), methyl α-D-glucopyranoside (c) and methyl β-D-glucopyranoside (d).

For D-*glucose derivative*, a doublet (δ = 4.63 ppm, J = 4 Hz) for the pyranose α anomeric proton and a doublet (δ = 4.38 ppm, J = 7 Hz) for the pyranose β anomeric proton which were present in approximately equal proportions (from integration values).

For D-*galactose derivative*, a doublet (δ = 4.35 ppm, J = 7 Hz) for the pyranose β anomeric proton, a doublet (δ = 4.92 ppm, J = 4 Hz) for the pyranose α anomeric proton and a doublet (δ = 5.08 ppm, J = 4 Hz) for a furanose anomeric proton. These were present in the ratio 4:2:1.

HPLC analysis

A system comprising an Altex 110A pump, Rheodyne 7125 sample injection valve fitted with a 20-μl sample loop, Cecil CE 272 spectrophotometer fitted with a 20-μl flow cell and a Watanabe Servocorder was used. A Partisil 5 column (25 cm

TABLE III

UV ANALYSIS

Derivative	λ_{max} *(nm)*	ε	*Derivative*	λ_{max} *(nm)*	ε
D-Glucose	254	880	Methyl α-D-	254	720
	260	1290	glucopyranoside	260	1050
	265	1250		265	989
	270	839		270	648
D-Galactose	254	916	Methyl β-D-	254	817
	260	1340	glucopyranoside	260	1170
	265	1310		265	1060
	270	900		270	733

× 4.6 mm I.D., Whatman) was eluted with ethyl acetate–hexane with different compositions (within the range 1:49–1:199) depending on the nature of the carbohydrate derivatised. Typically injections were of 3 μl if no concentration of the hexane extract was made. The retention times of the derivatives, relative to an unretained compound, k', and the isomeric compositions (based on the combined areas of peaks) are recorded in Tables IV–VII. Typical analyses of mixtures are shown in Figs. 3–5.

The same column has been used over several months giving excellent reproducibility in terms of k' and efficiency with various samples.

DISCUSSION

The use of silyl derivatives in the analysis of carbohydrates[2] is now well established, but the commonly used trimethylsilyl derivatives are not suitable for HPLC due to their high volatility, rapid hydrolysis and non-absorbance of UV light. Monoalkyl dimethylsilyl derivatives such as (1,1-dimethylethyl)dimethylsilyl derivatives[15] have been shown to be more stable towards hydrolysis and suitable intermediates and protecting agents in, for example, lipid synthesis. The use of an aromatic substituent such as a phenyl group provides intermediate stability whilst conferring UV absorbing characteristics on the derivative.

The initial method of preparing the derivative showed that, on the large (100-mg) scale, the degree of conversion to a fully derivatised material was high with only traces of partially derivatised material, as indicated by the absence of slower running bands on TLC (see Table II). This was further supported by the physicochemical methods described. The major impurity, present in all preparations, is a fast running band; the proportion of which is dependent on the amount of residual water present in the N,N-dimethylformamide. When sufficient water to react with the amount of phenyldimethylsilyl chloride present was added to the solvent the only product formed was that having the fast running band on TLC. This impurity is thought to be tetramethyldiphenylsiloxane. With solvent dried, and stored, over freshly activated molecular sieves, the amount of this impurity can be kept below the 10% level as recorded by fluorescence quenching densitometry of TLC separations.

TABLE IV

HPLC SEPARATION OF PERPHENYLDIMETHYLSILYL DERIVATIVES OF NEUTRAL MONOSACCHARIDES

Eluent: ethyl acetate–hexane (1:99)

Carbohydrate	*Isomer*							
	1		*2*		*3*		*4*	
	k'	*(%)*	*k'*	*(%)*	*k'*	*(%)*	*k'*	*(%)*
L-Arabinose	4.1	(31)	4.6	(34)	5.9	(35)		
D-Ribose	3.6	(34)	4.0	(66)				
D-Lyxose	4.1	(80)	4.9	(20)				
D-Xylose	2.9	(62)	4.1	(38)				
D-Altrose	2.6	(34)	3.7	(39)	5.5	(27)		
D-Galactose	3.0	(62)	3.3	(25)	3.8	(13)		
D-Glucose	2.4	(38)	2.8	(62)				
D-Mannose	3.0	(88)	3.5	(12)				
D-Fructose	2.3	(11)	3.1	(78)	3.2	(10)	3.9	(1)
D-Tagatose	1.9	(2)	2.3	(42)	2.8	(51)	3.2	(5)
L-Fucose	2.4	(2)	2.6	(44)	3.3	(44)	3.7	(10)
L-Rhamnose	2.8	(4)	3.2	(9)	3.7	(87)		
Methyl α-D-glucopyranoside	≈10							
Methyl β-D-glucopyranoside	3.4							
Methyl α-D-galactopyranoside	≈20							
Methyl β-D-galactopyranoside	≈10							

