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METHANOLYSIS STUDIES OF CARBOHYDRATES, USING H.P.L.C.*

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ABSTRACT

An h.p.l.c. system that separates carbohydrates as their methyl glycosides has been used to study the products obtained on treatment of various carbohydrates with methanolic hydrogen chloride. Results are presented for the monosaccharide composition of several polysaccharides, lactone and ester formation during the treatment of D-glucuronic acid, and relative rates of glycosidation *vs.* esterification during the treatment of D-galacturonic acid.

INTRODUCTION

Aqueous solutions of methyl glycosides are amenable[1,2] to h.p.l.c., and complete, or almost complete, resolution of many monosaccharide mixtures is possible. We now report on the application of h.p.l.c. for the quantitative analysis of mixtures obtained on methyl glycosidation of sugars and methanolysis of polysaccharides, in a manner analogous to the g.l.c. of trimethylsilylated methyl glycosides[3]. Methanolysis of polysaccharides causes less destruction of carbohydrate, including uronic acids, than does aqueous acid[3].

EXPERIMENTAL

A Waters Assoc. h.p.l.c. system was used, involving an M6000 pump, U6K injector, R401 refractive-index detector, radial compression module RCM100, and Model 730 Data Module. The column was a Waters Dextropak plastic cartridge (10 × 0.8 cm), which was compressed in the RCM 100 unit. Elution was effected with distilled water, at 2 mL/min unless specified otherwise. The Dextropak is a special, reversed-phase column developed for carbohydrate oligomers[4], but it can also be used for methyl glycosides[1,2]. Methanolic ~M hydrogen chloride was made by adding acetyl chloride (7.8 g) to dry methanol (92 mL), and methanolysis was carried out at 85° for 24 h, unless specified otherwise. After treatment of samples (typically 10 mg) with the above solution (2 mL), acid was removed by using Amberlite IRA-400

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(HCO_3^-) resin, methanol was removed by using a rotary evaporator at 40°, and a solution of the residue in water was filtered (0.45 μm, Millipore) and injected.

For quantitative analysis, samples (100 μL) of standard, aqueous solutions (0.5 mmol/mL) of each monosaccharide were dried *in vacuo* over P_2O_5 for 16 h at 60°, and then treated with methanolic HCl. Each solution was neutralised ($BaCO_3$) and concentrated, a solution of each residue in water (0.5 mL) was filtered, and a portion (20 μL) was injected. Molar response factors were calculated relative to that of D-glucose at 2 mL/min. Methyl D-glucuronate was obtained by deacetylation of its tetra-acetate (kindly provided by Dr. J. D. Stevens).

P.m.r. spectra (internal Me_4Si) were obtained for solutions in D_2O at 25° with a JEOL JNM-FX100 spectrometer.

Chemical ionisation (c.i.) mass spectrometry, with ammonia as the reagent gas, was performed with a GEC-AEI MS902 spectrometer fitted with a dual e.i.-c.i. source (Scientific Research Instruments CIS 2), under the following conditions: source temperature, 170°; probe temperature, 130°; filament emission current, 0.5 mA; ionising voltage, 550 eV, and accelerating voltage, 8 kV.

RESULTS AND DISCUSSION

Monosaccharides. — Treatment of monosaccharides with methanolic ~M hydrogen chloride at 80° for 24 h yielded the mixtures of glycosides shown in Table I

TABLE I

GLYCOSIDE COMPOSITION (%) OF THE PRODUCTS OBTAINED ON TREATMENT OF MONOSACCHARIDES AND SOME POLYSACCHARIDES WITH METHANOLIC ~M HCl AT 85° FOR 24 h

