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G.L.C. OF THE *O*-TRIMETHYLSILYL DERIVATIVES OF HEXURONIC ACIDS*

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ABSTRACT

The free acids, sodium salts, and lactones of several hexuronic acids have been studied as their *O*-trimethylsilyl derivatives by gas–liquid chromatography using SE-30 and XE-60 liquid phases. Silylation was best performed in methyl sulphoxide. The equilibrium between the various forms of a hexuronic acid in methyl sulphoxide was also studied by g.l.c. following silylation. The hexamethyldisilazane used in the silylation disturbed the equilibrium attained in the solvent, but this was overcome by premixing the hexamethyldisilazane with chlorotrimethylsilane. Methyl sulphoxide and the silylating reagents gave a two-phase system in which the derivative was favourably partitioned into the upper layer. Partition coefficients and stabilities of the derivatives were measured, and a g.l.c. method for the analysis of the hexuronic acids was thereby developed. The oximes of the hexuronic acids were studied as alternative derivatives for g.l.c., and their equilibrium compositions and g.l.c. retention times are recorded.

INTRODUCTION

The hexuronic acids are constituents of many biologically vital polysaccharides[1–3], and it is important that accurate methods for their determination be available. Decarboxylation procedures (assay of evolved carbon dioxide) and chromatography of polysaccharide hydrolysates have been used for this purpose. G.l.c. of volatile trimethylsilyl derivatives[4] has revolutionized the analysis of monosaccharides, and several techniques have been developed for the determination of the hexuronic acids. This has led to important discoveries such as the detection of both L-iduronic and D-glucuronic acids in heparin[5,6]. Perry and Hulyalkar[7] studied, as mixtures, the five hexuronic acids occurring naturally in polysaccharides, but their reduction of the hexuronic acid to the corresponding aldonolactone has the disadvantage of introducing another step into the analysis procedure.

*Dedicated to the memory of Professor Edward J. Bourne.

A more satisfactory, direct method of analysis[8] has emerged from studies of g.l.c. of the trimethylsilyl derivatives of the free acids, sodium salts, lactones, methyl glycosides, and methyl ester methyl glycosides. With pyridine as solvent, each uronic acid could best be determined as the trimethylsilyl derivative of the methyl ester methyl glycoside. Although there was much overlap of peaks, it was claimed that each hexuronic acid studied (L-iduronic acid was excluded) showed one well-separated peak which permitted identification and quantitation in a mixture. For D-mannuronic and L-guluronic acids, however, the relevant peak represented only a small part of the total hexuronic acid, and hence large errors were likely to arise. More recently, D-glucurono-6,3-lactone and D-galacturonic acid have been studied using wall-coated, open, tubular columns[9], but without prior equilibration of the various forms.

A variety of silylating reagents are available commercially, but many of them, particularly those supplied in the form of combined reagent plus solvent, do not permit anomerisation to proceed to a stable equilibrium. Since the anomeric composition of samples of hexuronic acids may vary, any sound analytical technique must be based on a known distribution of isomers. We now report on the equilibration and silylation of the five, naturally occurring hexuronic acids, D-galacturonic acid, D-glucuronic acid, L-guluronic acid, L-iduronic acid, and D-mannuronic acid, and their related forms, and the development of a rapid and accurate method for their analysis.

RESULTS AND DISCUSSION

Since each uronic acid can exist in five forms, the ratios of which depend on the equilibrium reached in a given solvent system, it was necessary to investigate the conditions of equilibration and the equilibrium reached in the solvents chosen for analysis. Pyridine is a solvent widely used for silylation reactions of sugars, but we experienced low degrees of conversion with the hexuronic acids. Although pyridine is a good solvent for the neutral sugars, it is a poor solvent for the hexuronic acids, and after several hours at 37° the concentration of sodium D-glucuronate attained was 0.6 mg/ml (Fig. 1) compared with concentrations >20 mg/ml attained rapidly in methyl sulphoxide. Tetrahydrofuran and *N,N*-dimethylformamide were also better solvents than pyridine (Fig. 1). Furthermore, studies in pyridine indicated a lower rate of silylation for the hexuronic acids and their salts compared to the lactones and a D-glucose standard, due to the formation of the trimethylsilyl ester. No partially silylated derivatives were found by g.l.c., suggesting that ester formation was a prerequisite for volatility. Silylation in methyl sulphoxide was immediate in all cases, and its use is recommended.

A two-phase system resulted when methyl sulphoxide was used as the solvent in the silylation reaction. Ellis[10] demonstrated the immiscibility of hexamethyldisilazane and methyl sulphoxide, but stated that addition of chlorotrimethylsilane effected miscibility; the subsequent rapid formation of hexamethyldisiloxane then re-formed the two-phase system. However, in the present study, the formation of a small amount of hexamethyldisiloxane, resulting from the presence of traces of water, was found to be of little importance.

