STRUCTURAL ELUCIDATION OF A POLYSACCHARIDE ISOLATED FROM CRUDE PAPAIN

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Dedicated to my mother and father

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Chandrasekaran et al. (1978) reported that two acidic polysaccharides isolated from crude papain, PP I and PP II (papain polysaccharide I and II) exhibited interesting biological properties when applied to normal human diploid fibroblast cells, viz., they effected a reduction in growth and nuclear area, aggregation and alignment of cells, and caused the cells to behave as if they were contact-inhibited. Determination of the structure of PP II, the more abundant and more active of the two polysaccharides, was undertaken because of the potential of thereby learning more about intercellular recognition and adhesion.

Complete hydrolysis of PP II indicated its composition to be L-arabinose (31.0%), L-rhamnose (13.3%), D-galactose (42.6%), D-glucuronic acid (10.3%), and 4-O-methyl-D-glucuronic acid (2.8%).

Methylation analysis of the carboxyl-reduced PP II indicated the presence of 2,3,5-tri-O-methylarabinose (31.7%),

2,4-di-O-methylrhamnose (10.4%), 2,3,4,6-tetra-O-methyl-galactose (9.0%), 2,3,6-tri-O-methylglucose (10.1%), 2,4-di-O-methylgalactose (19.3%), and 2-O-methylgalactose (10.5%), as the major components as well as less than 3% each of 2,3,4-tri-O-methylarabinose, 2,3,4,6-tetra-O-methyl-glucose, 2,4,6-tri-O-methylgalactose, 2,3,4-tri-O-methyl-galactose, and 2,6-di-O-methylgalactose. The highly branched structure of the polysaccharide is evident from the high percentage of end units and branch-point units.

Methylation analysis of products of partial hydrolysis led to the identification of the aldobiouronic acid fragment as 6-O-(D-glucopyranosyluronic acid)-D-galactose. Other fragments indicated that the backbone of the polysaccharide was made up of galactosyl units substituted at either the O-6 or O-3 positions. Still other fragments indicated that the rhamnosyl units were substituted at O-3 with either terminal arabinofuranosyl units or terminal galactopyranosyl units, and that the rhamnosyl units were themselves linked to glucuronic acid residues through O-4.

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LIST OF ABBREVIATIONS

PP I - papain polysaccharide I

PP II - papain polysaccharide II

PP II-R - carboxyl-reduced PP II

TMS - trimethylsilyl derivatives

PAAN - peracetylated aldononitrile

PMAA - partially methylated alditol acetate

MPP II-R - methylated PP II-R

Glc - gas-liquid chromatography

MS - mass spectrometry

INTRODUCTION

Two acidic polysaccharides were isolated and identified as contaminants in the crude papain (Sigma type II) used by Chandrasekaran et al.(1) in investigations of qualitative and quantitative changes in glycosaminoglycans of fibroblast cells as a function of age. These polysaccharides were labelled PP I and PP II (papaya polysaccharide II), a designation of their order of elution by a salt gradient from a DEAE-sephadex column. Other lots of crude papain contained additional polysaccharides. All these polysaccharides appeared to belong to a family, the individual member of which differed from each other primarily in charge density, i.e., amount of uronic acid.

The monosaccharide compositions of the two polysaccharides were found to be very similar, consisting of the same sugar units, viz., D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid and D-glucose (trace), but to differ slightly in the relative amounts of each sugar present, especially in the relative amounts of uronic acid. The papaya polysaccharides were reported to have some interesting biological properties. When applied to normal human diploid fibroblast cells, they effected a reduction in growth and in nuclear area and an aggregation and alignment of cells. They also caused aggregated diploid cells to behave as if they were contact-inhibited, but they did

not have any effect on aneuploid mouse embryo fibroblast cells which do not exhibit contact-inhibition of growth or movement. This effect shown by PP I and PP II could have far reaching consequences. It may help us to understand intercellular adhesion and communication.

Cell surfaces contain glycoproteins and glycolipids. There is considerable indirect evidence that the carbohydrate residues of these molecules which constitute the glycocalyx are involved in intercellular recognition and adhesion. Because PP I and PP II effect aggregation of fibroblast cells, it is possible that an elucidation of their structure might shed some light on the mechanism of this cell-cell recognition and adhesion, particularly on the nature of the carbohydrate residues involved.

Papain is obtained from the latex of green fruit and leaves of Carica papaya plants via salting out of proteins with NaCl or $(NH_4)_2SO_4$ and centrifugation. The filtrate is then spray dried at about 50^{o} (2) or vacuum dried (3). Although much work has been done on the isolation of papain from papaya and on the sugar content of the fruits during development, little has been done on the polysaccharides of papaya. In the only previous report of papaya polysaccharides published in 1969, Biwas and Roa (4) reported the isolation of a galactan from unripe papaya fruits; this galactan contained in addition to D-galactose, a consider-

able amount of D-galacturonic acid, a small amount of L-arabinose, and traces of L-rhamnose.

This study was undertaken to attempt an elucidation of the structure of PP II.

RESULTS

This section is divided into five subsections:

1. Isolation of PP II and complete acid-catalyzed hydrolysis of PP II and PP II-R
2. Methylation analysis of PP II-R
3. Partial acid-catalyzed hydrolysis of PP II-R
4. Mild acid-catalyzed hydrolysis of PP II-R without depolymerization
5. Aldobiouronic acids

1. Isolation of PP II and Complete Acid-catalyzed Hydrolysis of PP II and PP II-R

PP II was isolated by the procedure described by Chandrasekaran et al. (1).

About 1.5 g of a mixture of polysaccharides was obtained from 200 g of crude papain via fractionation on a DEAE-cellulose column (Fig. 1); about 0.7 g of pure PP II was obtained from the mixture. Although the amounts of PP II isolated varied from one sample to another, it was always the most abundant of the polysaccharides present in every sample of papain used. The relative amounts of the other polysaccharides present (designated PP I, PP III, etc.) varied greatly from one lot to another.

The optimum hydrolysis time for PP II was found to be 6 h in 0.5 M H_2SO_4 at 100° (Fig. 2). A paper chromatogram

of the neutralized, deionized hydrolyzate showed the presence of five components, three monosaccharides and two uronic acid-containing oligosaccharides. The monosaccharides were identified by the characteristic color produced by reaction with p-anisidine·HCl and by comparison of R_f values with standards. The monosaccharide with the largest R_f value developed a yellowish-green color characteristic of 6-deoxyhexoses and was identified as rhamnose. The component with the next largest R_f value developed a cherry-red color characteristic of pentoses and was identified as arabinose. The monosaccharide component with the smallest R_f value developed a yellowish-brown color characteristic of hexoses and was identified as galactose and/or glucose. The faster moving uronic acid-containing oligosaccharide developed a light brown color; while the slowest moving component developed a very dark brown color.

The uronic acid-containing fragments were separated from the neutral sugars by adsorption onto anion-exchange resins and purified by preparative paper chromatography, while the monosaccharides were further characterized by glc of their trimethylsilyl (TMS)-oxime and peracetylated aldononitrile (PAAN) derivatives.

Hydrolysis of the carboxyl reduced PP II (PP II-R) in 0.5 M H_2SO_4 at 100° was completed after 4 h. A paper chromatogram of the neutralized hydrolyzates indicated the

presence of only monosaccharides; no oligosaccharides or uronic acids were present.

Initially, TMS-oxime derivatives of the neutral monosaccharides obtained by hydrolysis of PP II and PP II-R were prepared, but because each sugar component gives more than one TMS-oxime derivative (Fig. 3), the gas-liquid chromatogram of these derivatives had too many peaks for convenient analysis (Fig. 4 and 5). Whereas the PP II hydrolyzate gave a relatively simple chromatogram in which all peaks could be identified by comparison with standards (Fig. 5), the PP II-R hydrolyzate had peaks due to both glucoseand galactose which overlap extensively (Fig. 4), causing computational problems. It was, therefore, necessary to find derivatives which give single well-separated peaks for each of the components of PP II and PP II-R. PAAN derivatives met this requirement (Figs. 6 and 7).

Comparison of gas-liquid chromatograms of PAAN derivatives of the sugars of PP II (and PP II-R) hydrolyzates with those of standards conclusively identified the sugar components of PP II as (L-)arabinose, (L-)rhamnose, (D-)glucuronic acid, and (D-)galactose. (The specific D and L configurations of the sugars was not determined but assumed on the basis of the enantiomers found in other plant polysaccharides.) (D-)Glucuronic acid was identified by comparison with a standard and also by the fact that

glucose was absent from the neutral monosaccharides isolated from a PP II hydrolyzate and present in the hydrolyzate of PP II-R. The mole % of each monosaccharide residue in PP II and PP II-R (Table I) was determined by integration of the areas under the peaks.

Table I

Mole % Calculated From Integration of PAAN Derivative Peaks

	Mole %				
	L-Rhamnose	L-Arabinose	D-Galactose	D-Glucose	4-O-MeGlc
PP II	17.53	40.10	42.37	-	-
PP II-R	13.29	30.98	42.56	10.33	2.78

2. Methylation Analysis of PP II-R

Attempted methylation analysis of PP II was unsuccessful because methylation resulted in the formation of the products of base-catalyzed degradation of uronic acid-containing polysaccharides (5). Therefore, methylation was restricted to PP II-R and fragments obtained from PP II-R by partial hydrolysis.

Complete methylation of PP II-R was confirmed by absence of hydroxyl absorption in the infrared spectrum.

After hydrolysis, reduction, and subsequent derivitization to form alditol acetates, the partially methylated alditol acetates (PMAA) formed from methylated PP II-R (MPP II-R) were subjected to gas-liquid chromatography (glc) for separation and identification.

Gas-liquid chromatographs of PMAA derivatives of MPP II-R (Fig. 8) indicated eleven components. Studies by Bjorndal et al. (6) and more recently by Jansson et al.(7) on gas-liquid chromatography and mass spectrometry (glc-ms) of PMAA derivatives of a large number of sugars provided useful references for both retention times and fragmentation patterns of these derivatives. Conclusive identification of the eleven PMAA derivatives obtained from MPP II-R was possible by comparisons of retention times and fragmentation patterns with available data for known standards. Mass spectra of all eleven compounds are presented in Figs. 9-19. The glc-ms data for PMAA derivatives obtained from MPP II-R are summarized in Table 2.

3. Partial Acid-catalyzed Hydrolysis of PP II-R

Paper chromatography of the neutralized hydrolyzate obtained after treatment of PP II-R with 0.5 M H_2SO_4 at $100^{o}$for 2 h indicated the presence of rhamnose, arabinose, galactose, and a number of oligomers. Two oligosaccharides were isolated by preparative paper chromatography and

Table 2

Glc-ms Data For Partially Methylated Alditol Acetates of PP II-R on 3% OV225 (1/8" x 7')

Methylated Alditol	T_r^1	Peak	T_r	MS^2 fragmentation(m/e)
2,3,5-Tri-O-methyl-arabinitol	0.41	A	0.48	161,129,117,101,87,71 45,43
2,3,4-Tri-O-methyl-arabinitol	0.45	B	0.57	161,117,101,43
2,4-Di-O-methyl-rhamnitol	0.94	C	0.89	233,201,173,159,131 117,99,43
2,3,4,6-Tetra-O-methylglucitol	1.00	D	1.00	205,161,145,129,117 101,87,71,45,43
2,3,4,6-Tetra-O-methylgalactitol	1.19	E	1.17	205,161,145,129,117 101,87,71,45,43
2,4,6-Tri-O-methyl-galactitol	2.03	F	1.94	233,201,161,129,117 101,87,45,43
2,3,6-Tri-O-methyl-glucitol	2.32	G	2.21	233,173,161,131,129 117,113,101,99,71,45 43
2,3,4-Tri-O-methyl-galactitol	2.89	H	2.71	233,189,173,161,129 117,101,99,87,43
2,6-Di-O-methyl-galactitol	3.14	I	2.97	305,231,203,143,117 45,43
2,4-Di-O-methyl-galactitol	5.10	J	4.81	233,201,189,159,129 117,87,43
2-O-Methylgalactitol	N.A.	K	5.36	333,259,117,43

[1] T_r=relative retention time as reported by Lindberg et al.(7) under similar conditions, i.e., isothermal at 170°.

[2] MS data were obtained from a temperature-programmed chromatogram (from 160° to 210° at 2°/min, then held at 210°).

labelled F3 and P1 in order of decreasing R_f value. P1, was however a mixture of two larger fragments. Only P1 was isolated in quantity sufficient for structural analysis.

Glc of PAAN derivatives of a hydrolyzate of P1 indicated the presence of only glucose and galactose(Fig.20).

Each oligosaccharide was methylated and hydrolyzed. The products of hydrolysis were converted into their respective PMAA derivatives. Because relative retention times and respective mass spectral data was already available for all MPP II-R components, PMAA derivatives of MF3 and MP1(Fig.20a) were identified by comparison of their relative retention times with those available from MPP II-R (Table 3).

Table 3

Relative Retention Times of Partially Methylated Alditol Acetates on an OV225 Column Held at 165° for 2 min, Then Linear Temperature Programmed at 2°/min to 210°, Then Held at That Temperature for 5 min

Peak	MPP II-R	MPP II-RA	MP1	MF3
A	0.58	0.57	-	-
B	0.66	-	-	-
C	0.93	0.92	-	-
D	1.00	1.00	1.00	1.00
E	1.11	1.10	1.10	-
F	1.48	-	1.48	-
G	1.58	1.56	-	-
H	1.74	1.74	1.74	1.72
I	1.81	-	-	-
J	2.25	2.23	2.23	-
K	2.42	2.40	-	-

A separate mass spectrum for each of the components of MF3, and MP1 was unnecessary since the relative retention times provided rather conclusive identification.

Mole % values were calculated for each PMAA component (Table 4).

Table 4

Mole % of PMAA Components of Fragments From PP II-R

		Mole %		
Component	Mol.wt.	MPP II-R	MPP II-RA	MP1
A	**278**	31.7	17.8	-
B	**278**	1.7	1.1	-
C	**320**	10.4	6.8	-
D	322	2.7	7.5	21.1
E	322	9.0	6.7	13.0
F	350	1.7	2.0	10.5
G	350	10.1	9.0	-
H	350	0.9	8.1	38.3
I	378	1.5	-	-
J	378	19.6	28.1	11.2
K	406	10.5	2.9	-

4. Mild Acid-catalyzed Hydrolysis of PP II-R Without Depolymerization

When subjected to paper chromatography, the neutralized hydrolyzate from a 2-h hydrolysis of PP II-R in 0.05 M H_2SO_4 at 100° indicated the presence of rhamnose, arabinose, small

amounts of galactose, a disaccharide labelled D1, and a polymeric material remaining at the origin, labelled PP II-RA.