In order to provide a method of preparation suitable for the analysis of many samples on the 0–10-mg scale, we modified our original method to use a single quantity of reagent, etc., rather than a calculated amount and investigated various parameters of the reaction to give a versatile method which requires the minimum of attention. Where analyses are required rapidly, the reaction can be completed by heating at 100°C, but, for convenience, allowing the reaction to stand overnight prior to extraction with hexane causes no loss of product and allows the use of push fit stoppered vials.

From TLC data (see Table I), it was evident that a system based on ethyl acetate–hexane was suitable for HPLC analysis, but in order to provide detailed resolution of the various anomeric forms and carbohydrate ring types present in the derivatisation mixture of a single monosaccharide and maximum separation from the impurity component, a system having a much lower polarity must be used. Initially an eluent comprising ethyl acetate–hexane (1:99) was selected for HPLC (Table IV), but alditols (Table V) were found to require a less polar solvent system (ethyl acetate–hexane, 1:199) and disaccharides (Table VI) and methyl glycosides (Table VII) required more polar systems (ethyl acetate–hexane, 3:197 and 1:49 respectively) to allow quantitation of the results. Resolution was such that mixtures of carbohydrates could be resolved (see Figs. 4 and 5) and quantitated although not all peaks were fully resolved. 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 and methods for analysing and quantitating overlapping peaks have been described[18].

TABLE V

HPLC SEPARATION OF PERPHENYLDIMETHYLSILYL DERIVATIVES OF ALDITOLS

Eluent: ethyl acetate–hexane (1:199)

Carbohydrate	*k'*
Erythritol	1.7
Ribitol	1.5
Xylitol	1.7
D-Arabinitol	2.1
D-Glucitol	1.5
Galactitol	1.5
D-Mannitol	2.3

TABLE VI

HPLC SEPARATION OF PERPHENYLDIMETHYLSILYL DERIVATIVES OF DISACCHARIDES

Eluent: ethyl acetate–hexane (3:197)

Carbohydrate	*Isomer*			
	1		*2*	
	k'	*(%)*	*k'*	*(%)*
Lactose	2.8	(27)	4.4	(73)
Maltose	2.5	(62)	3.7	(38)
Sucrose	2.7	(100)		

Good separation of anomeric pairs of methyl glycosides was obtained (Fig. 3) and such is the sensitivity of the system that it was possible to detect the equivalent of 440 ng of methyl β-D-glucopyranoside with detection at 254 nm or 250 ng at 260 nm using maximum electronic amplification available in order to obtain a peak height of 10% full scale deflection which was also greater than three times the deflections due to "noise" on the chart recorder. This sensitivity was obtained by accurate dilution of the product obtained by derivatising 10 mg of the glycoside and extracting

TABLE VII

HPLC SEPARATION OF PERPHENYLDIMETHYLSILYL GLYCOPYRANOSIDES

Eluent: ethyl acetate–hexane (1:49)

Carbohydrate	*k'*
Methyl α-D-glucopyranoside	5.6
Methyl β-D-glucopyranoside	2.3
Methyl α-D-galactopyranoside	8.4
Methyl β-D-galactopyranoside	3.8
Methyl α-D-xylopyranoside	6.4
Methyl β-D-xylopyranoside	3.6

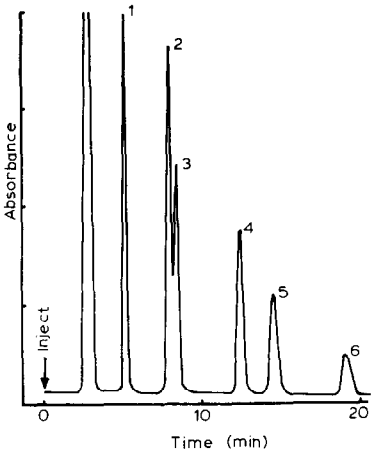
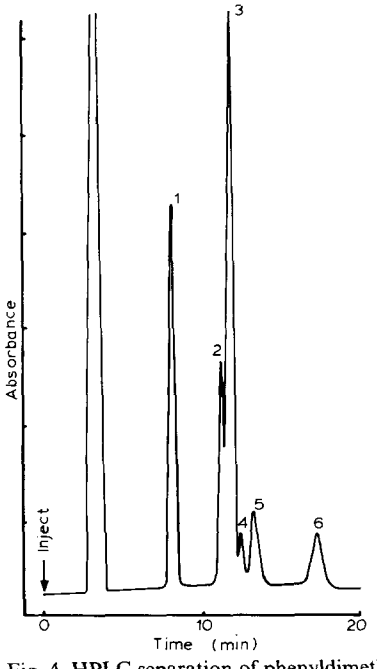