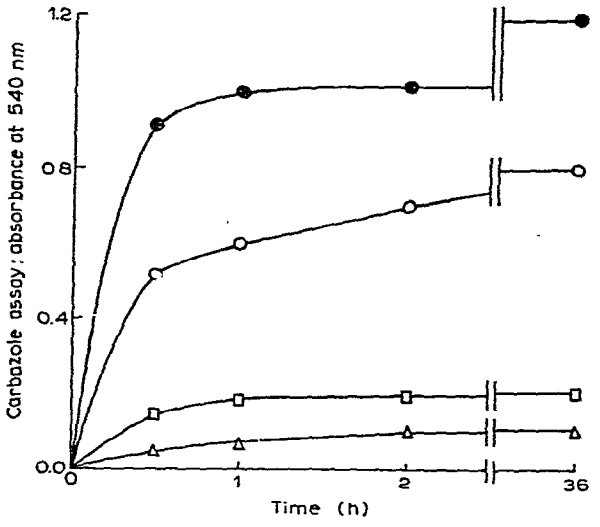


Fig. 1. Rate of dissolution of sodium D-glucuronate in methyl sulphoxide (—●—), tetrahydrofuran (—○—), *N*,*N*-dimethylformamide (—□—), and pyridine (—△—).

The infrared spectra of hexamethyldisilazane and the upper layer from the silylation reaction mixture were almost identical. A strong absorption at 1180 cm^{-1} (Si–N) was found in both spectra, while the Si–O absorption at 1050 cm^{-1} was observed, as a weak absorption, in the spectrum of the upper layer only. Addition of water resulted in complete loss of the Si–N band and the production of a strong Si–O absorption. G.l.c. analysis (direct insertion on SE-30) confirmed that the upper layer was mainly hexamethyldisilazane together with traces of methyl sulphoxide and impurities, and indicated that the lower layer was mainly methyl sulphoxide together with hexamethyldisilazane and chlorotrimethylsilane.

The trimethylsilyl derivatives of the hexuronic acids and lactones were favourably partitioned into the upper layer, and the sensitivity of the technique was thereby increased. The data in Table I show that the lactones were only partially partitioned, whereas complete partitioning was observed for the parent acids and salts. The acid molecules, with five trimethylsilyl groups, present a surface of non-polar methyl groups, and are partitioned to the hexamethyldisilazane layer which similarly presents a surface of methyl groups. For the same reason, complete partition was also noted for neutral sugars processed in the same way. The lactones, however, with only three trimethylsilyl groups, present a slightly polar surface, particularly at the lactone group, and are partially retained in the more-polar methyl sulphoxide layer. With the exception of D-mannurono-6,3-lactone, the hexurono-6,3-lactones possessed similar partition coefficients, the values of which reduced the g.l.c. detection sensitivity to ~30% of that of the corresponding acids. For D-mannurono-6,3-lactone, the amount detected was dependent on the distribution of isomers, as their partition coefficients were different. For the other lactones, the partition coefficients for the two isomers

TABLE I

DISTRIBUTION OF THE TRIMETHYLSILYL DERIVATIVES OF HEXURONIC ACIDS BETWEEN THE TWO LIQUID PHASES FORMED ON SILYLATION IN METHYL SULPHOXIDE

Substrate	T (*SE-30*)	*Amount in upper layer* (%)[a]	*Amount in lower layer* (%)[a]	*Amount in new upper layer after resilylation of lower layer* (%)[a]	*Partition coeff. (upper/lower)*
D-Glucurono-6,3-lactone	1.20, 1.27	33	12.2	28	3.0 ±0.5
D-Glucuronic acid	1.73, 2.10, 2.53	100	0	0	∞
Sodium D-glucuronate	1.73, 2.10, 2.53	100	0	0	∞
D-Galacturonic acid	1.48, 1.89, 2.02, 2.60	100	0	0	∞
Sodium D-galacturonate	1.48, 1.89, 2.02, 2.60	100	0	0	∞
D-Mannurono-6,3-lactone	1.22, 1.48	46	6.1	37	4.3 ±0.5, 7.0 ±1.0
Sodium D-mannuronate	1.52, 2.11, 2.21, 2.54	100	0	0	∞
L-Idurono-6,3-lactone	4.14, 4.32[b]	63	5.8	39	9.6 ±1.0
Sodium L-iduronate	1.48, 1.80, 2.00	100	0	0	∞
L-Gulurono-6,3-lactone	0.86, 1.15	72	0	0	∞
Sodium L-guluronate	1.60, 1.91, 2.42, 2.67	100	0	0	∞

[a]Measured relative to amount of D-glucuronic acid detected. [b]On XE-60.

were identical, and therefore the isomer distribution was the same as that in the original solution.