Monosaccharide	*Glycoside peaks*[a,b]	*Polysaccharide*[c]	
D-Glucose	99(1.0) 1.0(1.35)	Amylose, dextran, and pullulan all yielded a 99:1 ratio	
D-Galactose	68.1(0.78) 17.1(0.96) 14.8(1.10)	Locust-bean gum	70.6:13.4:16.0
		Guar gum	70.4:11.2:18.4
D-Mannose	7.9(1.31) 92.1(1.51)	Locust-bean gum	7.6:92.4
		Guar gum	8.6:91.4
D-Xylose	2.3(1.05) 2.5(1.15) 35(1.40) 60.2(1.62)		
L-Arabinose	7.5(1.07) 22.2(1.21) 70.3(1.39)		
D-Ribose	6.0(1.25) 31.6(1.42) 9.0(1.93) 53.4(2.32)		
L-Rhamnose	10.1(3.02) 89.9(3.32)		
L-Fucose	5.8(1.70) 15.8(2.17) 52.2(3.15) 26.3(3.54)		
D-Glucuronic acid	32.7(1.06) 5.1(1.16) 15.2(4.54) 47(5.22)		
D-Galacturonic acid	49.9(3.94) 50.1(4.39)[d]	"Polygalacturonic acid"	50.2:49.8
		Gum tragacanth	48:52

[a]Starting from monosaccharides. For identities of most peaks, see ref. 1. [b]Figures in brackets are retention times (R_G) relative to that of methyl α-D-glucopyranoside. [c]Peak-area ratios for the monosaccharide appearing in column 1. [d]This peak had a large shoulder on the trailing edge, which is included in the area (Fig. 7).

The identity of each peak is not essential knowledge in the present context, but most peaks have been identified[1], and some comments are warranted.

At equilibrium, D-glucose and D-xylose afforded mainly the methyl pyranosides (99 and 95.2%, respectively), as expected by the fewer non-bonded interactions relative to furanosides[5]. Higher proportions of the galactofuranosides were found (31.9%), as the furanoid conformations (E_3) are comparable in stability to the $^4C_1$ pyranoid forms[5]. L-Arabinose, presumably in the favourable E_2 conformation, also gave a large proportion of furanosides (29.9%).

Methanolysis of amylose, dextran, and pullulan gave the same mixture of glycosides (Table I). Similarly, the mixture of glycosides obtained from galacturonic acid was similar to that obtained on methanolysis of "polygalacturonic acid" and gum tragacanth. The data for galactose and mannose in guar and locust-bean gums were more variable, and these polysaccharides were troublesome to analyse (see below).

The molar response factors based on total glycoside peak-areas are shown in Table II and were similar, with the exception of D-ribose which gave a low and unexplained value. Calibration curves over the range 45–450 μg for D-glucose (coefficient of correlation, 0.998), D-galactose (0.992), and L-arabinose (0.985) indicated linearity of response for the refractive-index detector. These curves were based on one glycoside peak only, namely, that which was least likely to overlap with those of other sugars.

TABLE II

MOLAR RESPONSE FACTORS OF SUGARS AS THEIR METHYL GLYCOSIDES (REFRACTIVE-INDEX DETECTOR)

D-Glucose	1.0	D-Xylose	0.91
D-Galactose	1.01	L-Arabinose	0.92
D-Mannose	1.00	D-Ribose	0.58
L-Fucose	0.98	D-Galacturonic acid	0.98
L-Rhamnose	0.88	D-Glucuronic acid	1.01

TABLE III

ANALYSIS (MOLAR RATIOS) OF MONOSACCHARIDES WITH SOME OVERLAPPING METHYL GLYCOSIDE PEAKS

Mixture	*Actual ratios*	*Found*
D-Galactose/D-glucose	1:1	1:1.01
	1:2	1:1.90
	1:3	1:2.95
L-Arabinose/D-xylose	1:1	1:1.08
	1:2	1:1.98
	1:3	1:3.04
	1:4	1:3.68