Portions of both D1 and PP II-RA, isolated by preparative paper chromatography, were hydrolyzed; the hydrolysis products were converted into PAAN derivatives and subjected to glc (Figs. 21 and 22). D1 showed the presence of only two components, arabinose and galactose, which were identified by R_f values and relative retention time comparisons with standards. PP II-RA was shown by glc to contain four component sugars: arabinose, rhamnose, glucose, and galactose. Paper chromatography of this mixture however, indicated the presence of only three components; because glucose and galactose have very similar R_f values and develop the same color with p-anisidine hydrochloride, they appear as a single spot. The amounts of the various components in mole % are reported in Table 5.

Table 5

Mole % of PAAN Derivatives of Hydrolyzates from Fragments of PP II-R

	PP II-R	PP II-RA	P1	D1
L-Arabinose	31.0	18.9		50
L-Rhamnose	13.3	13.3		
D-Glucose	10.3	11.9	15	
D-Galactose	42.6	55.8	85	50

Complete methylation of PP II-RA gave MPP II-RA. The methylated sugars released upon hydrolysis of MPP II-RA were reduced and acetylated to convert them to PMAA derivatives. Glc of the PMAAs indicated the presence of eight components (Fig. 23) which were identified by comparison of relative retention times with those obtained for components of MPP II-R under the same conditions (Table 4).

5. Aldobiouronic Acids

Two aldobiouronic acids were isolated by preparative paper chromatography from the 4-h hydrolyzate of PP II and labelled UA1 and UA2, UA1 being the one with the smaller R_f value. The aldobiouronic acids were reduced and a portion hydrolyzed; the monosaccharides obtained were converted into PAAN derivatives. The other portion of each was methylated before hydrolysis; and PMAA derivatives of the products of hydrolysis were prepared. Glc's of both the PAAN and the PMAA derivatives (Figs. 24 and 25) showed that only glucose and galactitol were present in the reduced aldobiouronic acids, in equimolar ratios.

Gas-liquid chromatography and mass spectral analysis of PMAA derivatives of sugar components of UA1 were identical with those of UA2 (Table 6). Both their retention times and their mass spectral fragmentation patterns were identical (Figs. 26a and b). The fragmentation pattern for the

component that elutes first is identical with that of 6-O-acetyl-1,2,3,4,5-penta-O-methylgalactitol and that of the component that elutes last is identical with the fragmentation pattern of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol.

Table 6

Glc-ms Data for Partially Methylated Alditol Acetates of UA1 and UA2 on 3% OV225 (1/8" x 7') Run Isothermally at 185°

Methylated alditol	mole%		MS fragmentation (m/e)
	MUA1	MUA2	
1,2,3,4,5-Penta-O-methylgalactitol	50	50	249,205,177,161,145,117 101,89,45,43
2,3,4,6-Tetra-O-methylglucitol	50	50	205,161,145,129,117,101 87,45,43

EXPERIMENTAL

Materials

Papain (Sigma, type II), standard sugars, DEAE-cellulose, and 3-(3-diethylaminopropyl)-1-ethylcarbodiimide hydrochloride were obtained from Sigma Chemical Company. Whatman chromatography paper (No.1, 0.16-mm thickness, medium flow rate; No.4, 0.2-mm thickness, fast flow rate; No. 3MM, 0.38-mm thickness, medium flow rate) was obtained from Scientific Products. Dialysis membrane Spectra/por.6 with molecular weight cut off of 50,000 daltons was obtained from Fisher Scientific Company. All other chemicals used were of reagent grade or the purest available.

General Analytical Techniques

Paper chromatograms were irrigated with ethyl acetate-acetic acid-formic acid-water (18:3:1:4 v/v) in a tank saturated with vapors of the same mixture. Descending paper chromatography was the method of choice. Developed chromatograms were dried, sprayed with a 3-4%(w/v) solution of p-anisidine hydrochloride in n-butanol-ethanol-water (4:1:1 v/v) containing a trace of stanous chloride for stabilization, allowed to dry, and heated over a hot plate until the spots appeared (5 min.).

Gas-liquid chromatography (glc) was done with a Varian Series 200 instrument, equipped with a temperature programmer

a flame ionization detector, and a Varian aerograph(model 20) recorder. Integration was performed with a Hewitt-Packard 3390 A recorder.

Glc of TMS-oxime derivatives was done on a 1/8" x 30' stainless steel column packed with chromsorb W (a diatomaceous earth support) with a 3% (w/w) coating of SE 52 (a 5% phenyl, 95% methyl silicone with temperature limits of 50^{o} - 300^{o}). The gas flow rates were as follows: helium 35 mL/min, hydrogen 45 mL/min, and air 200 mL/min. The column temperature was programmed at 2^{o}/min from 100^{o} to 250^{o}; the injector port temperature was 200^{o}, while the detector temperature was 290^{o}.

Both PAAN and PMAA derivatives were chromatographed on stainless steel columns (1/8" x 7') packed with Gas Chrom Q (a diatomaceous earth support) with a 3% (w/w) coating of OV225 (a 25% phenyl, 25% cyanopropyl, 50% methyl silicone). Gas flow rates were the same as those used for the glc of the TMS-oxime derivatives. For PAAN derivatives, the column temperature was increased at 2^{o}/min from 190^{o} to 215^{o} and held at 215^{o} for a total run time of 20 min. For PMAA derivatives, the column temperature was increased at 2^{o}/min from 165^{o} to 215^{o} and held at 215^{o} for a total run time of 30 min. An injection port temperature of 225^{o} and a detector block temperature of 240^{o} was used for both derivatives.

Mass spectra were done at the NIH Resource of

Biomedical Mass Spectrometry, Washington University School of Medicine, St. Louis, Missouri, Supported by NIH Grant No. RR00954 from the Division of Research Resources.

Isolation of PP II from Crude Papain

PP II was isolated by procedure of Chandrasekaran et al.(1). Crude papain (100 g) was dissolved in 1 L of 0.04 M cysteine (1 g/10 mL), and the pH of the mixture was adjusted to 9 with 1 M NaOH. The grey precipitate of contaminating latex material was removed by centrifugation at 4^{o}. The supernatant was adjusted to 0.4 saturation with solid ammonium sulfate (saturation at 4^{o} = 71.93 g/100 g, 0.4 saturation = 22.74 g/100 g) and kept at 4^{o} for 1 h. Centrifugation at 4^{o} removed the white precipitate which contained enzyme activity. The supernatant was dialyzed overnight against running tap water, then 24 h against several changes of distilled water. The dialyzate was then made to contain 10% trichloroacetic acid by the addition of trichloroacetic acid, and the resulting precipitate of protein was removed by centrifugation at 4^{o}. The supernatant was again dialyzed overnight against running tap water, then 24 h against several changes of distilled water. After concentration to a small volume, residual protein was removed by passing the dialyzate through a 200-mL bed volume cation-exchange column packed with Dowex 50 (H^{+}) cation-exchange resin equilibrated with 0.1 M acetate buffer (pH 4.0). The column was washed

with the same buffer. The eluant was dialyzed exhaustively, first against running tap water overnight, then against several changes of distilled water 24 h, before evaporation to dryness on a rotary evaporator at 50°; yield of polysaccharide, 1.5 g.

The mixture of polysaccharides was dissolved in 0.04 M NaCl and subjected to fractionation on a 5 x 41-cm DEAE-cellulose column (bed volume = 660 mL) which had been equilibrated with 0.04 M NaCl. The column was eluted with a 0.04 M—0.4 M linear gradient of NaCl achieved by placing 1 L of 0.4 M NaCl in a reservoir tank and 1 L of 0.04 M NaCl in a mixing chamber. Fractions (14 mL) were collected at the rate of 14 mL/h using a Gilson fraction collector together with a Gilson Mini-Puls 2 pump.

Fractions were analyzed for the presence of uronic acids. Plots of fraction number vs. absorbance at 520 nm and fraction number vs. conductance were made to determine the fractions that contained PP II. Fractions 45 to 53 were pooled, dialyzed exhaustively concentrated to a small volume, and lyophilized to give pure PP II; yield, 0.68 g.

Uronic Acid Determination

The presence of uronic acids was determined by the procedure discribed by Blumenkrantz and Asboe-Hansen (8). A 20-µL aliquot was added to 480 µL of water in an ice bath;

3.0 mL of 0.0125 M sodium tetraborate ($Na_2B_4O_7.10\ H_2O$) in conc. H_2SO_4 was added. After mixing, the solution was heated at 100° (boiling water bath) for 5 min, then cooled in an ice bath for 5 min before addition of 50 µL of a solution of 0.15% (w/v) m-hydroxydiphenyl in 0.5% (w/v) NaOH. After thorough mixing, the solution was allowed to stand for 30 min; then the absorbance at 520 nm was determined. Appearance of a pink color in the mixture indicated the presence of uronic acid.

Optimum Hydrolysis Time for PP II

PP II (63 mg) was dissolved in 45 mL of 0.5 M H_2SO_4. Portions (2.0 mL) were placed in each of 21 centrifuge tubes which were then capped and heated in a boiling water bath. After 2 h, three tubes were withdrawn; three more tubes were withdrawn every hour thereafter until a total hydrolysis time of 8 h was reached. Reducing sugar content of the tubes was determined by the Somogyi micro copper reduction method (9).

The contents of each tube were neutralized with solid $BaCO_3$, centrifuged, and filtered. The solid was washed twice with 1.00-mL portions of water, and both washings and filtrate were combined and diluted to 5.0 mL with distilled water. Copper reagent[1] (5.0 mL) was added, and the mixture was heated in a boiling water bath for 0.5 h, then cooled

before addition of 3.0 mL of 1 M H_2SO_4. After thorough mixing, liberated iodine[2] was titrated with 0.005 M sodium thiosulfate until the solution was pale yellow, when starch indicator was added and titration was continued until the disappearance of the starch-iodine color. A plot was made of the hydrolysis time vs. mL of thiosulfate required to titrate liberated iodine equivalent to the amount of reducing sugar present. Three blanks were also run.

[1]Copper reagent was prepared as for the Somogyi micro method for the determination of reducing sugars as follows: Potassium sodium tartrate (Rochelle salt, 30 g), anhydrous sodium carbonate (30 g), and 1 M NaOH (40 mL) were added to 200 mL of hot distilled water. A solution of 8 g of $CuSO_4.5H_2O$ in 80 mL of water was added, and the mixture was boiled and cooled before adding it to the previous solution. The two solutions were mixed well. An aqueous solution of 8 g of KI and 25 mL of 0.2 aq KIO_3 were added, and the mixture was made up to 1 L with cold boiled water. The reagent was allowed to stand at room temperature for 3 days before use.

[2]Iodine was liberated by the acid-catalyzed redox reaction of KI/KIO_3. The reducing sugars present reduce Cu^{2+} to Cu^{+} which precipitates as the reddish-brown Cu_2O. The cuprous oxide reduces iodine to iodide. Thiosulfate ion is used to determine the amount of unreacted iodine. By subtraction of the volume of thiosulfate required to titrate the excess-iodine from the volume required for the blanks

(those containing no reducing sugars), a measure of the amount of reducing sugars present was obtained.

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$
$$Cu_2O + 2H^+ + I_2 \longrightarrow 2Cu^{2+} + 2I^- + H_2O$$
$$I_2 + 2S_2O_3^{--} \longrightarrow 2I^- + S_4O_6^{--}$$

Complete Hydrolysis of PP II and PP II-R

PP II or PP II-R (75 mg) was dissolved in 3.0 mL of 0.5 M H_2SO_4. The solution was heated at 100° (oven) for 6 h. The hot solutions were neutralized with solid $BaCO_3$ to pH 6, filtered, deionized with Dowex 50(H^+) cation-exchange resin, passed through a column (8 x 260 mm) of Amberlite IR-45 (OH^-) anion-exchange resin to remove the uronic acid-containing components, and evaporated to dryness under reduced pressure at 50°.

Isolation of Uronic Acid Residues in PP II

PP II (500 mg) was heated at 100° in 25 mL of 0.5 M H_2SO_4 for 4 h. After neutralization and deionization, the uronic acid-containing fragments were isolated by preparative descending paper chromatography on Whatman 3MM paper (24 h); yield of UA1, 30 mg; yield of UA2, 10 mg. These components were reduced and hydrolyzed as described below.

Reduction of Uronic Acid Residues in PP II

Reduction of PP II was achieved by the method described by Taylor and Conrad (10). PP II (100 mg) was dissolved in 2.0 mL of water, and 300 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added. The pH of the solution was maintained at 4.5 for 1 h at room temperature by dropwise addition of 0.1 M HCl. Sodium borohydride (500 mg) was added slowly with stirring, and the mixture was kept at 50° for 2 h. Excess borohydride was destroyed by dropwise addition of 4 M HCl with strirring until no more effervescence occured. The mixture was exhaustively dialyzed, first against running tap water overnight, then against several changes of distilled water for 24 h, and lyophilized; yield of PP II-R 86 mg.

Reduction of Uronic Acid-Containing Fragments

Uronic Acid-containing fragments were reduced as described for PP II except that the excess borohydride was destroyed by addition of Dowex 50(H^+) cation-exchange resin. After filtration to remove the resin, the filtrate was concentrated to dryness at 50° under reduced pressure several times with MeOH to remove borate ions as methyl borate.

Partial Hydrolysis of PP II-R

Partial hydrolysis was achieved by heating PP II-R in 0.5 M H_2SO_4 at 100° for 2 h. The mixture was worked up as before, and the components were isolated by preparative paper chromatography.

Partial hydrolysis with minimal molecular weight reduction was achieved by heating PP II-R in 0.05 M H_2SO_4 at 100° for 2 h. Workup was the same as before. Isolation of components was achieved by preparative descending paper chromatography.