The trimethylsilyl derivatives of the lactones were stable in the silylation mixture for up to 4 h, and the acid and salt derivatives for more than 8 h, thus permitting satisfactory analysis. The relative instability of the lactone derivatives was thought to be due to their appreciable retention in the methyl sulphoxide layer.

Sweeley *et al.*[4] stated that the silylation reaction had no effect on the anomeric equilibrium for a monosaccharide in a particular solvent, but they did not study any hexuronic acids. Using the standard procedure (consecutive addition of hexamethyldisilazane and chlorotrimethylsilane to a solution of the hexuronic acid in pyridine, Method *A*) of these workers, we found a small, but significant, difference in the measured ratio of isomers compared to that found using hexamethyldisilazane in pyridine (Table II). With methyl sulphoxide as solvent, a far greater difference was observed, both in the initial and final distribution of isomers. A comparison of the measured isomer ratios found using each method with that expected in the crystalline hexuronic acid indicated that Method *B* (premixing of the silylation reagents) gave a more reproducible result (Table III), and one which more accurately represented the state in the solid; *e.g.*, D-glucuronic acid gave 90% of one isomer; D-glucurono-6,3-lactone, 100% of one isomer; and D-galacturonic acid 98% of one isomer. Method *A* produced variable mixtures similar in isomer ratios to that eventually found at equilibrium. This disturbance of the true isomer ratios in solution usually resulted in the preferential formation of the derivatives of a particular isomer in the upper layer, as shown in a comparison of the two methods of silylation on the unequilibrated hexuronic acids (Table III). Less effect was noted on the equilibrated solutions, although method *B*, which results in immediate silylation, gave more reproducible results. Since silylation appeared to occur immediately after the addition of chlorotrimethylsilane, the hexamethyldisilazane must have been responsible for the disturbance. It is therefore possible that both a preferential silylation and a changed

TABLE II

DETERMINATION OF THE EFFECT OF HEXAMETHYLDISILAZANE ON THE EQUILIBRIUM ISOMERIC COMPOSITION OF SODIUM D-GLUCURONATE IN PYRIDINE

Incubation time (min)	*Isomeric composition before addition of chlorotrimethylsilane*			*Extent of silylation (%)*[a]	*Isomeric composition after addition of chlorotrimethylsilane*			*Extent of silylation (%)*[a]
	1 T *1.73*	2 *2.10*	3 *2.53*		1 T *1.73*	2 *2.10*	3 *2.53*	
5	—	—	—	0	5	43	52	100
30	10	36	54	61	12	36	52	100
60	18	31	51	85	21	30	49	100
120	17	31	52	100	21	31	48	100

[a]Based on combined areas of peaks: analysis on SE-30.

TABLE III

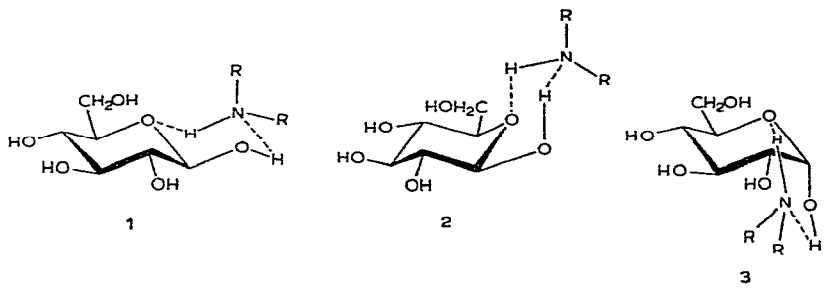
EFFECT OF SILYLATION UPON THE ISOMER DISTRIBUTION OF HEXURONIC ACIDS IN METHYL SULPHOXIDE

Substrate	*State of isomer equilibrium*	*Method of silylation*	*Amount of each isomer present (%)*[a]			
			1[b]	2[b]	3[b]	4[b]
D-Glucurono-6,3-lactone[c]	Unequilibrated	*A*	13	87		
		B	0	100		
	Equilibrated	*A*	13	87		
		B	8	92		
D-Glucuronic acid	Unequilibrated	*A*	27	23	50	
		B	0	10	90	
	Equilibrated	*A*	41	15	44	
		B	4	44	52	
Sodium D-glucuronate	Unequilibrated	*A*	44	18	38	
		B	13	18	69	
	Equilibrated	*A*	27	31	42	
		B	31	24	35	
D-Galacturonic acid	Unequilibrated	*A*	34	17	19	30
		B	1	0	98	1
	Equilibrated	*A*	51	13	14	22
		B	57	12	16	15
Sodium D-galacturonate	Equilibrated	*A*	80	20		
		B	82	18		
D-Mannurono-6,3-lactone[c]	Unequilibrated	*A*	63	37		
		B	10	90		
	Equilibrated	*A*	76	24		
		B	75	25		
Sodium D-mannuronate	Equilibrated	*A*	56	36	6	2
		B	60	27	13	0
L-Idurono-6,3-lactone[c,d]	Unequilibrated	*A*	94	6		
		B	96	4		
	Equilibrated	*A*	90	10		
		B	93	7		
Sodium L-iduronate	Equilibrated	*A*	38	52	10	
		B	36	54	10	
L-Gulurono-6,3-lactone[c]	Equilibrated	*A*	85	15		
		B	87	13		
Sodium L-guluronate	Equilibrated	*A*	43	30	9	18
		B	86	8	6	0