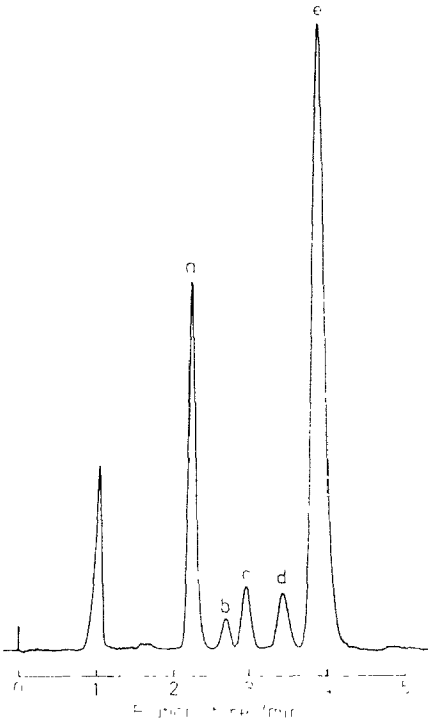

Fig. 1. H.p.l.c. of methanolysis products from locust-bean gum: *a*, methyl α- and β-D-galactopyranosides; *b*,*c*, methyl α- and β-D-galactofuranosides; *d*, methyl α-D-mannopyranoside; *e*, methyl β-D-mannopyranoside and α- and β-D-mannofuranosides.

The analysis of mixtures containing overlapping peaks was explored by using standard combinations of D-glucose/D-galactose and D-xylose/L-arabinose (Table III). The results indicate that reasonable accuracy is obtainable.

Monosaccharide composition of polysaccharides. — Several polysaccharides were methanolysed and the products were subjected to h.p.l.c. The methyl mannosides and galactosides from the galactomannan of locust-bean (carob) gum (Fig. 1) were cleanly separated and quantification indicated a galactose–mannose ratio of 1:2.8 (literature value[6], ~1:4). The sample was an unpurified, commercial product and this could account for the discrepancy[7]. Hydrolysis with 2M trifluoroacetic acid followed by treatment with methanolic HCl gave a galactose–mannose ratio of 1:3.0. It appeared that methanolysis may not have effected complete depolymerisation. Xanthan gum (Fig. 2) yielded methyl glycosides of glucose, mannose, and glucuronic acid in the ratios of 3:2.9:1 (lit.[11], 2:2:1). Thus, the release of glucuronic acid is low. Preliminary hydrolysis with trifluoroacetic acid failed to improve the yield of glucuronic acid, probably because conversion of the calcium salt into the free acid was incomplete. "Polygalacturonic acid" (Fig. 3) gave methyl glycosides of rhamnose, arabinose, and galactose (17% of total), and galacturonic acid (83%), as would be expected for a pectic substance. Apple pectin was shown to contain somewhat higher

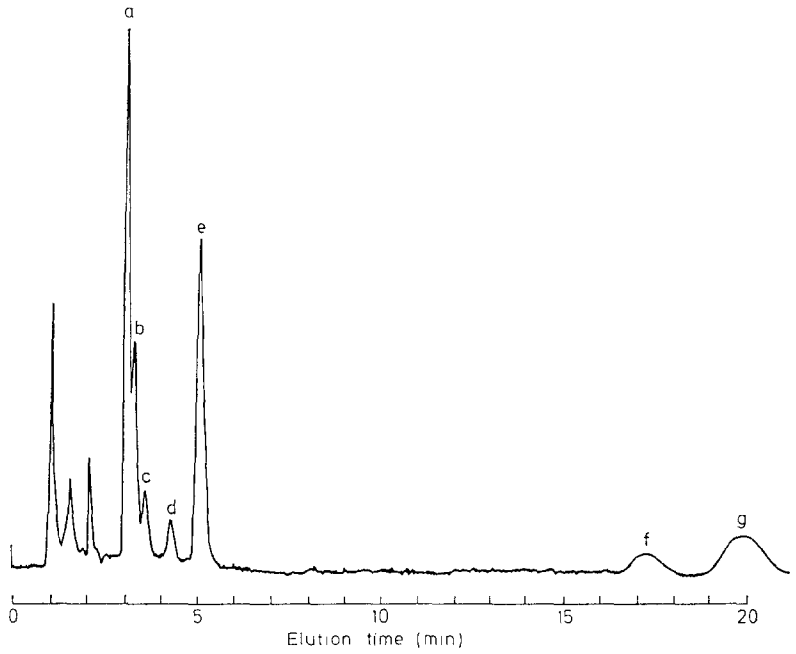

Fig. 2. H.p.l.c. of methanolysis products from xanthan gum: *a*, methyl D-glucopyranosides; *b*, methyl β-D-glucofuranosidurono-6,3-lactone; *c*, methyl α-D-glucofuranosidurono-6,3-lactone; *d*, methyl α-D-mannopyranoside; *e*, methyl β-D-mannopyranoside and methyl α- and β-D-mannofuranosides; *f*, methyl (methyl β-D-glucopyranosid)uronate; *g*, methyl (methyl α-D-glucopyranosid)uronate.