Fig. 3. HPLC separation of phenyldimethylsilyl derivatives of methyl glycosides (eluent, ethyl acetate–hexane, 1:49 at 1.5 ml/min). Peaks: 1 = methyl β-D-glucopyranoside; 2 = methyl β-D-xylopyranoside; 3 = methyl β-D-galactopyranoside; 4 = methyl α-D-glucopyranoside; 5 = methyl α-D-xylopyranoside; 6 = methyl α-D-galactopyranoside.



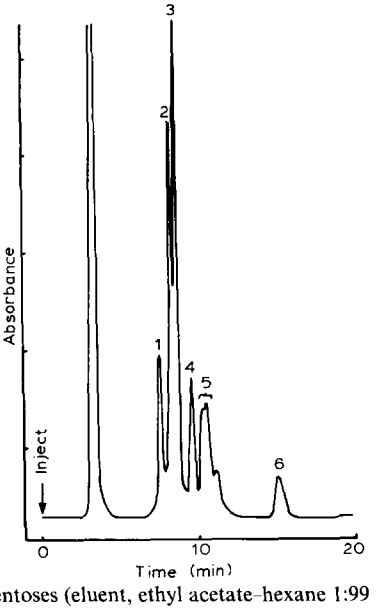


Fig. 4. HPLC separation of phenyldimethylsilyl derivatives of pentoses (eluent, ethyl acetate–hexane 1:99 at 1.5 ml/min). Peaks: 1 = D-xylose; 2 = D-ribose; 3 = L-arabinose, D-ribose, D-lyxose, D-xylose; 4 = L-arabinose; 5 = D-lyxose; 6 = L-arabinose.

Fig. 5. HPLC separation of phenyldimethylsilyl derivatives of hexoses (conditions as in Fig. 4). Peaks: 1 = D-glucose; 2 = D-altrose, D-glucose; 3 = D-galactose, D-mannose; 4 = D-galactose; 5 = D-altrose, D-galactose, D-mannose; 6 = D-altrose.

into a total volume of 1.0 ml hexane. Obviously, if the hexane solution is concentrated prior to analysis and larger injection volumes used (up to 20 μl) the sensitivity can be further increased. A further increase in sensitivity can also be obtained if detection at 210 nm can be made, but this will require the use of purer solvents to ensure that the "noise" level of the system is kept low.

REFERENCES

1 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497–2507.
2 J. F. Kennedy, S. M. Roberston and M. Stacey, *Carbohydr. Res.*, 49 (1976) 243–258.
3 J. F. Kennedy, *Biochem. Soc. Trans.*, 2 (1974) 54 64.
4 J. F. Kennedy and J. E. Fox, *Carbohydr. Res.*, 54 (1977) 13–21.
5 J. K. Palmer, *Anal. Lett.*, 8 (1975) 215–224.
6 E. Tarelli and C. A. White, *J. Biol. Stand.*, 10 (1982) 95–103.
7 C. A. White, P. H. Corran and J. F. Kennedy, *Carbohydr. Res.*, 87 (1980) 165–173.
8 R. Macrae and J. Dick, *J. Chromatogr.*, 210 (1981) 138–145.
9 H. Binder, *J. Chromatogr.*, 189 (1980) 414–420.
10 G. B. Wells and R. L. Lester, *Anal. Biochem.*, 97 (1979) 184–190.
11 C. A. White, J. F. Kennedy and B. T. Golding, *Carbohydr. Res.*, 76 (1979) 1–10.
12 F. Nachtmann and K. W. Budna, *J. Chromatogr.*, 136 (1977) 279–287.
13 R. M. Thompson, *J. Chromatogr.*, 166 (1978) 201–212.
14 D. D. Perrin, W. L. F. Armarego and D. R. Perrin, *Purification of Laboratory Chemicals*, Pergamon, Oxford, 1966.
15 G. H. Dodd, B. T. Golding and P. V. Ioannou, *J. Chem. Soc., Perkin Trans I*, (1976) 2273–2277.
16 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 43 (1978) 2923–2925.
17 H. Jacin and A. R. Mishkin, *J. Chromatogr.*, 18 (1965) 170.
18 J. F. Kennedy, *Chromatographia*, 3 (1970) 316–319.