[a]Measured on SE-30 unless otherwise stated. [b]In order of increasing *T*, see Table VI. [c]% Values measured at 180° using upper layer. [d]Measured on XE-60.

equilibrium, catalysed by hexamethyldisilazane and resulting from the methyl sulphoxide–hexamethyldisilazane solvent system, were responsible.

Incubation of a solution of D-glucuronic acid in methyl sulphoxide with hexamethyldisilazane and monitoring of the upper, hexamethyldisilazane layer by g.l.c. revealed preferential silylation to produce a particular peak, T 1.73. A similar result was obtained with D-galacturonic acid, considerably increased formation of the peak at T 1.48 being observed (Table III). G.l.c. analysis of the lower, methyl sulphoxide layer showed no derivative to be present, but silylation of this layer by method *B* indicated that the isomer ratios of the underivatised D-glucuronic acid had been altered by the initial addition of hexamethyldisilazane. The presence of catalytic quantities of hexamethyldisilazane in the uronic acid solution failed to produce a major distortion of the isomer ratios. It is thus possible that the change in isomer ratios found above was the result of a new equilibrium due to the presence of hexamethyldisilazane in the methyl sulphoxide solvent system. The speed of attainment may have resulted from an interaction with a single molecule of hexamethyldisilazane [two possibilities (**1** and **2**) for the β anomer; one (**3**) for the α anomer].



Hexamethyldisilazane was found to be a poor silylating reagent, requiring several hours to effect full derivatization. The addition of chlorotrimethylsilane, which acted both as silylating agent and catalyst, caused immediate derivatization. Resilylation of the derivative-containing upper layer indicated complete silylation.

Having established that *B* is the best silylation method for assessment of the hexuronic acid isomers, the attainment of equilibrium of each hexuronic acid was studied (Figs. 2 and 3). Equilibrium had been reached by all samples 4 h after dissolution in methyl sulphoxide. This finding is in contrast to evidence presented by Jacin *et al.*[11] that methyl sulphoxide, being aprotic, allows little or no mutarotation. Although our studies on the neutral sugars supported this statement, the hexuronic acids underwent mutarotation, and initial evidence from kinetics indicated autocatalysis. This cannot, however, be the whole explanation, as the presence of a hexuronic acid caused no increase in the rate of mutarotation of α-D-glucose in methyl sulphoxide. Also, intramolecular catalysis by the carboxylic proton was not possible, as the lactones and sodium salts also mutarotated.

The use of lithium perchlorate[12] increases the rate of mutarotation of sugars in pyridine and methyl sulphoxide, an incubation of 2 h at 37° being necessary to achieve

complete equilibration. Lithium perchlorate was not used in the present studies, as the process of equilibration was being investigated. In routine analysis, however, it should be included.

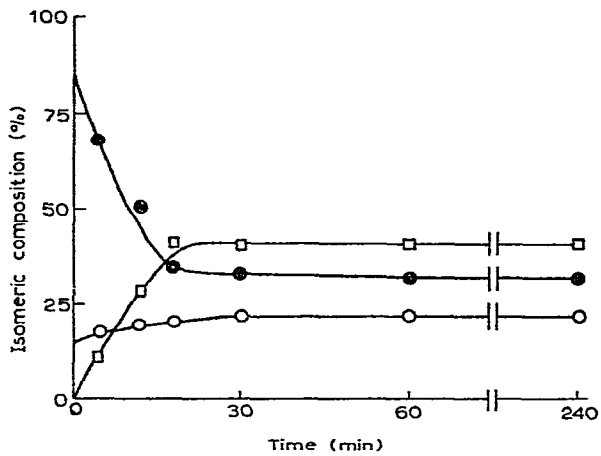


Fig. 2. Variation of isomer distribution on incubation of a solution of sodium D-glucuronate in methyl sulphoxide, determined *via* silylation: —●—, isomer *T* 2.53; —○—, isomer *T* 2.10; —□—, isomer *T* 1.73 (analysis on SE-30).