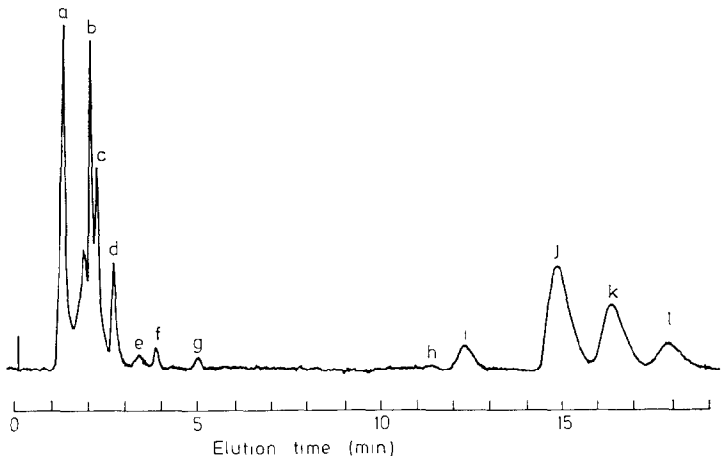

Fig. 3. H.p.l.c. of methanolysis products from "polygalacturonic acid": *a*, salts, D-galacturonic acid; *b,c*, unidentified material (see Results and Discussion); *d*, methyl α- and β-D-galactopyranosides; *e,f*, methyl α- and β-D-galactofuranosides; *f*, methyl α- and β-L-arabinofuranosides; *g*, methyl α- and β-L-arabinopyranosides; *h,i*, methyl L-rhamnosides; *j*, methyl (methyl α- and β-D-galactofuranosid)uronates; *k,l* methyl (methyl α- and β-D-galactopyranosid)uronates.

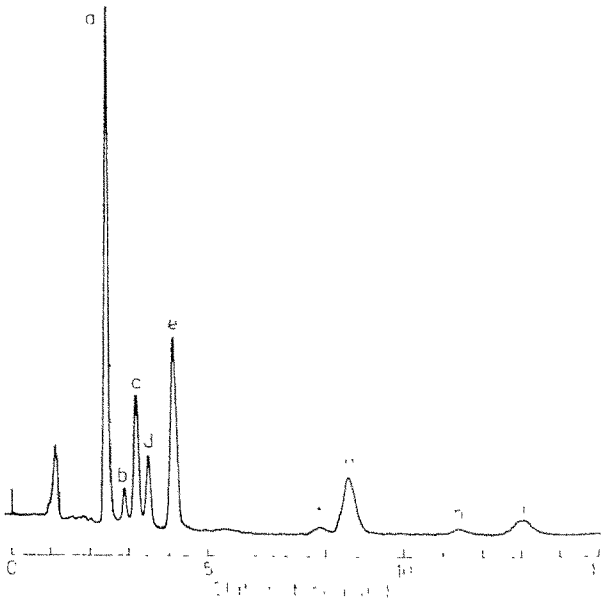

Fig. 4. H.p.l.c. of methanolysis products from gum acacia. *a*, methyl α-D-galactopyranoside; *b,c*, methyl β-D-galactopyranoside and methyl α- and β-D-galactofuranosides; *c,d*, methyl α- and β-L-arabinofuranoside; *e*, methyl α- and β-L-arabinopyranosides; *f,g*, methyl L-rhamnosides; *h*, methyl (methyl β-D-glucopyranosid)uronate; *i*, methyl (methyl α-D-glucopyranosid)uronate.