Preparation of Peracetylated Aldononitriles (PAAN)

PAAN derivatives were prepared as described by Lance and Jones (II). Dried neutral sugars (40 mg) from the PP II hydrolyzate were dissolved in 0.5 mL of dry pyridine (distilled over CaH_2 and stored over KOH pellets); 40 mg of hydroxylamine hydrochloride was added, and the mixture was heated in a capped vial at 90°(oven) for 1 h. Acetic anhydride (0.5 mL) was then added, and heating was continued at 90° for another 1 h. The reaction mixture was evaporated to dryness with the aid of toluene to remove pyridine. The residue was dissolved in chloroform; and the resulting solution was washed three times with water, dried over anhydrous $MgSO_4$, concentrated to a small volume (1.0 mL), and subjected to glc on OV225.

Preparation of Trimethylsilylated-Oxime (TMS-oxime) Derivatives

Neutral sugars from hydrolyzed PP II (40 mg) were dissolved in 4.0 mL of a solution of hydroxylamine hydrochloride in pyridine (30 mg/mL). The solution was heated in a water bath at 75° for 0.5 h, then allowed to cool to room temperature before addition of 4.0 mL of hexamethyldisilazane (HMDS), followed by addition of 0.4 mL of trifluoroacetic acid. After the white precipitate had settled, the clear supernatant was subjected to glc on SE 52.

Methylation of PP II-R

PP II-R was methylated as described for polysaccharides by Jansson et al. (7). Dried PP II-R (500 mg; dried at 50° in the presence of P_2O_5 under reduced pressure overnight) was dissolved in 3.0 mL of dry methyl sulfoxide (distilled under reduced pressure over CaH_2 and stored over molecular sieves, 4 Å) at 60°. The flask was stoppered with a serum cap and flushed with dry argon via two injection needles. A 2 M solution of sodium methyl sulfinylate in methyl sulfoxide (5.0 mL) was added to the stirred mixture in a dropwise fashion through a syring, using a second needle as a vent. After stirring at room temperature for 1 h, the needles were removed, but stirring was continued at room temperature overnight. The vial was then cooled in an ice-

water bath before dropwise addition of methyl iodide (5.0 mL) through a syringe with a second needle as vent. Stirring was continued at room temperature for 1 h; excess methyl iodide was removed by evaporation under reduced pressure, and the residue was exhaustively dialyzed, first against running tap water overnight, then against several changes of distilled water for 24 h. The dialyzate was lyophilized; yield of MPP II-R, 54 mg.

Oligomeric Material

In the case of oligomeric material, the reaction mixture was not dialyzed but was instead diluted with five times its volume of water and extracted with four 5.0-mL portions of chloroform. The chloroform extracts were washed with four 5.0-mL portions of water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure.

Preparation of Sodium Methyl Sulfinylate (Sodium Dimsylate 2M *in* DMSO (7))

Sodium hydride (50% suspension in oil, 5 g) was weighed into a 100-mL flask and washed three times with 25-mL portions of hexane. The sodium hydride was allowed to settle, and the solvent was decanted. The flask was flushed with dry argon to dry the solid while keeping it in an

atmosphere of dry argon. Dry methyl sulfoxide (vacuum distilled over CaH_2 and stored over molecular sieves, 4 Å; 50 mL) was added. The flask was sealed with a serum cap and flushed with dry argon via two injection needles. The flask was heated to 60° with stirring and evolved hydrogen was vented through an injection needle. After the evolution of hydrogen had ceased, a dark greenish-grey solution of 2 M sodium dimsylate remained. The reagent was stored sealed with a serum cap in a refrigerator and was stable for up to 4 weeks.

Acid-Catalyzed Hydrolysis of Methylated Polysaccharide and Transformation into Partially Methylated Alditol Acetates (PMAA)

Methylated PP II-R (MPP II-R; 50 mg) was treated with 10 mL of 88% formic acid at 100° (oven) for 1 h. The acid was removed under reduced pressure; the residue was dissolved in 15 mL of 0.13 M H_2SO_4, and heating was continued at 100° overnight. Excess acid was neutralized with solid $BaCO_3$; the solids were removed by filtration and washed twice with 5-mL portions of water. Both washings and filtrate were combined and treated with Dowex 50(H^+) cation-exchange resin to remove residual Ba^{2+} ions. The resins were removed by filtration, and the filtrate was concentrated to 5.0 mL at 50° under reduced pressure.

Sodium borohydride (500 mg) was carefully added, and the mixture was kept at 50° for 2 h. Excess borohydride was destroyed by addition of Dowex 50(H^+) cation-exchange resin, which was then removed by filtration. The filtrate was evaporated to dryness three times with 15-mL portions of methanol to remove borate ions as methyl borate.

Acetylation of the partially methylated alditols was achieved by treatment with 6 mL of a 1:1 v/v pyridine-acetic anhydride mixture. (The pyridine was dried over KOH pellets. After 1 h at 100°, the solvents were removed by evaporation under reduced pressure at 40° with the aid of toluene. The residue was dissolved in 5.0 mL of dichloromethane. The solution was washed three times with 3-mL portions of water, dried over anhydrous $MgSO_4$, concentrated to 1.0 mL, and subjected to glc on an OV225 column.

Transformation into PMAA derivatives was done in the same fashion for all residues of fragments isolated from partial hydrolysis of PP II-R as well as the uronic acid-containing fragments.

DISCUSSION

Gas-liquid chromatography (glc) offers a fast, versatile method for separation and quantitative analysis of sugars. However, before it can be used, the sugars must be suitably derivatized to make them volatile. Derivatives that preserve the anomeric center present a problem because they give rise to multiple peaks, for example α- and β-pyranose peaks and α- and β-furanose peaks. Thus, derivatives such as alditol acetates, which give a single peak, produce much simpler chromatograms.

Gas-liquid chromatography alone, however, does not identify unequivocally any component; but together with complimentary information from mass spectral fragmentation patterns, unambiguous identification is possible. Glc-ms has been used extensively for characterization of substituted and unsubstituted sugars. Glc-ms has been used to identify peracetylated aldononitriles (PAAN) (12 - 15), trimethylsilylated derivatives (TMS) (16-18), and partially methylated alditol acetates (PMAA) (6,19,20,21). In this investigation, alditol acetates (PMAA derivatives) were the derivatives of choice in the methylation analysis, not only because they give single peaks, but also because sufficient data for conclusive identification as available (7).

Complete hydrolysis of PP II indicated that the only sugars present in a hydrolyzate of the polysaccharide were rhamnose, arabinose, galactose and an aldobiouronic acid which was separated from the neutral sugars by adsorption onto an anion-exchange resin. The presence of small amounts of glucuronic acid and glucuronolactone were also evident on paper chromatograms of the PP II hydrolyzate. Complete hydrolysis of PP II-R indicated the presence of rhamnose, arabinose, glucose, and galactose.

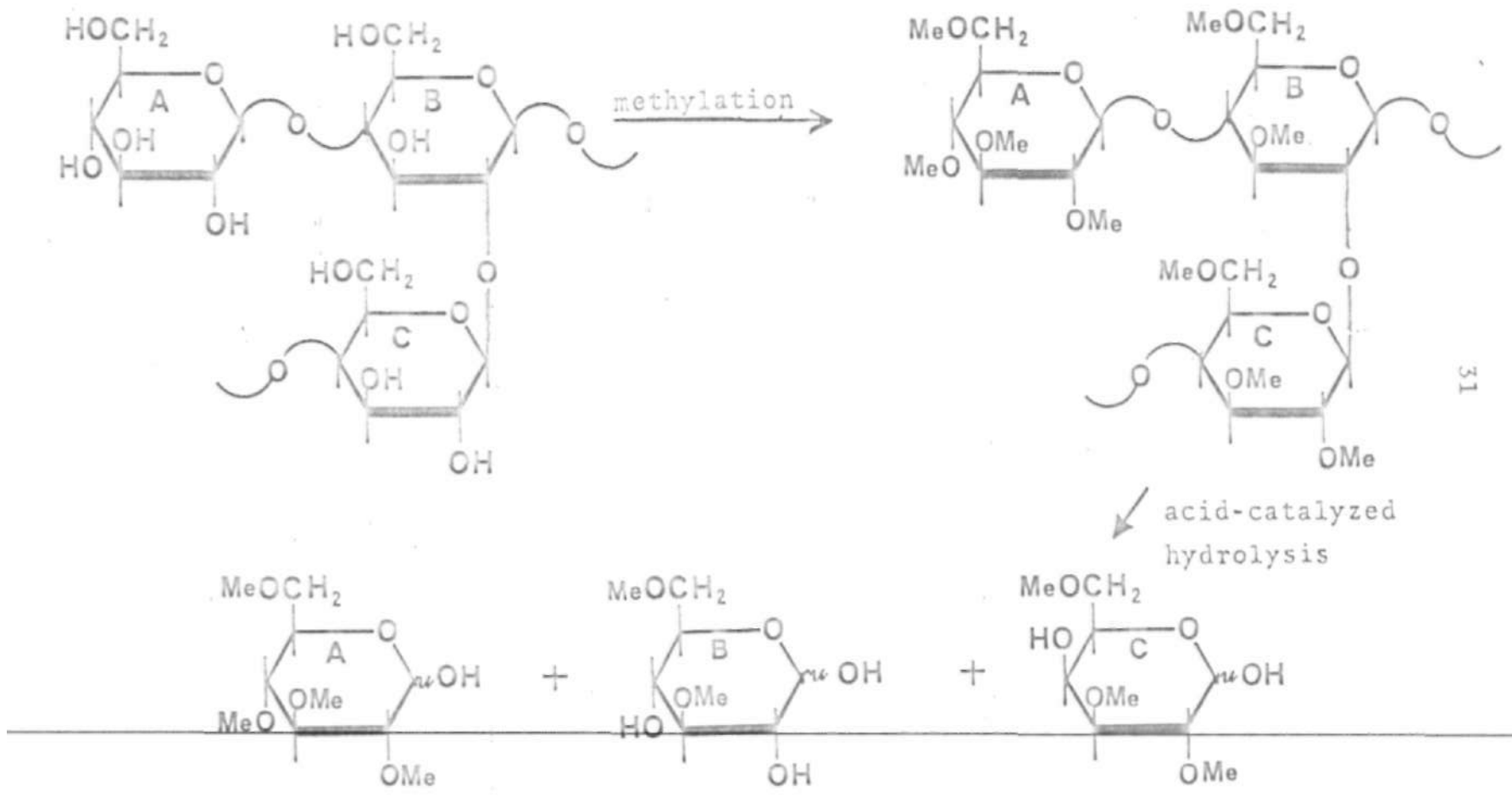
The total absence of glucose in the neutral sugars of hydrolyzed PP II and its presence in the neutral sugar of PP II-R indicated that the uronic acid component of pp II was glucuronic acid. The minor peak in Figure 7 may be that derived from 4-O-methylglucuronic acid. Methylation analysis of the aldobiouronic acids isolated from the hydrolyzate revealed that the reducing end unit was galactose, the uronic acid at the nonreducing terminus being glucuronic acid, in the major aldobiouronic acid and 4-O-methylglucuronic acid in the minor aldobiouronic acid.

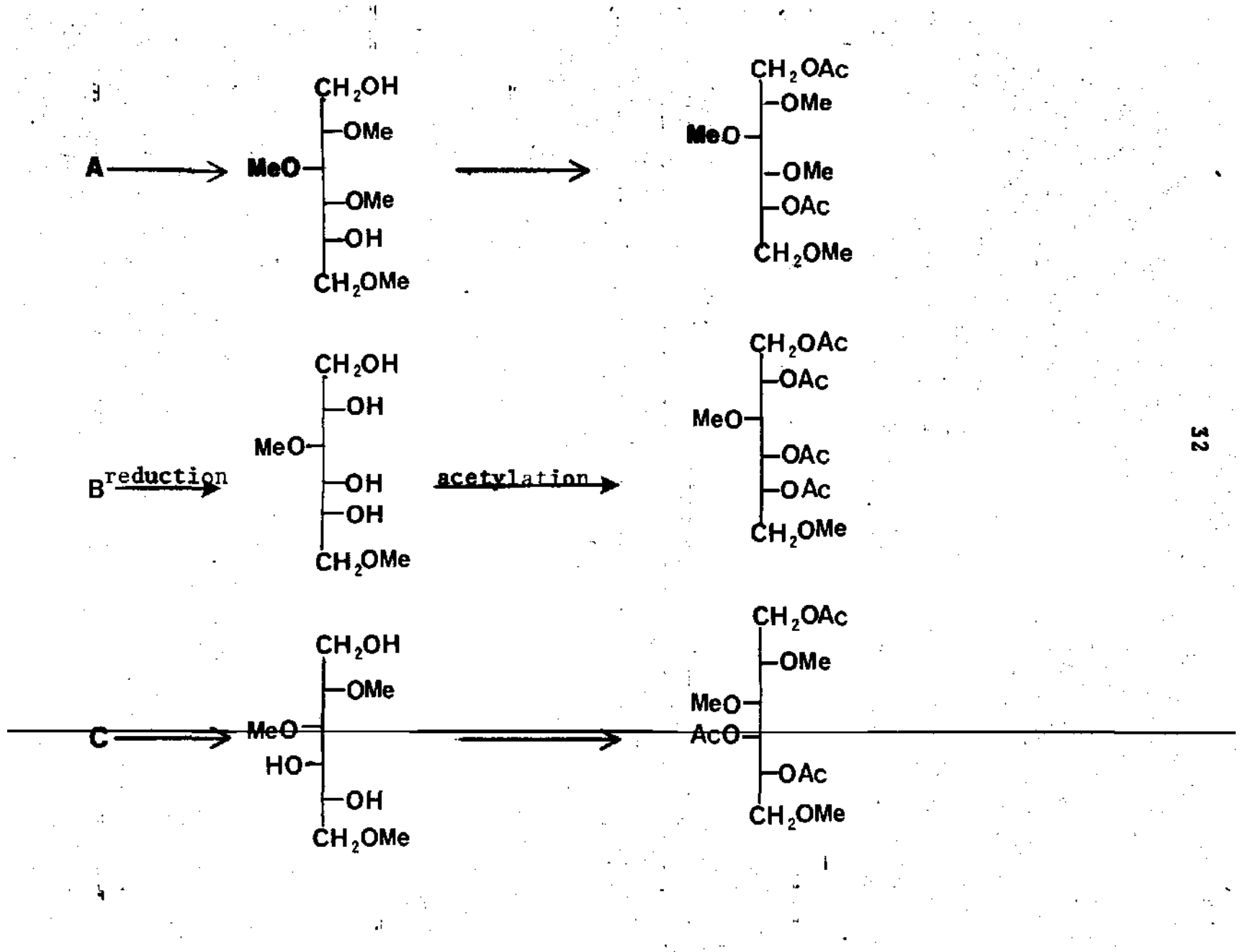
Methylation analysis is an invaluable tool in the determination of linkages between the monosaccharide units of oligosaccharides. The method involves methylation of all free hydroxyl groups by the Hakomori procedure ($CH_3SOCH_2^-$ Na^+-CH_3I) (22) which has now repaced the Haworth ($NaOH$, $(CH_3)_2SO_4$) and Purdie (Ag_2O, CH_3I) methods (23). Acid-catalyzed