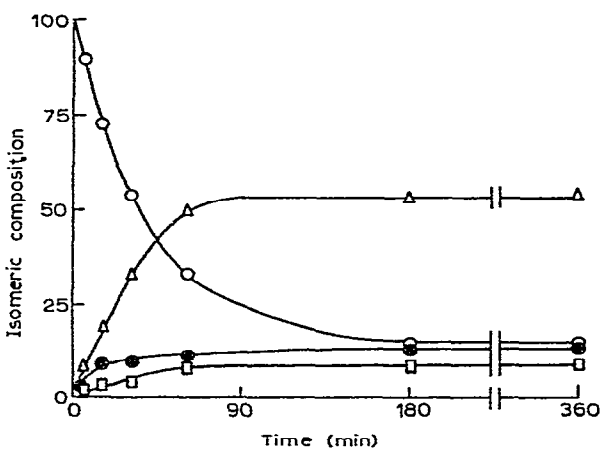


Fig. 3. Variation of isomer distribution on incubation of a solution of D-galacturonic acid in methyl sulphoxide, determined *via* silylation: —●—, isomer *T* 2.60; —○—, isomer *T* 2.02; —□—, isomer *T* 1.89; —△—, isomer *T* 1.48 (analysis on SE-30).

A reduced flame-ionisation response per unit weight of sample was noted for the hexuronic acid and the lactone derivatives at solution concentrations greater than ~1 and ~4 mg/ml respectively, the upper layers of the silylation mixtures being analysed. At no time was hexuronic acid derivative found in the lower layer. Resilylation of the lower layer at the higher concentrations revealed underivatized hexuronic acid, suggesting that the original silylation was incomplete. Further incubation produced no increase in derivative formation, and it appeared that a maximum concentration had been achieved in the upper layer. Since no derivative could exist in the methyl sulphoxide layer, attainment of this maximum effectively prevented further silylation. On the other hand, the hexurono-6,3-lactones, the derivatives of which were partitioned between two layers, were silylated quantitatively over a greater range.

The effect of concentration on the equilibrium of the hexuronic acids was sometimes important. D-Galacturonic acid showed a marked change over the concentration range 0–1 mg/ml (Fig. 4), the peak at *T* 1.48 occurring in greater proportion for lower concentrations. A similar trend was observed for sodium D-glucuronate (Fig. 4). Since the equilibrium should be independent of total concentration, these

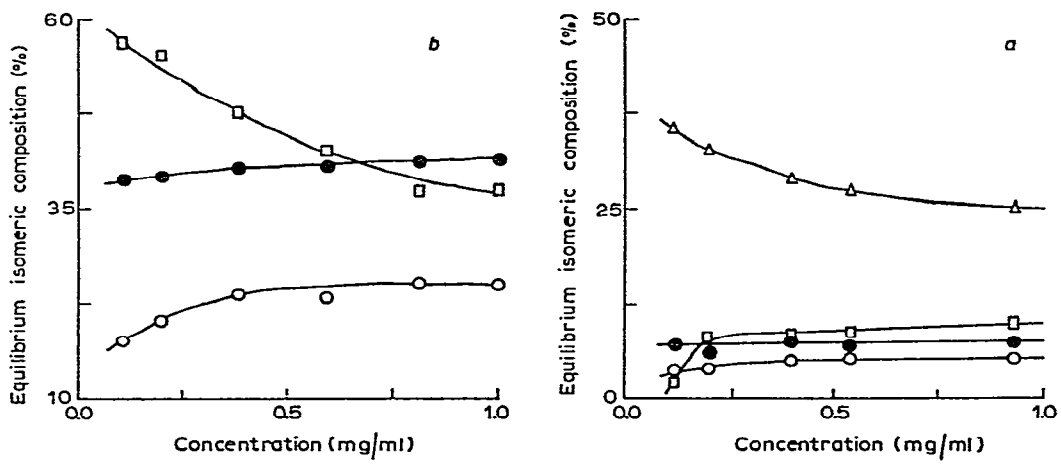


Fig. 4. The effect of concentration of hexuronic acid upon the apparent equilibrium in methyl sulphoxide, as measured *via* silylation: (*a*) D-Galacturonic acid: —●—, isomer *T* 2.60; —○—, isomer *T* 2.02; —□—, isomer *T* 1.89; —△—, isomer *T* 1.48 (analysis on SE-30). (*b*) Sodium D-glucuronate: —●—, isomer *T* 2.53; —○—, isomer *T* 2.10; —□—, isomer *T* 1.73 (analysis on SE-30).

changes must be a result of the silylation reaction. However, the changes were only small in most cases (Table IV).