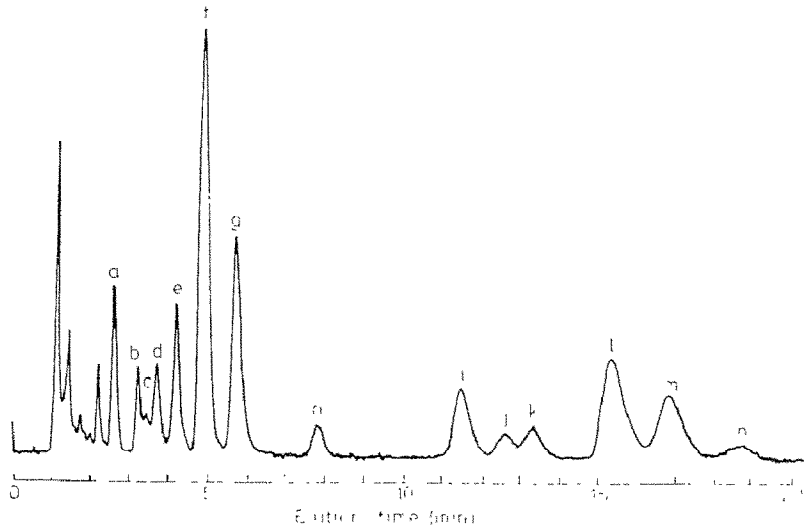

Fig. 5. H.p.l.c. of methanolysis products from gum tragacanth. *a*, methyl α-D-galactopyranoside; *b,d*, methyl β-D-galactopyranoside and methyl α- and β-D-galactofuranosides; *f*, methyl α- and β-D-glucopyranosides; *d,e*, methyl α- and β-L-arabinofuranosides; *f*, methyl α- and β-L-arabinopyranosides; *e*, methyl α- and β-D-xylofuranosides; *f*, methyl α-D-xylopyranoside; *g*, methyl β-D-xylopyranoside; *h,i,k*, methyl L-fucosides; *i,j*, methyl L-rhamnosides; *l*, methyl (methyl α- and β-D-galactofuranosid)-uronates; *m,n*, methyl (methyl α- and β-D-galactopyranosid)uronates

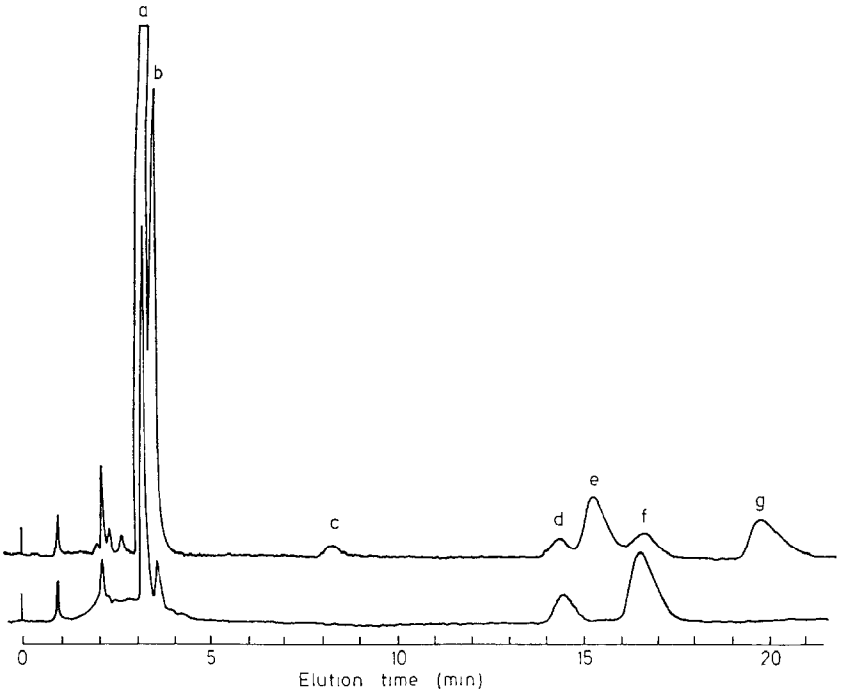

Fig. 6. H.p.l.c. of the products obtained from D-glucuronic acid on treatment with methanolic HCl: upper trace, 0.02M acid at room temperature for 3 days, lower trace, M acid at 85° for 24 h; *a*, methyl β-D-glucofuranosidurono-6,3-lactone; *b*, methyl α-D-glucofuranosidurono-6,3-lactone; *c*, methyl α,β-D-glucopyranosidurono-6,3-lactones; *d*, methyl (methyl β-D-glucopyranosid)uronate; *f*, methyl (methyl α-D-glucopyranosid)uronate; *e,g*, methyl (methyl α,β-D-glucofuranosid)uronates.

amounts of rhamnose, arabinose, and galactose, plus a substantial proportion of glucose.