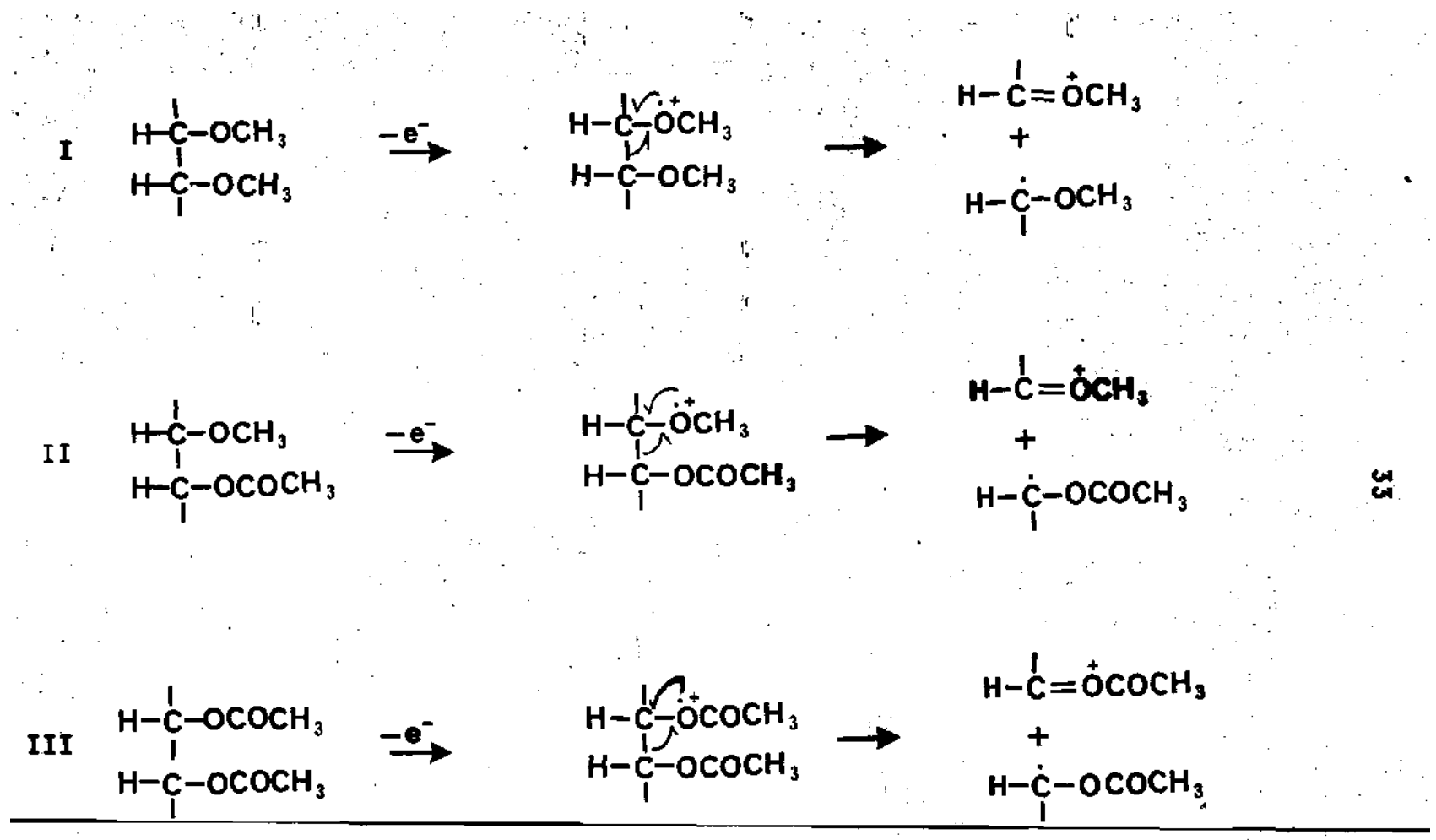
hydrolysis of the methylated polysaccharide, followed by reduction and acetylation of the free hydroxyl groups gives the partially methylated alditol acetates (PMAA, Scheme 1) in which the unmethylated hydroxyl groups indicate positions of substitution before hydrolysis.

The PMAA derivatives are separated by glc (24) and characterized by their mass spectral fragmentation patterns (6). The positions of the acetyl groups which indicate the positions of chain linkages and ring cyclization are determined by examination of mass spectral fragmentation patterns. Although molecular ions are rarely observed, they may be determined by addition of a pair of fragments. Because fragmentation depends only on the substitution patterns, stereoisomers give identical fragmentation patterns and cannot be identified solely on the basis of their ms data. However, a combination of glc and ms differentiates between stereoisomers.

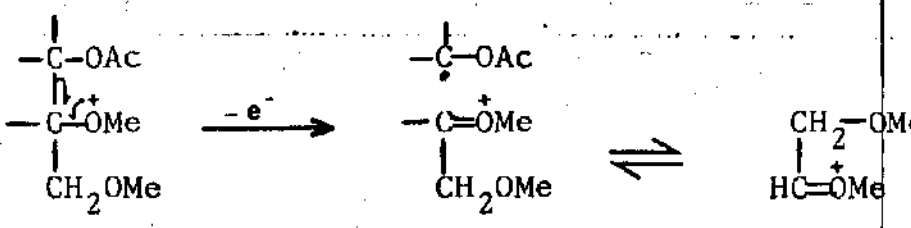
Fragmentation of PMAA derivatives occurs via primary cleavage between carbon atoms in the sugar chain. Fragmentation of PMAA derivatives in the mass spectrometer has been extensively studied and preferential fissions have been established (6,19,25). α-Cleavage between two methoxylated carbon atoms (I) is more likely than cleavage between a methoxylated carbon atom and an acetoxylated


HOCH2
A
OH
HO
OH
HOCH2
B
OH
O
HOCH2
C
OH
methylation
MeOCH2
A
OMe
MeO
OMe
MeOCH2
B
OMe
O
MeOCH2
C
OMe
acid-catalyzed
hydrolysis
MeOCH2
A
OMe
MeO
OMe
OH
MeOCH2
B
OMe
HO
OH
OH
MeOCH2
HO
C
OMe
OH
OMe


A
MeO
CH2OH
OMe
OMe
OH
CH2OMe
CH2OAc
OMe
MeO
OMe
OAc
CH2OMe
B reduction
MeO
CH2OH
OH
OH
OH
CH2OMe
acetylation
MeO
CH2OAc
OAc
OAc
OAc
CH2OMe
C
MeO
HO
CH2OH
OMe
OH
CH2OMe
MeO
AcO
CH2OAc
OMe
OAc
CH2OMe


I
II
III
$-e^-$

carbon atom (II); the least abundant cleavage is one between two actoxylated carbon atoms (III) (Scheme 2). An exception is the formation of the primary fragment m/e 89, when cleavage II is favored over cleavage I in 1,2-di-O-methylalditols (Scheme 3), probably owing to stabilization of the positive charge by both methoxyl groups.

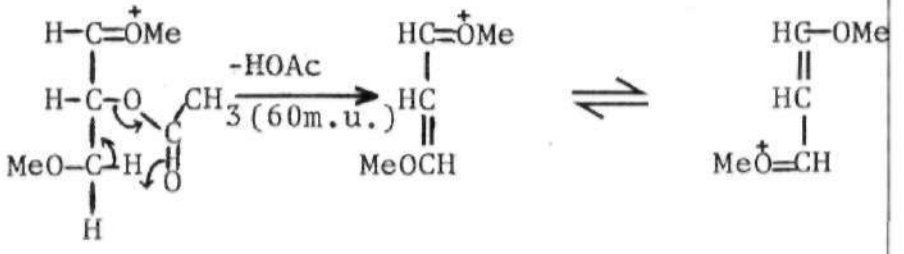


Scheme 3

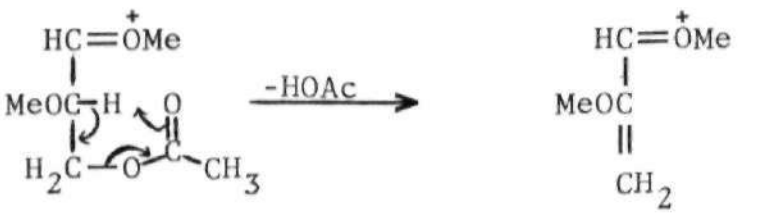
The primary fragment m/e 117 is obtained whenever C-1 is acetoxylated and C-2 is methoxylated while the lowest mass primary fragment m/e 45 is obtained in greater than 5% of base peak when C-1 is methoxylated and C-2 is acetoxylated.

Primary fragments may undergo a single fragmentation or a number of consecutive secondary fragmentations by β-elimination of small molecules such as acetic acid (60 m.u.), ketene (42 m.u.), methanol (32 m.u.), or formaldehyde (30 m.u.), giving rise to secondary fragments. The base peak in the mass spectra of all alditol acetates is the acetylium ion m/e 43 ($CH_3C\overset{+}{=}O$).

Acetic acid can be eliminated when an acetoxyl group is in either the alpha or beta position to the carbon atom bearing the formal positive charge or to a carbonyl group (Scheme 4).



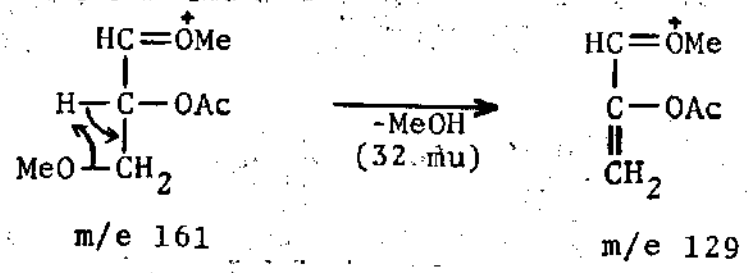
m/e 161 m/e 101



m/e 101

Scheme 4

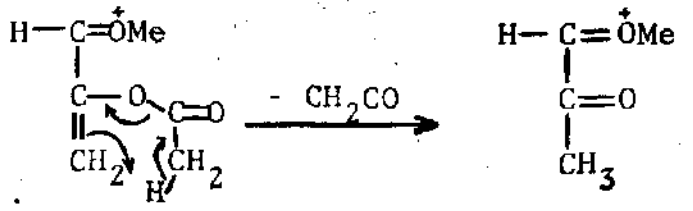
Methanol, on the other hand, can only be eliminated when it is beta to the carbon atom bearing the positive charge or to a carbonyl group (Scheme 5).



m/e 161 m/e 129

Scheme 5

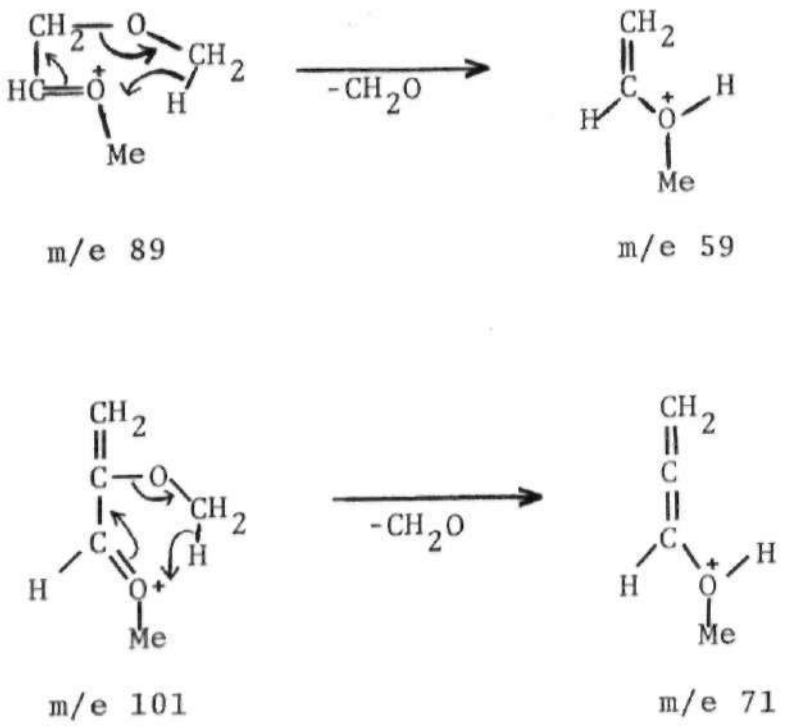
Ketene is subsequently lost from vinyl acetoxylated fragments formed by loss of methanol or acetic acid from some primary fragments (Scheme 6).



m/e 129 m/e 87

Scheme 6

Formaldehyde is lost from a fragment bearing adjacent methoxylated carbon atoms (Scheme 7).



m/e 89 m/e 59

m/e 101 m/e 71

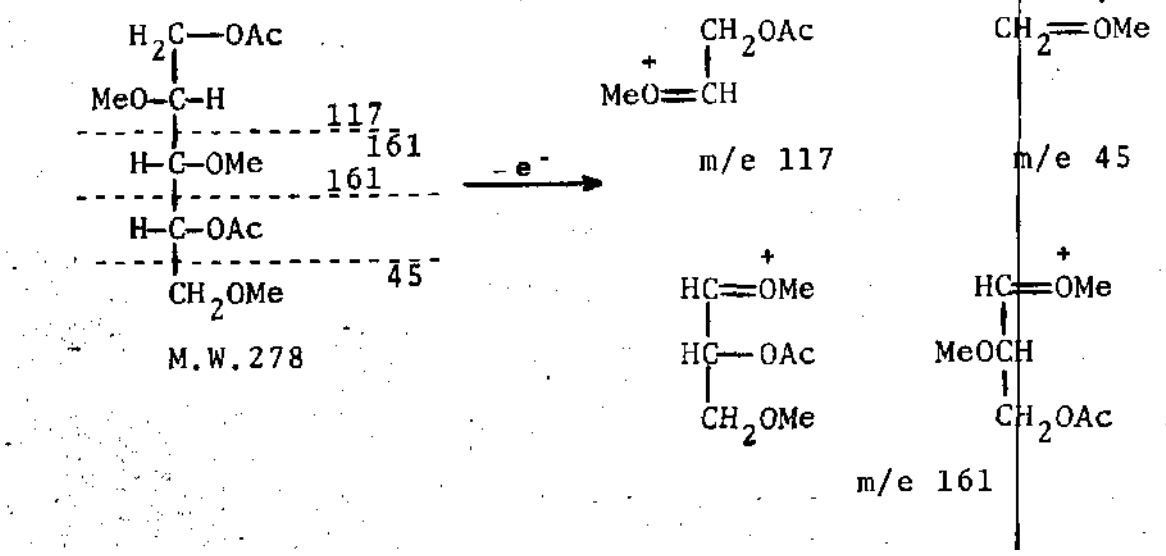
Scheme 7

Application of the knowledge of fragmentation behavior and retention times of PMAA derivatives led to the identification of all eleven components of the hydrolyzate of MPP II-R.