The presence of 0.4% of water in the methyl sulphoxide caused a serious loss of quantitation by decreasing the formation of the trimethylsilyl derivatives; 2% solutions caused complete decomposition in only 3 h. The presence of water produced an increase in the volume of the upper layer with a consequent loss of measured peak

TABLE IV

THE EFFECT OF CONCENTRATION OF HEXURONIC ACID UPON THE APPARENT EQUILIBRIUM IN METHYL SULPHOXIDE, AS MEASURED VIA SILYLATION

Substrate	*Concentration (mg/ml)*	*Amount of each isomer present (%)*[a]			
		1[b]	2[b]	3[b]	4[b]
Sodium D-galacturonate	1.0	82	16	2	1
	0.1	86	14	1	1
D-Glucurono-6,3-lactone	1.0	8	92		
	0.1	8	92		
D-Glucuronic acid	1.0	7	40	53	
	0.1	6	44	50	
L-Iduronic acid	0.5	30	57	13	
	0.1	32	56	13	
D-Mannurono-6,3-lactone	1.0	79	21		
	0.2	83	17		
Sodium D-mannuronate	1.6	45	45	7	3
	0.1	41	52	6	1

[a]Based on combined areas of peaks: analysis onSE-30. [b]Isomers in order of increasing T (See Table VI)

intensity from an analysis sample of fixed volume. However, the total loss of detected derivative could not be entirely explained by the decrease in derivative concentration in the upper layers, and the general instability caused by the presence of water makes it necessary to use rigorously anhydrous conditions.

The effects of the volume of silylation reagent on the eventual volume of the upper layer and consequently on the derivative detection were studied (Table V). The use of smaller volumes of silylating reagents gave smaller upper-layer volumes and, consequently, higher detector responses for the same solution concentration. This was despite a lack of complete derivatization with the small volumes. The optimum silylating conditions, giving complete silylation with highest detection response, were hexamethyldisilazane and chlorotrimethylsilane volumes of 100 and 25 μl or 50 and 100 μl for 250 μl of methyl sulphoxide solution; the former ratio is preferred, as hexamethyldisilazane is more stable.

Retention times (T) were measured for each isomer against a standard ribitol sample, both on SE-30 (10%) and XE-60 (4%) liquid phases (Table VI). Analytical data are also presented for the neutral monosaccharides using method B (Tables VII and VIII). Having defined the various optimum working conditions, the isomer distributions and retention times for the trimethylsilyl derivatives of hexuronic acids, salts, and lactones were recorded (Table VI).

The trimethylsilyl derivatives of the C-5 epimer pairs, D-glucuronic acid/L-iduronic acid and D-mannuronic acid/L-guluronic acid could be separated on SE-30. Analysis on XE-60, however, showed greater potential for uronic acid separation. Contrary to the behaviour observed on SE-30, the derivatives of the lactones were

TABLE V

THE EFFECT OF VARIATION OF VOLUME OF SILYLATING REAGENTS ON SENSITIVITY OF DETECTION[a]

Volume of chlorotrimethylsilane (μl)	*Volume of hexamethyldisilazane (μl)*								
	200			*100*			*50*		
	Total peak-area detected[b]	*Volume of upper layer (μl)*	*Yield*[c] (%)	*Total peak-area detected*[b]	*Volume of upper layer (μl)*	*Yield*[c] (%)	*Total peak-area detected*[b]	*Volume of upper layer (μl)*	*Yield*[c] (%)
200	11.2	500	100	—	—		—		
100	12.6	220	83	28.7	120	103	40.8	80	97
50	17.2	160	82	33.5	100	100	44.8	65	87
25	—			40.0	85	101	60.0	40	82
10	—			48.4	65	94	88.5	30	79

[a]For a 250-μl solution of hexuronic acid etc. in methyl sulphoxide: analysis on SE-30. [b]For a fixed (10 μl) sample of the upper layer. [c]As a percentage of yield for 100 μl of hexamethyldisilazane plus 50 μl of chlorotrimethylsilane, for which conditions complete derivatization was demonstrated.

TABLE VI

RETENTION TIMES AND ISOMERIC COMPOSITION OF THE TRIMETHYLSILYL DERIVATIVES OF THE HEXURONIC ACIDS ON 10% OF SE-30 AND 4% OF XE-60

Substrate	*Isomer composition and retention time*[a]											
	Isomer 1			*Isomer* 2			*Isomer* 3			*Isomer* 4		
	%	(*SE-30*) T	(*XE-60*) T	%	(*SE-30*) T	(*XE-60*) T	%	(*SE-30*) T	(*XE-60*) T	%	(*SE-30*) T	(*XE-60*) T
D-Glucurono-6,3-lactone	8	1.20	7.18	92	1.27	9.13						
D-Glucuronic acid	7	1.73	2.52	43	2.10	3.26	50	2.53	4.17			
Sodium D-glucuronate	41	1.73	2.52	20	2.10	3.26	39	2.53	4.17			
D-Galacturonic acid	55	1.48	2.04	17	1.89	2.73	12	2.02	4.06	16	2.60	4.69
Sodium D-galacturonate	85	1.48	2.04	14	1.89	2.73	1	2.02	4.06	1	2.60	4.69
D-Mannurono-6,3-lactone[b]	80	1.22	10.10	20	1.58	16.73						
Sodium D-mannuronate	41	1.52	2.46	51	2.11	3.51	6	2.21	3.96	2	2.54	—
L-Idurono-6,3-lactone	93	1.00	4.14	7	1.00	4.32						
Sodium L-iduronate	30	1.48	2.09	57	1.80	2.51	13	2.00	2.94			
L-Gulurono-6,3-lactone	87	0.86	—	13	1.15	—						
Sodium L-guluronate	78	1.60	2.75	19	1.91	2.97	2	2.42	4.06	1	2.67	—