Gum arabic (acacia) (Fig. 4) and gum tragacanth (Fig. 5) gave the approximate monosaccharide compositions reported[8-10], although the amount of glucuronic acid in acacia gum was lower than expected.

Uronic acids. — Treatment of D-glucuronic acid with methanolic M HCl at 85° for 24 h yielded the products shown in Fig. 6 (lower trace). A similar product mixture was obtained from D-glucurono-6,3-lactone. Peaks *a* and *b* were shown by c.i. mass spectrometry and p.m.r. spectroscopy to contain a mixture of the methyl α- and β-glycosides of D-glucurono-6,3-lactone. Comparison with the synthetic methyl β-glycoside[12] showed that peak *a* consisted of this compound. Borohydride reduction of peak *a* and *b* material followed by h.p.l.c. showed the presence of methyl α- and β-D-glucofuranosides. Peaks *a* and *b* reached the proportions shown in Fig. 6 in ~16 h. Reaction times up to 2 days did not alter the composition of the product mixture, which was concluded to reflect an equilibrium for these conditions. The lactone-glycoside peaks *a* and *b* were close to the major glucoside peak, and to minor glycoside peaks of galactose, xylose, and arabinose (Table I). This makes quantifica-

tion of glucuronic acid in the presence of glucose difficult, *e.g.*, in the case of xanthan gum (Fig. 2). Peaks *d* and *f* were shown by c.i. mass spectrometry and p.m.r. spectroscopy to be the methyl ester methyl glycosides of glucuronic acid The pyranoid-ring form was confirmed by the products formed on reduction with sodium borohydride.

Treatment of D-glucuronic acid with methanolic 0.02M HCl at room temperature for up to 3 days yielded a series of patterns best summarised by the upper trace in Fig. 6. Initially, large amounts of the lactone glycosides (peaks *a* and *b*) occurred together with smaller peaks at 2.3 and 2.7 min. These smaller peaks, which had virtually disappeared after 10 h, were assumed to be the methyl esters of D-glucuronic acid, but insufficient was formed to allow isolation and identification. Authentic methyl D-glucuronate gave two peaks having the above retention times. Peaks *c*, *e*, and *g* appeared after ~8 h, and peaks *a*–*g* were present after 24 h. As peaks *d* and *f* had been shown to be the methyl glucopyranosiduronic esters, and previous work[1] had shown that furanosides were formed initially, peaks *e* and *g* were assumed to be methyl glucofuranosiduronic esters. These peaks did not appear during treatment with methanolic ~M HCl at 85 for 24 h (Fig. 6, lower trace). The material in peak *c* was subjected to c.i. mass spectrometry and p.m.r. spectroscopy. It had a molecular weight (190) corresponding to a lactone glycoside and contained a glycosidic methyl group. The n.m.r. spectrum differed from those of the compounds in peaks *a* and *b*. Borohydride reduction gave methyl α- and β-D-glucopyranosidurono-6,3-lactone, whereas peaks *a* and *b* contained methyl α- and β-D-glucofuranosidurono-6,3-lactones.

D-Galacturonic acid, when subjected to the standard methanolysis conditions, yielded 3 peaks (Fig. 7). No lactone-glycoside peaks were present: the configuration of galacturonic acid precludes the formation of a furanoside-6,3-lactone The formation of a pyranoside-6,3-lactone is also unlikely, as it would involve the $^1C_4$ conformation[13]. Borohydride reduction of the material in peak *a* gave methyl α- and β-D-galactofuranosides; the material in peaks *b* and *c* similarly gave methyl α- and β-D-galactopyranosides.