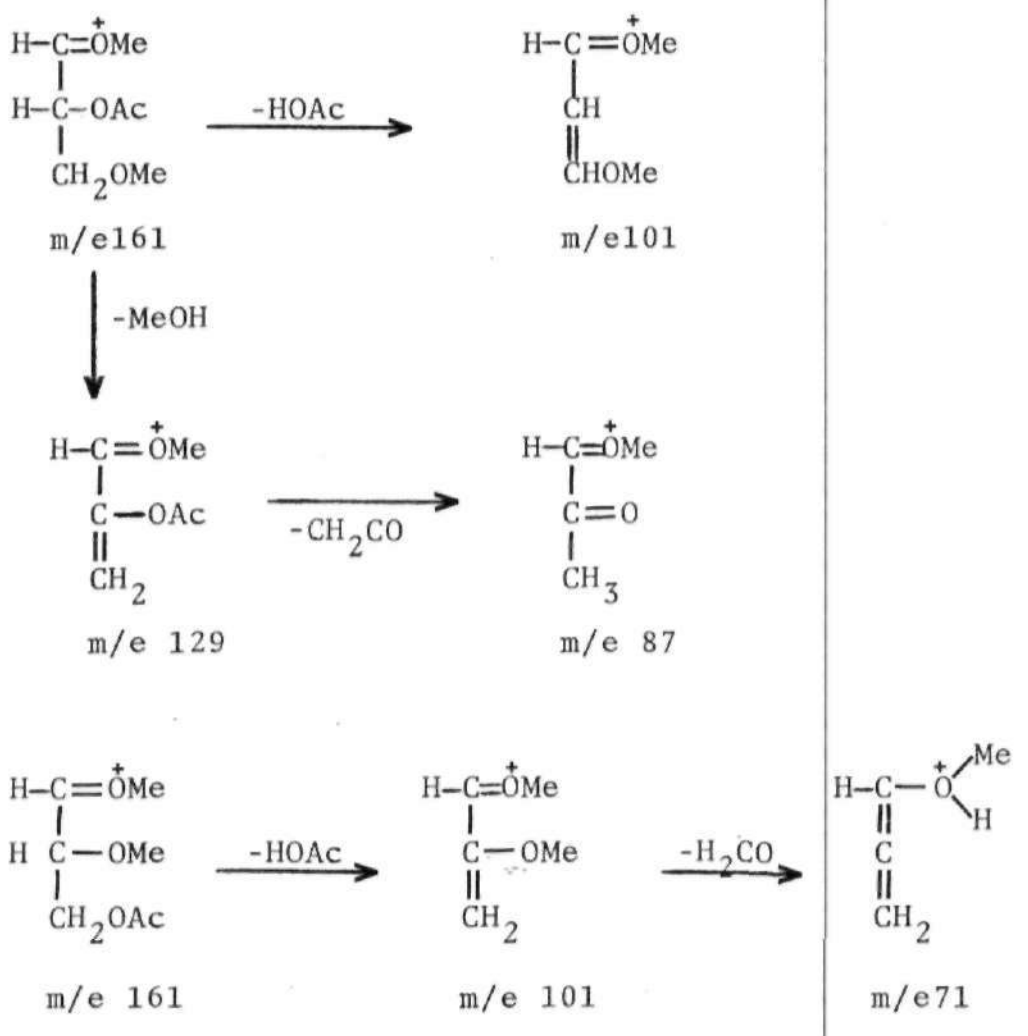
Peak A

1,4-Di-O-acetyl-2,3,5-tri-O-methylarabinitol

Primary fragmentation



Secondary fragmentation

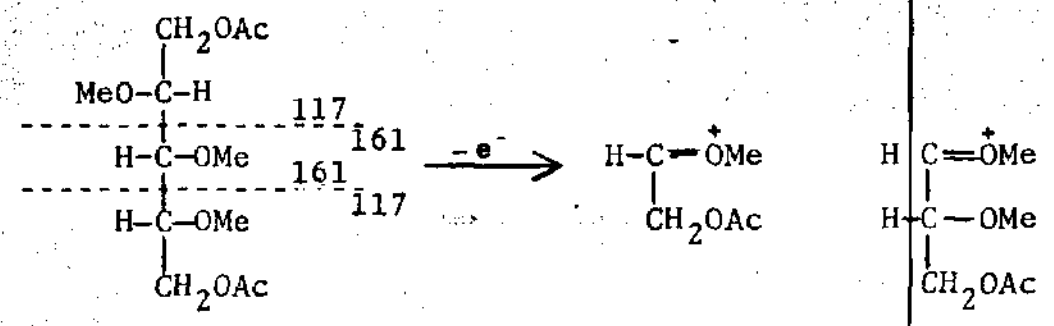


Scheme 8

Peak B

1,5-Di-O-acetyl-2,3,4-tri-O-methylarabinitol

Primary fragmentation



M.W.278 m/e117 m/e161

Secondary fragmentation as in scheme 8

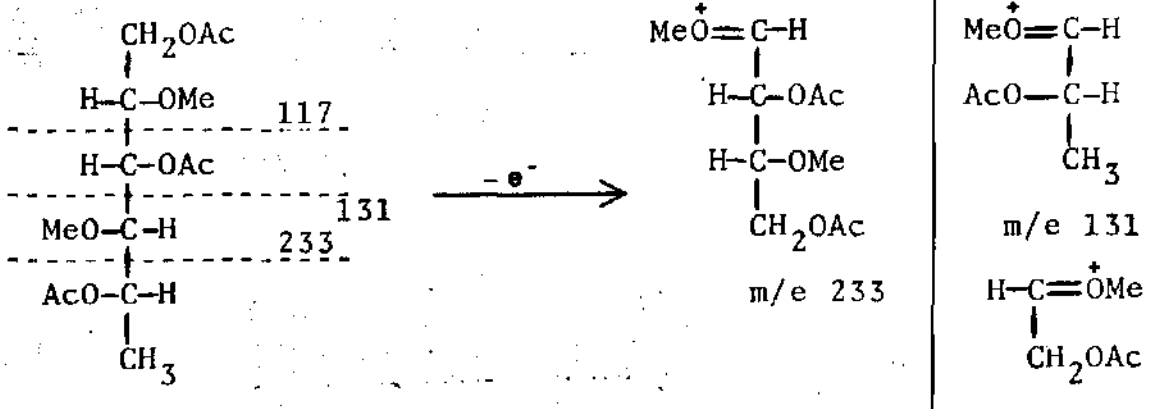
m/e 161 $\xrightarrow{-HOAc}$ m/e 101 $\xrightarrow{-H_2CO}$ m/e 71

Scheme 9

Peak C

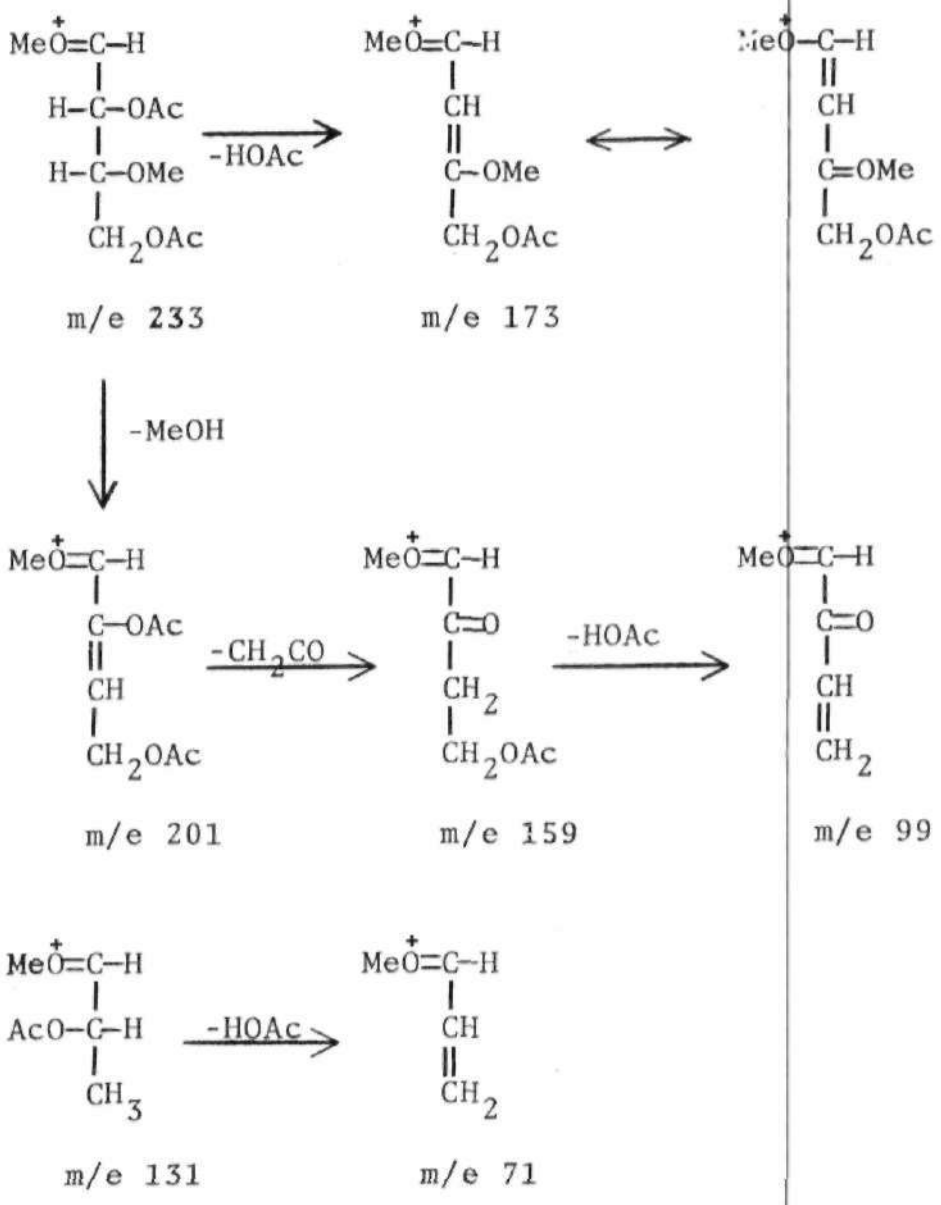
1,3,5-Tri-O-acetyl-2,4-di-O-methylrhamnitol