[a]Equilibria for a solution concentration of 0.5 mg/ml. [b]Actual equilibria before silylation: *T* 1.22, 71%; *T* 1.58, 29%. Measured values are different because of differing partitions (see Table I).

TABLE VII

RETENTION TIMES AND ISOMERIC COMPOSITION OF TRIMETHYLSILYL DERIVATIVES OF NEUTRAL MONOSACCHARIDES ON 10% OF SE-30

Neutral carbohydrate	*Isomer composition and retention time*							
	Isomer 1		*Isomer* 2		*Isomer* 3		*Isomer* 4	
	T	%	T	%	T	%	T	%
D-Glucose	1.48	2	1.80	48	2.91	50		
D-Galactose	1.36	28	1.60	34	1.90	38		
D-Mannose	1.36	85	1.92	15				
D-Ribose	0.71	7	0.74	83	0.80	10		
L-Arabinose	0.64	53	0.71	42	0.78	5		
D-Xylose	0.64	1	0.68	2	0.91	43	1.08	54
D-Lyxose	0.60	75	0.74	16	0.87	9		
D-Fructose	1.35	85	1.43	15				
L-Fucose	0.68	6	0.81	64	0.93	30		
L-Rhamnose	0.67	80	0.89	20				
2-Amino-2-deoxy-D-glucose	1.63	39	2.01	57	2.24	4		

TABLE VIII

RETENTION TIMES OF NEUTRAL MONOSACCHARIDES ON 4% OF XE-60

Neutral carbohydrate	T *values*			
D-Glucose	2.31	4.17		
D-Galactose	1.78	2.30	2.75	3.04
D-Mannose	1.55	2.88		
D-Ribose	1.05	1.23		
L-Arabinose	0.72	0.89	1.09	
D-Xylose	0.85	1.05	1.59	
D-Lyxose	0.88	1.02		
D-Fructose	1.69	2.04		
L-Fucose	0.76	1.07	1.21	1.34
L-Rhamnose	0.83			
2-Amino-2-deoxy-D-glucose hydrochloride	2.66	3.49	4.05	
2-Amino-2-deoxy-D-galactose hydrochloride	3.21	3.30	3.42	

eluted well after their acid counterparts. This is not surprising, as the trimethylsilylated lactone is slightly more polar than the derivatized acid, and thus more likely to interact with the polar XE-60 liquid phase. Thus, on XE-60, the lactones of L-iduronic, D-glucuronic, and D-mannuronic acids were well separated, both from themselves and from D-galacturonic acid, the only non-lactone-forming uronic acid studied. Since the hexuronic acids readily form lactones on evaporation of their solutions to dryness, they can readily be analysed as the lactones.

It is thus recommended that, in general, analyses should be carried out on XE-60 to achieve the best separations for the hexuronic acids. The sugar-containing

solution should first be vigorously evaporated to dryness to ensure lactone formation. Lower temperatures have to be used because of the instability of the liquid phase, and analysis times are therefore greater than for SE-30. Determination of the hexuronic acids in the presence of neutral monosaccharides was achieved adequately on 10% of SE-30 using Method *B*.

The method has been successfully applied to an acidic soil polysaccharide that contains D-glucuronic and D-galacturonic acids in small proportions, to an alginic acid containing D-mannuronic and L-guluronic acids, and to heparan sulphate which yielded well-separated peaks for D-glucurono-6,3-lactone and 2-amino-2-deoxy-D-glucose.

Attempts were made to simplify the chromatographs by the formation of the oximes of each uronic acid. G.l.c. of the trimethylsilyl derivatives of the hexuronic acid oximes gave peaks corresponding to the *syn* and *anti* forms. However, the loss of the furanose/pyranose ring-structures seemed to remove the means by which the hexuronic acids were separated, as the peaks for the *syn* and *anti* forms overlapped on both SE-30 and XE-60. Despite the excellent separation of the lactones from the acids, the method is therefore of little use as an analytical tool. G.l.c. of trimethylsilyl derivatives of hexuronic acid oximes on other liquid phases, for example OV-17 (Ref. 13), also gave separations poorer than those achieved by the present, recommended procedure. Similar work by Sweeley *et al.*[4] on neutral sugars was unsuccessful, decreased separations again being noted.