A very small pair of peaks centred at R_G 1.11 was observed in the chromatogram of the galacturonic acid products. Treatment with methanolic 0.02M HCl at room temperature showed that these peaks were formed initially (Fig. 8 upper trace,

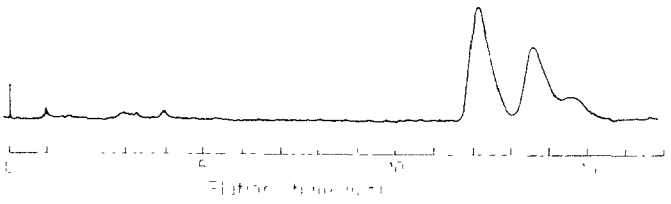

Fig. 7. H.p.l.c. of the products obtained from D-galacturonic acid by treatment with methanolic M HCl at 85 for 24 h: *a*, methyl (methyl α,β-D-galactofuranosid)uronates; *b*,*c*, methyl (methyl α,β-D-galactopyranosid)uronates

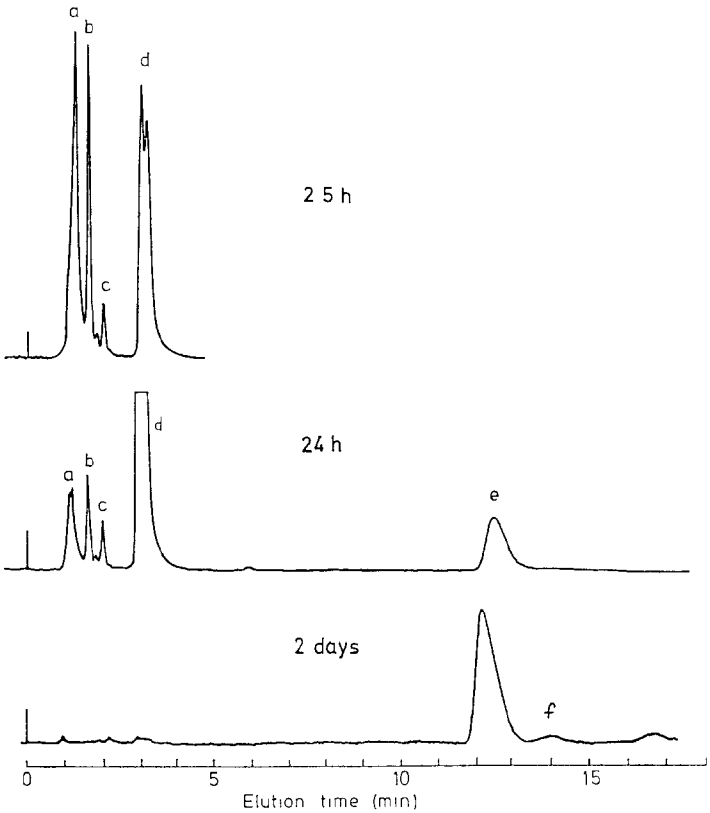

Fig. 8. H.p.l.c. of the products obtained from D-galacturonic acid by treatment with methanolic 0.02M HCl at room temperature for 2.5 h (upper trace), 24 h (middle trace), and 2 days (lower trace); *a*, D-galacturonic acid; *b,c*, unidentified peaks (see Results and Discussion); *d*, methyl D-galacturonates; *e*, methyl (methyl α,β-D-galactofuranosid)uronates; *f*, methyl (methyl α-D-galactopyranosid)uronate.

peaks *d*). They were shown to contain the methyl esters of galacturonic acid by c.i. mass spectrometry and p.m.r. spectroscopy. After ~4 h, the methyl galactofuranosiduronic methyl esters appeared; after 24 h, the pattern was as shown in Fig. 8, middle trace. The final composition after 2 days is shown in Fig. 8, lower trace. The identities of peaks *b* and *c* were not confirmed, as the material they contained could not be isolated pure, but could have contained unstable pyranoside-lactones. The rate of formation and proportions of galacturonic ester/ester glycosides were close to those obtained by Jansen and Jang[14] by measurement of peak areas in a series of samples up to 24 h. The methyl galactofuranosiduronic esters occur to the extent of ~50%, an even higher furanoside proportion than that for galactose. This finding was not possible by the procedure used by Jansen and Jang, which involved titration of the acidity and determination of reducing power.

The ability to monitor the formation and disappearance of methyl glycosides, and to collect small amounts (up to 5 mg per injection can be handled) of materials for identification or as standards, is a most useful attribute of this h.p.l.c. system.

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