Primary fragmentation



M.W.320

Secondary fragmentation

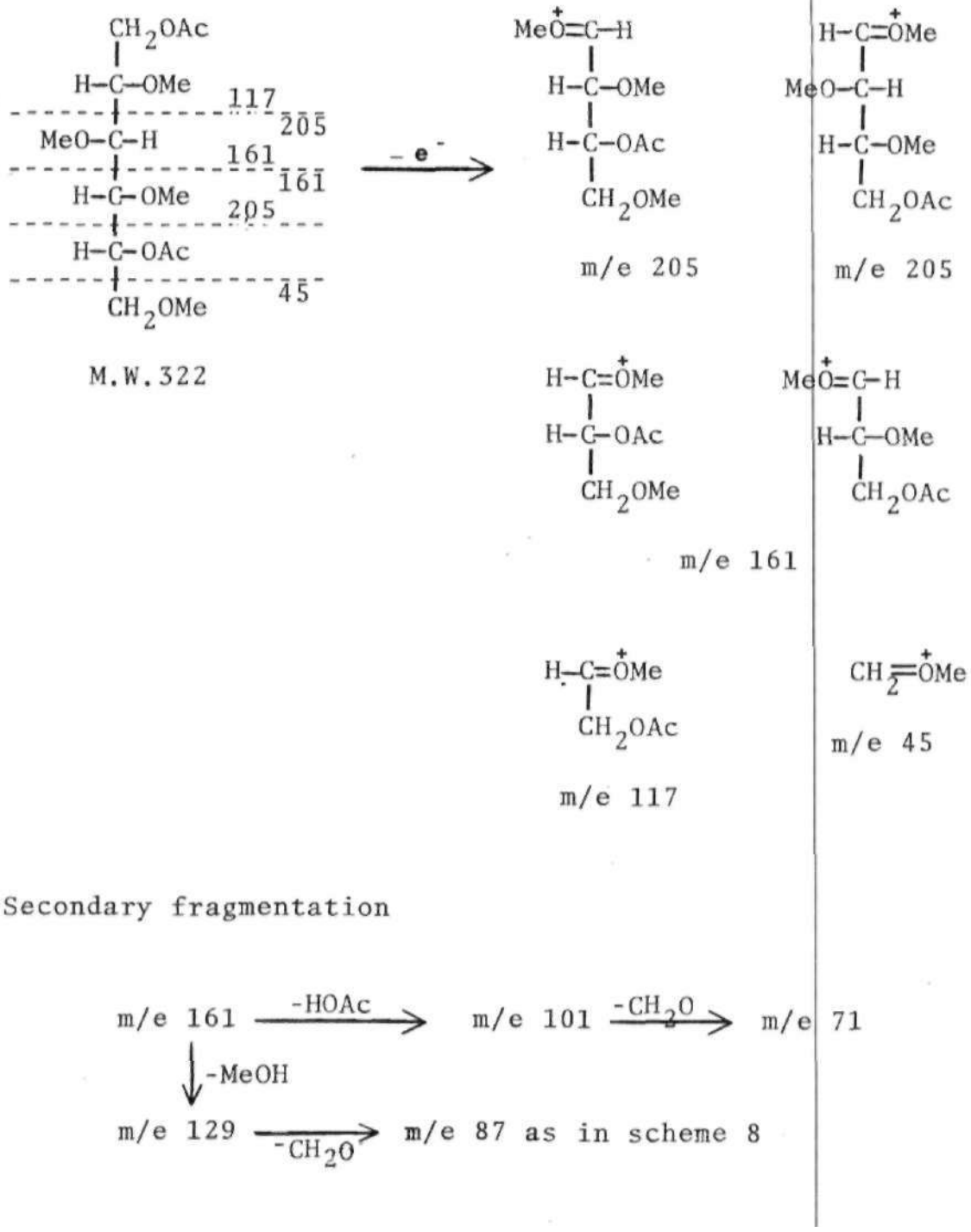


Scheme 10

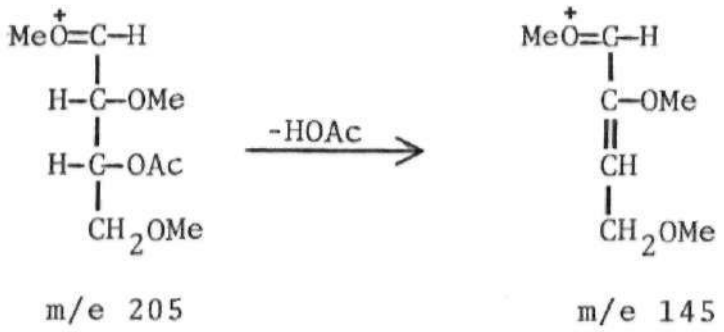
Peaks D and E

1,5-Di-O-acetyl-2,3,4,6-tetra-O-methylhexitol

Primary fragmentation



Secondary fragmentation

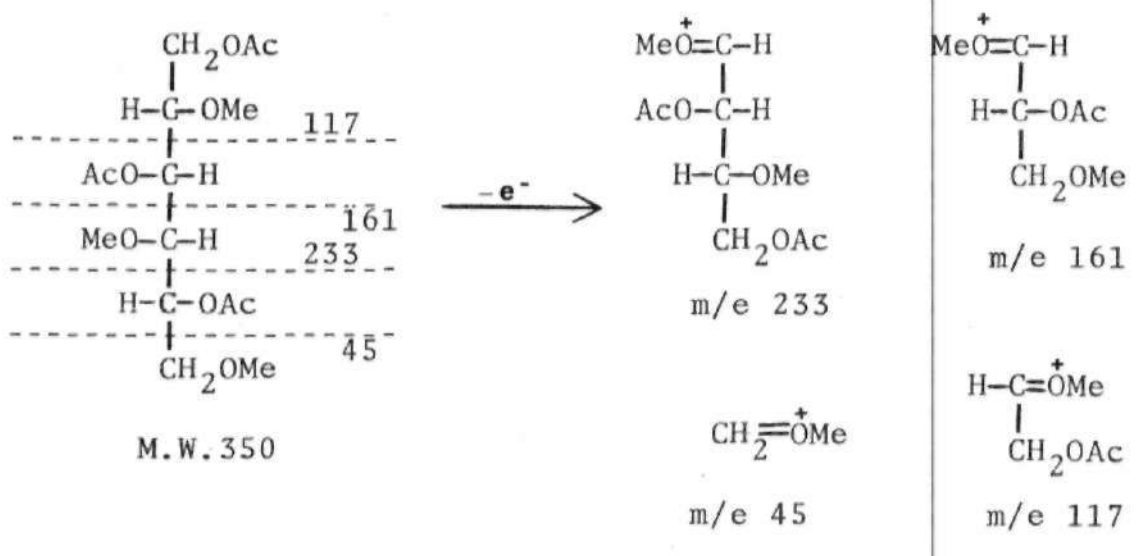


Scheme 11

Peak F

1,3,5-Tri-O-acetyl-2,4,6-tri-O-methylgalactitol

Primary fragmentation



Secondary fragmentation

m/e 233 $\xrightarrow{-MeOH}$ m/e 201 as in scheme 10

m/e 161 $\xrightarrow{MeOH}$ m/e 129 $\xrightarrow{-CH_2CO}$ m/e 87

$\downarrow$ -HOAc

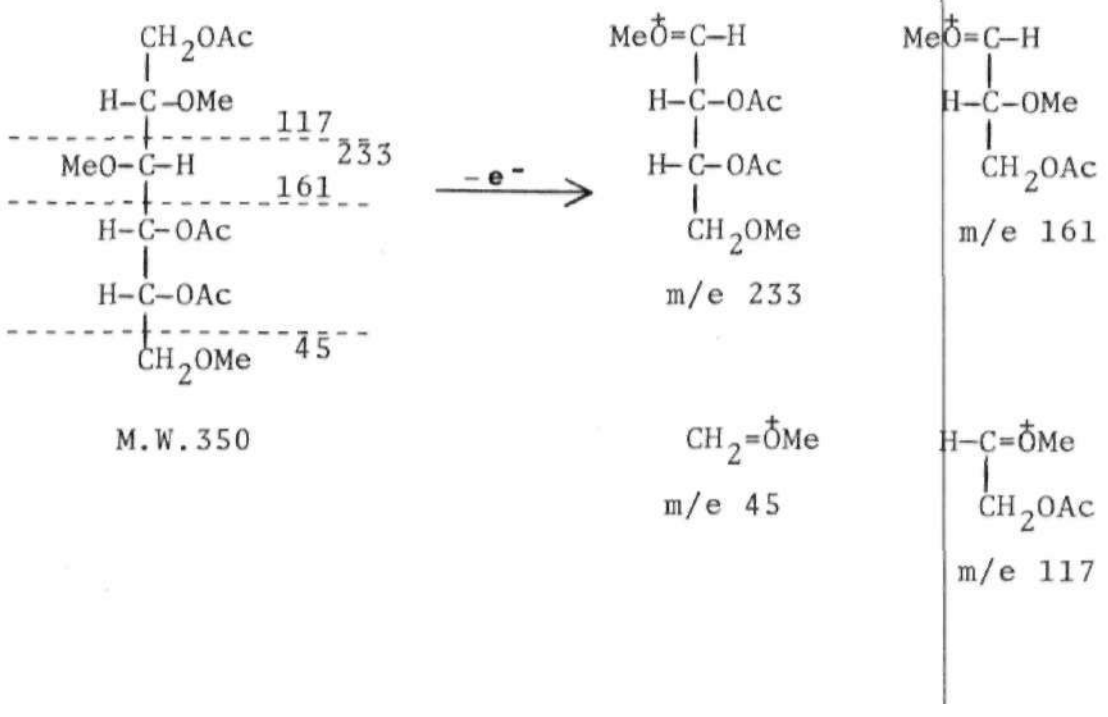
m/e 101 as in scheme 8

Scheme 12

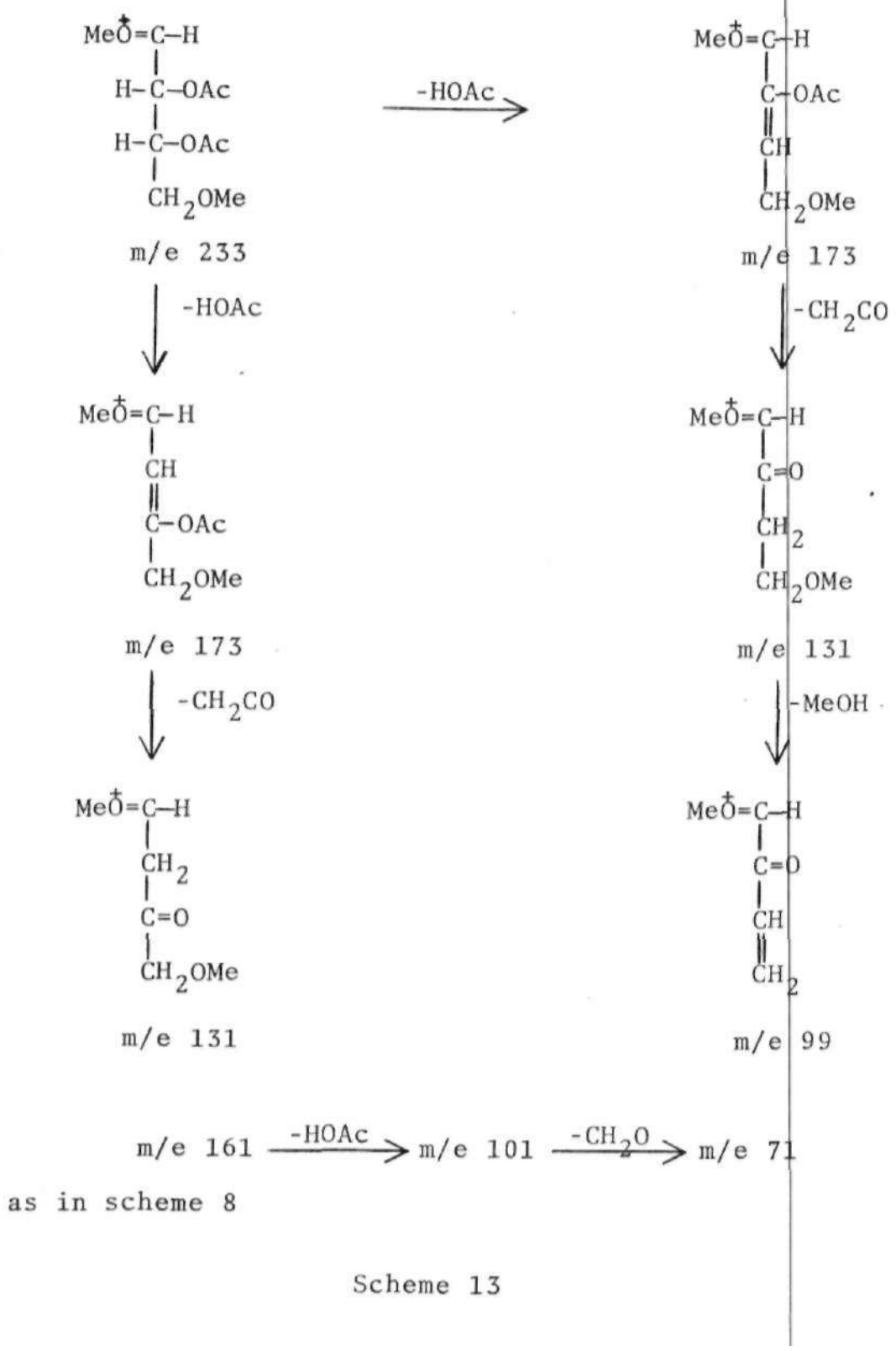
Peak G

1,4,5-Tri-O-acetyl-2,3,6-tri-O-methylglucitol

Primary Fragmentation



Secondary fragmentation

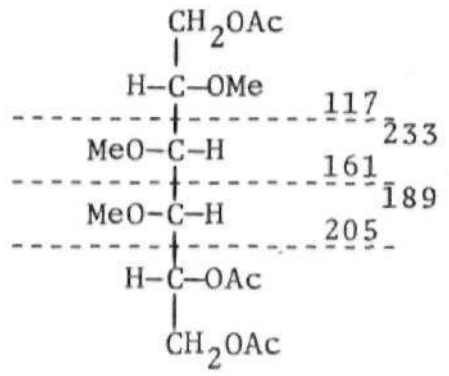


Scheme 13

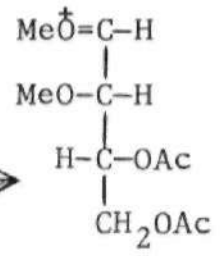
Peak H

1,5,6-Tri-O-acetyl-2,3,4-tri-O-methylgalactitol

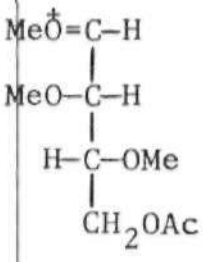
Primary fragmentation



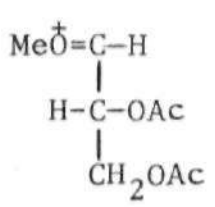
M.W. 350



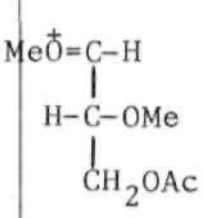
m/e 233



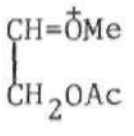
m/e 205



m/e 189

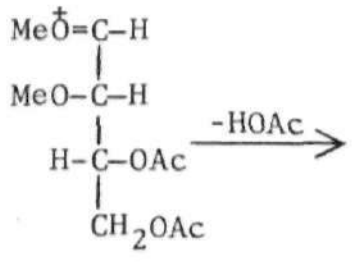


m/e 161