EXPERIMENTAL

Materials. — D-Glucurono-6,3-lactone, sodium D-glucuronate, D-galacturonic acid, and D-mannurono-6,3-lactone were commercial samples. L-Guluronic acid was kindly supplied by Dr. A. Haug (Institute of Seaweed Research, Trondheim, Norway) as an aqueous solution, and L-idurono-6,3-lactone as the 1,2-*O*-isopropylidene derivative by Professor D. Horton (Ohio State University, U.S.A.). L-Idurono-6,3-lactone was prepared from the foregoing derivative (1 mg) by hydrolysis with M hydrochloric acid (1 ml) at 100° for 3 h, removal of hydrogen chloride by vacuum evaporation with aqueous sodium hydroxide in the receiver, and evaporation to dryness. Sodium D-galacturonate, D-mannuronate, and L-iduronate were prepared by careful addition of equivalent amounts of M sodium carbonate to aqueous solutions of D-galacturonic acid, D-mannurono-6,3-lactone, and L-idurono-6,3-lactone (1 mg/ml). Mildly alkaline solutions were used to avoid decomposition of the alkali-labile lactones.

Hexamethyldisilazane and chlorotrimethylsilane, silylation grade, were obtained from Pierce Chemicals Co. All solvents were dried and redistilled before use.

Preparation of samples for derivatization. — A solution of the hexuronic acid (0–1 mg) in the solvent being tested (1 ml) was incubated at 37°, and, periodically, aliquots (500 μl) were removed for derivatization. When studies on the rate of equilibration were undertaken, larger volumes were used to facilitate the withdrawal

of a number of aliquots. Peak areas were taken as a quantitative measure of carbohydrate present.

Preparation of O-trimethylsilyl derivatives. — *Method* A. Hexamethyldisilazane (100 μl) and then chlorotrimethylsilane (50 μl) were added to the solution of the hexuronic acid (1 mg/ml, 500 μl). Following silylation, solutions in pyridine were centrifuged in order to remove any precipitated ammonium chloride, and a sample (10 μl) was then analysed by g.l.c. following incubation at 37°. For solutions in methyl sulphoxide, a sample (10 μl) of the upper layer was analysed by g.l.c.

Method B. Hexamethyldisilazane (100 μl) and chlorotrimethylsilane (50 μl) were premixed before the addition of the hexuronic acid solution (500 μl). The solutions were then treated as in method *A*.

G.l.c. instrumentation. — A Pye 104 instrument fitted with a dual flame-ionization detector was used in all analyses. Analyses were performed with columns (152 cm × 4 mm) of SE 30 (10%) or XE 60 (4%) on Celite (100–120 mesh). Analyses of hexuronic acid derivatives were performed at column temperatures of 175° (XE 60) or 200° (SE 30) using nitrogen as carrier gas at a flow rate of 40 ml/min. Detector-oven temperatures were 50° above the column-oven temperature. Analyses of solvents/reagents were conducted at 50° on SE 30. Ribitol was used as an internal standard because the peak for its Me_3Si derivative was well-separated from those for the derivatives of the uronic acids. The response given by the flame ionization detector was the same for each hexuronic acid derivative, the sum of the areas under the peaks per unit loading being independent of hexuronic acid structure.

Solubility measurements. — Sodium D-glucuronate (20 mg) was stirred with the solvent (1 ml) at 37°. An aliquot (20 μl) was removed at various time intervals, following centrifugation of the undissolved solid. Each aliquot was diluted with water (200 μl), and tested by the carbazole assay[14] as modified by Bitter and Muir[15] (Fig. 1).

Determination of the effects of water. — Hexuronic acid was dissolved in solutions of water (0–2%) in methyl sulphoxide and silylation performed as in method *B*. The volumes of the upper and lower layers were measured with respect to time. Aliquots (10 μl) of the upper layer were taken at times of 0–4 h and analysed by g.l.c. on SE-30.

Variation of volumes of silylating reagents. — Samples of hexuronic acid in methyl sulphoxide were silylated as in method *B* using various volumes of hexamethyldisilazane and chlorotrimethylsilane. The volumes of the upper and lower layers were measured, and the upper layer was analysed by g.l.c. on SE 30 (Table V).

Preparation of oximes. A sample of the monosaccharide (1 mg) was added to a 1% w/v hydroxylamine solution in dry pyridine (1 ml). The solution was incubated at 100° for 1 h and then cooled, hexamethyldisilazane (200 μl) and chlorotrimethylsilane (100 μl) were added, and the solution was incubated at 37° for 1 h before g.l.c. analysis.

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