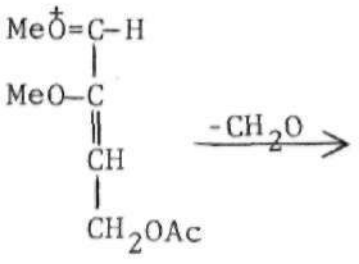


m/e 117

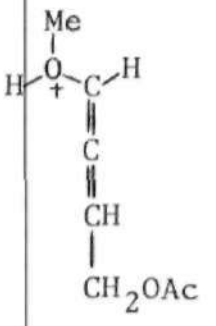
Secondary fragmentation



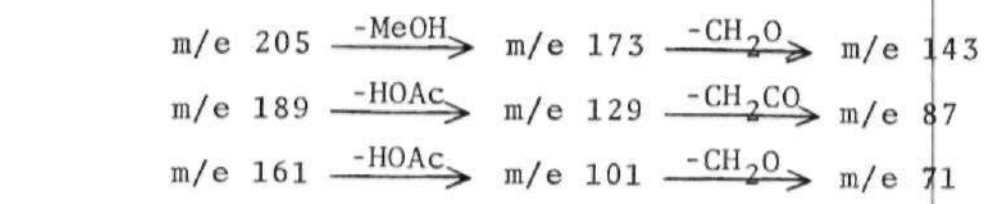
m/e 233



m/e 173



m/e 143

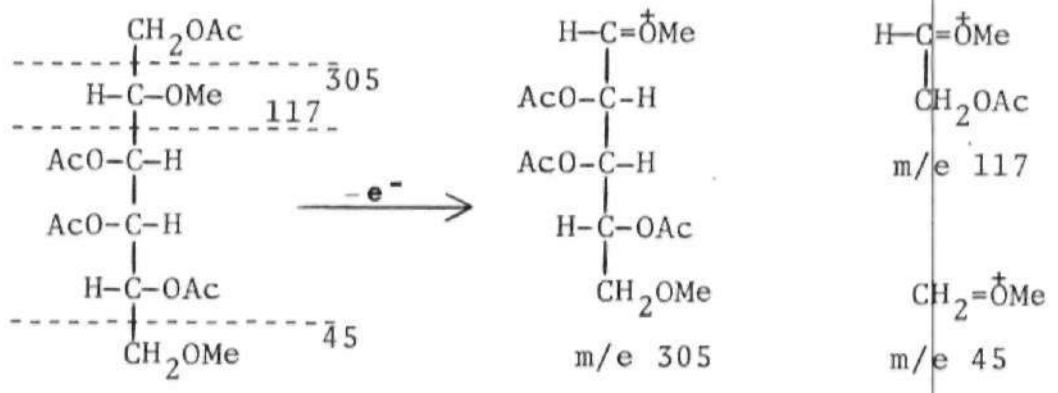


Scheme 14

Peak I

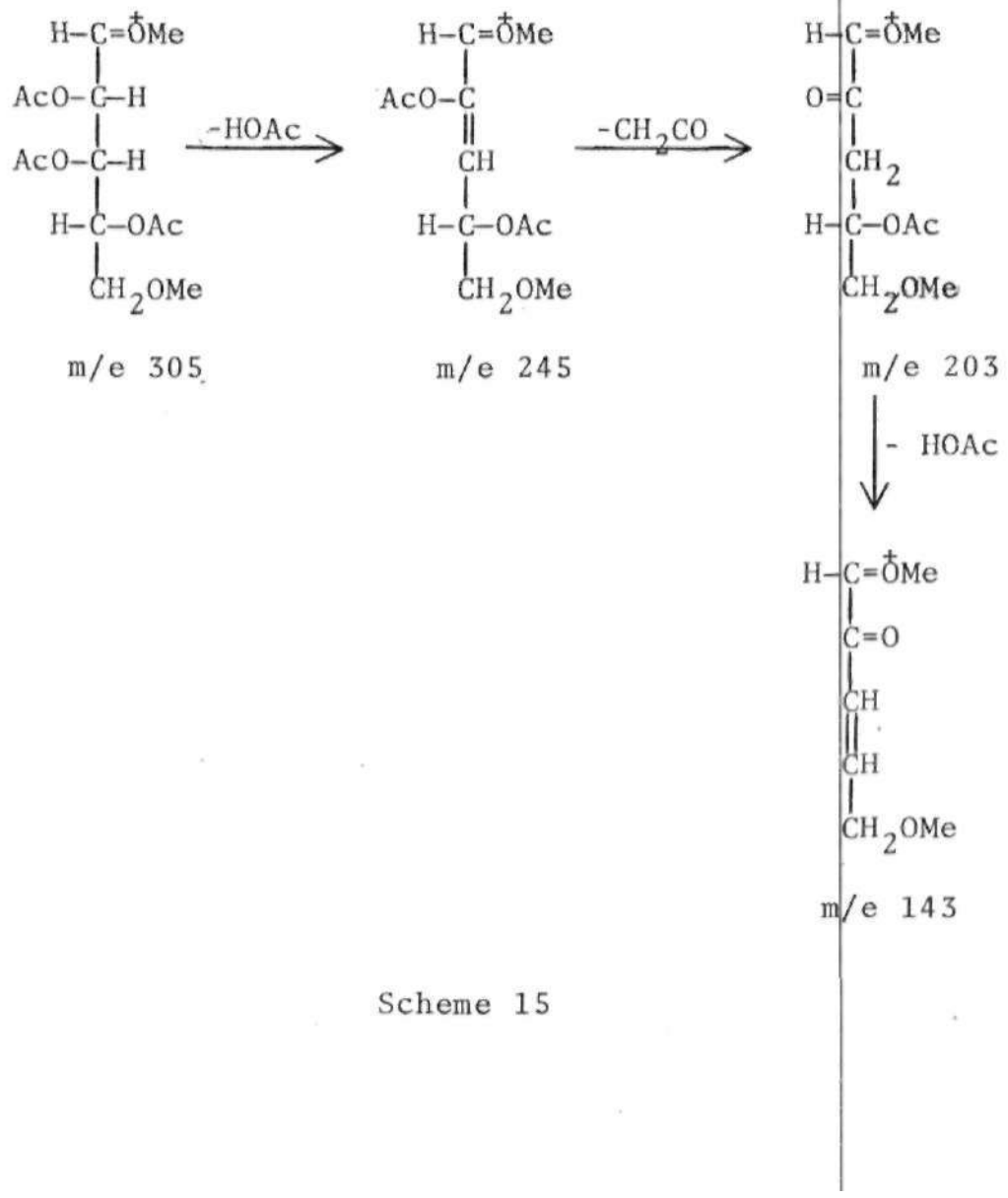
1,3,4,5-Tetra-O-acetyl-2,6-di-O-methylgalactitol

Primary fragmentation



M.W. 378

Secondary fragmentation

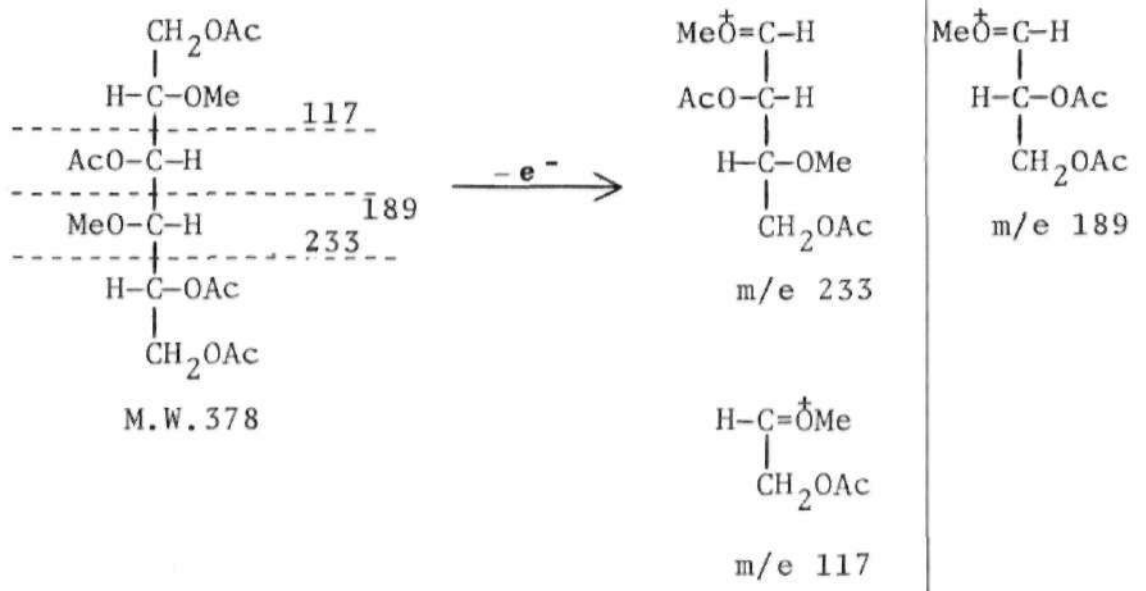


Scheme 15

Peak J

1,3,5,6-Tetra-O-acetyl-2,4-di-O-methylgalactitol

Primary fragmentation



Secondary fragmentation

m/e 233 $\xrightarrow{-MeOH}$ m/e 201 $\xrightarrow{-CH_2CO}$ m/e 159 $\xrightarrow{-HOAc}$ m/e 99 (scheme 10)

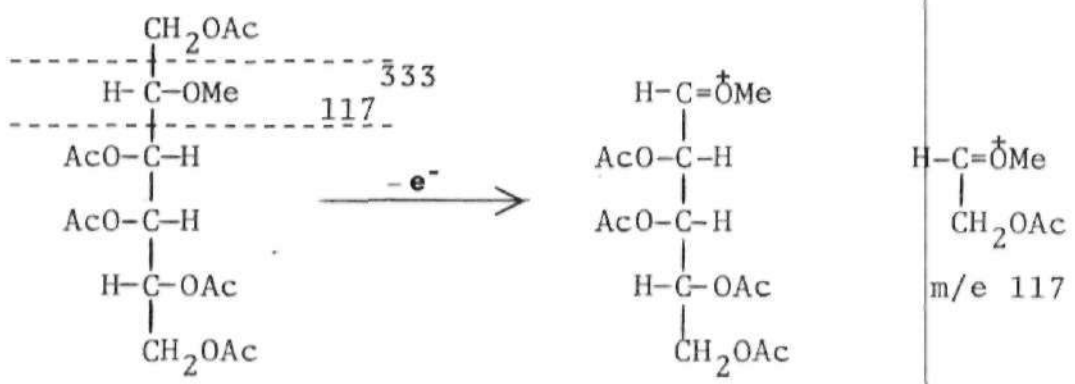
m/e 189 $\xrightarrow{-HOAc}$ m/e 129 $\xrightarrow{-CH_2CO}$ m/e 87 (scheme 14)

Scheme 16

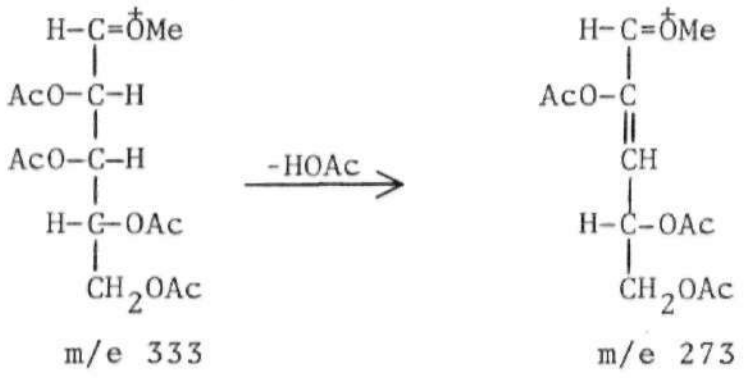
Peak K

1,3,4,5,6-Penta-O-acetyl-2-O-methylgalactitol

Primary fragmentation



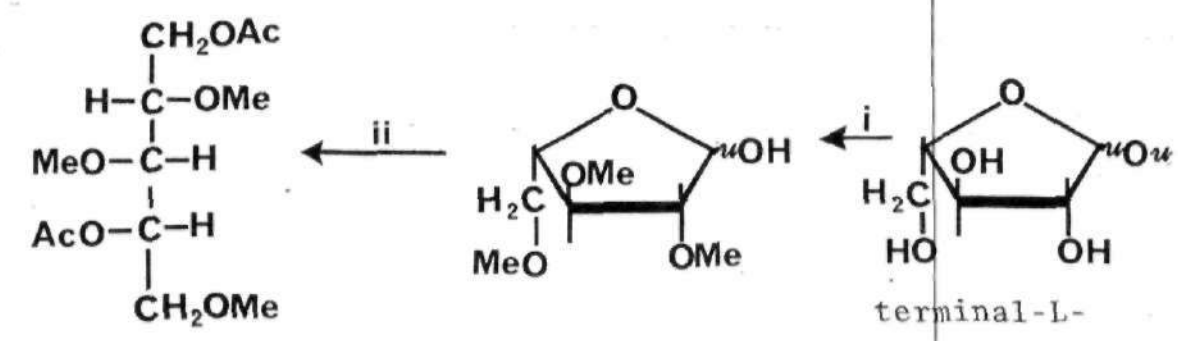
Secondary fragmentation



Scheme 17

The glc-ms data indicates the positions of acetoxyl groups in each PMAA derivative, which in turn indicates the positions of ring closure and chain linkage. Thus, the linkages in PP II-R may be derived as follows:-

Peak A

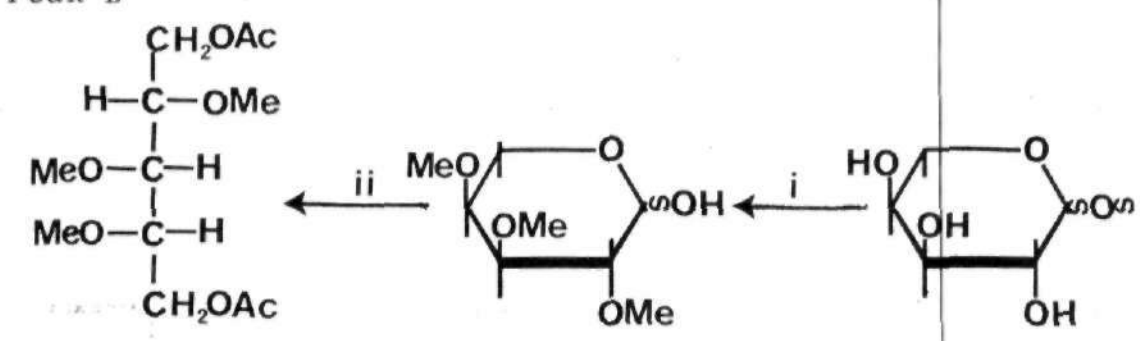


Scheme 18

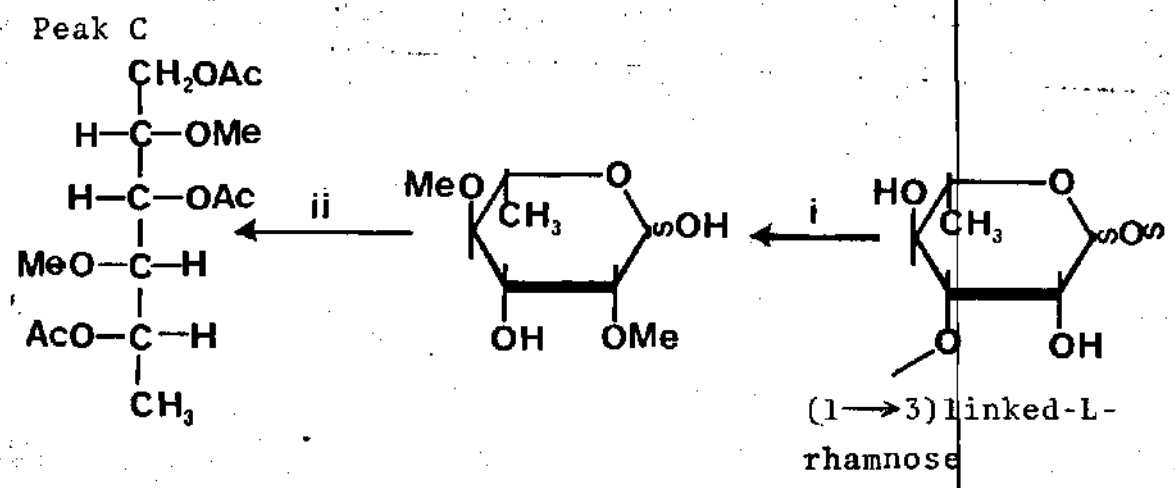
i methylation followed by acid-catalyzed hydrolysis

ii reduction followed by acetylation

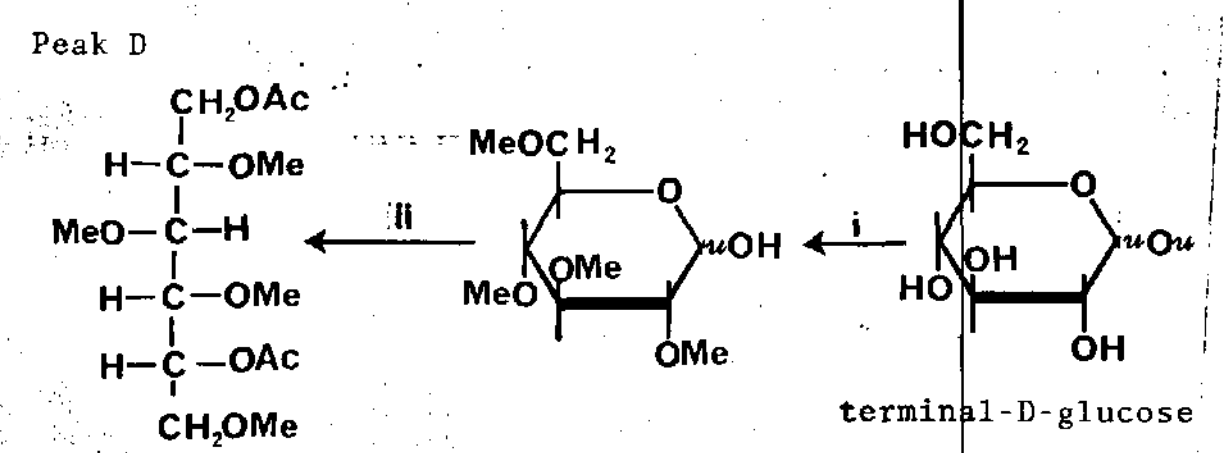
Peak B



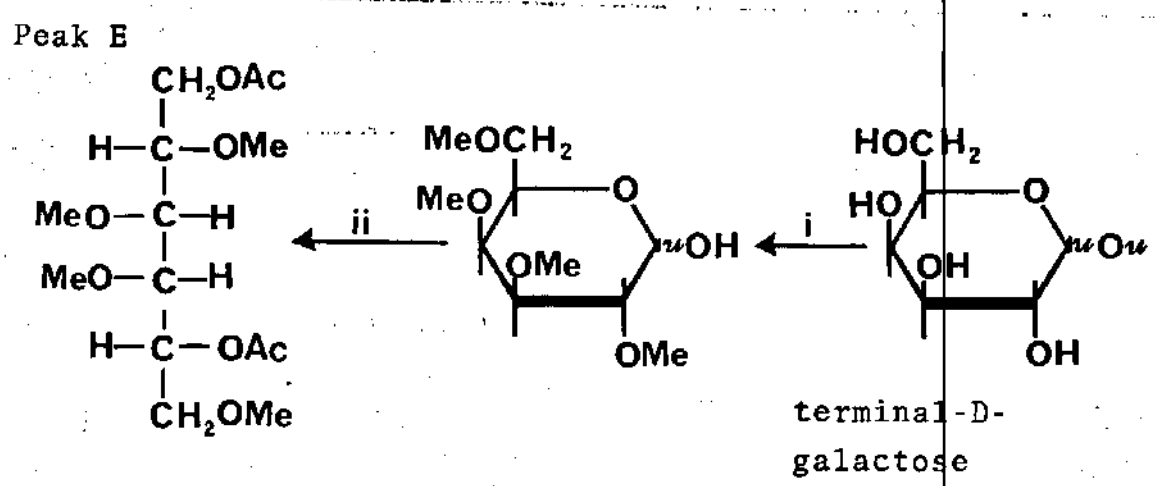
Scheme 19



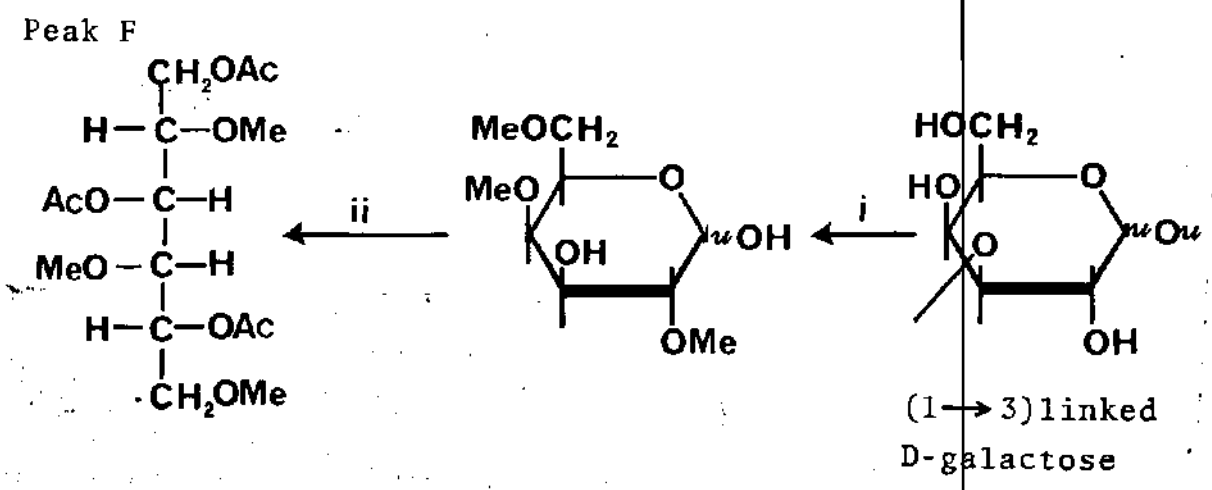
Scheme 20



Scheme 21

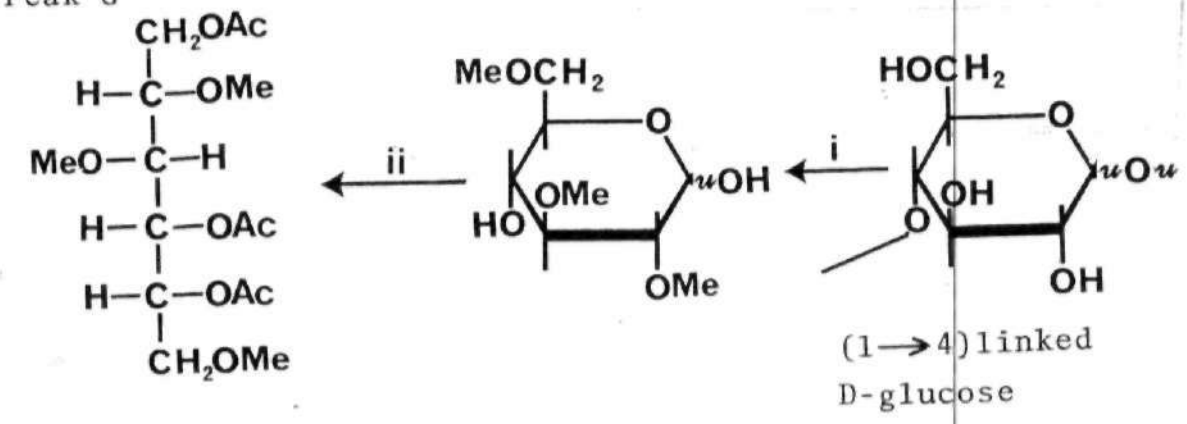


Scheme 22



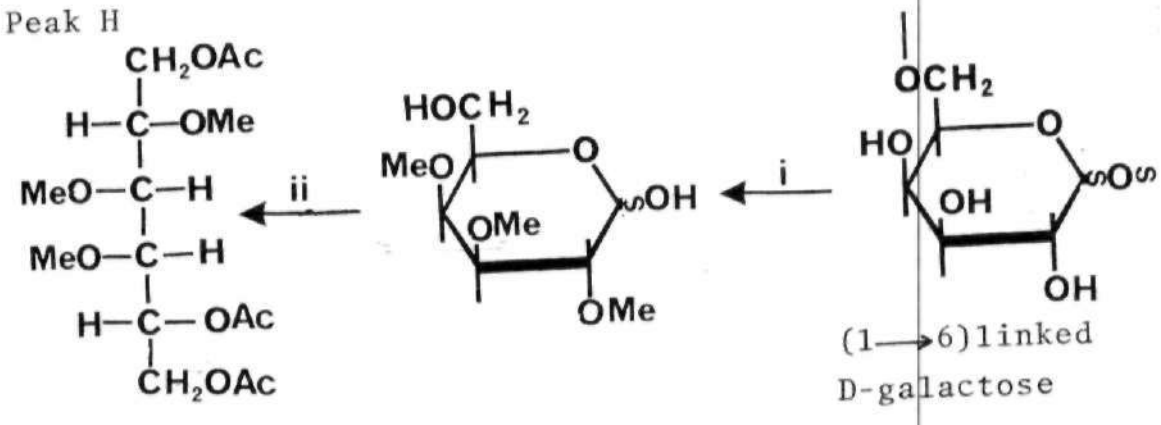
Scheme 23

Peak G

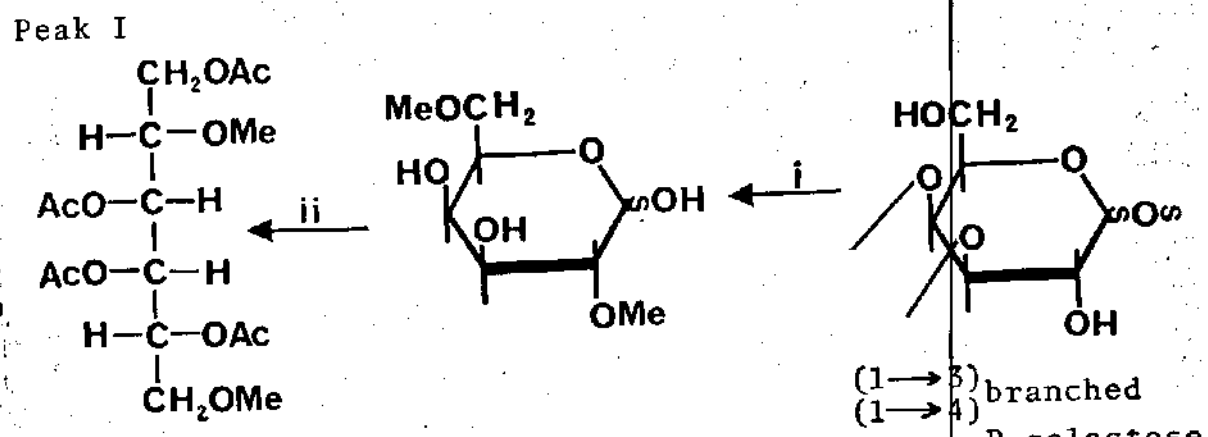


Scheme 24

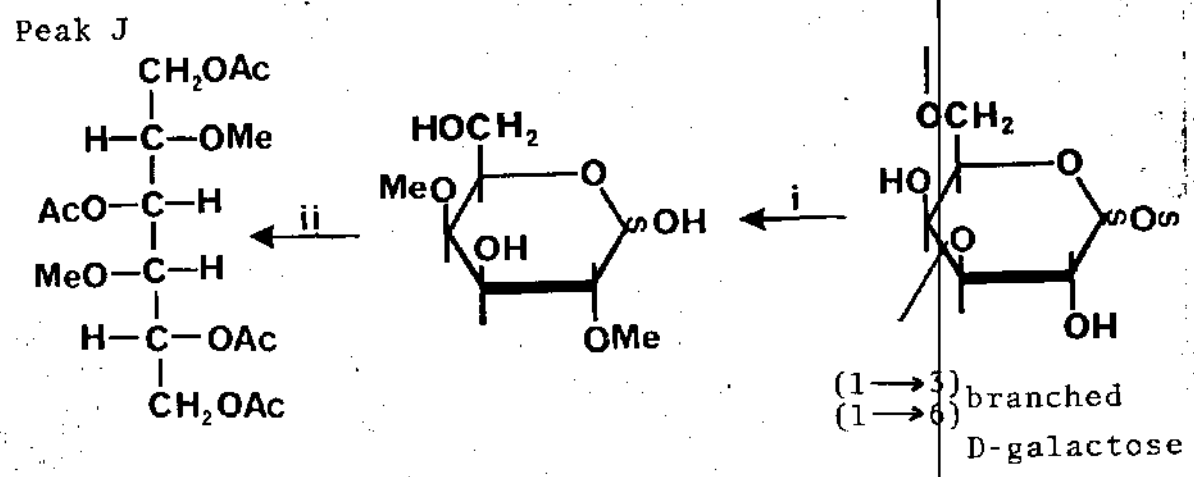
Peak H



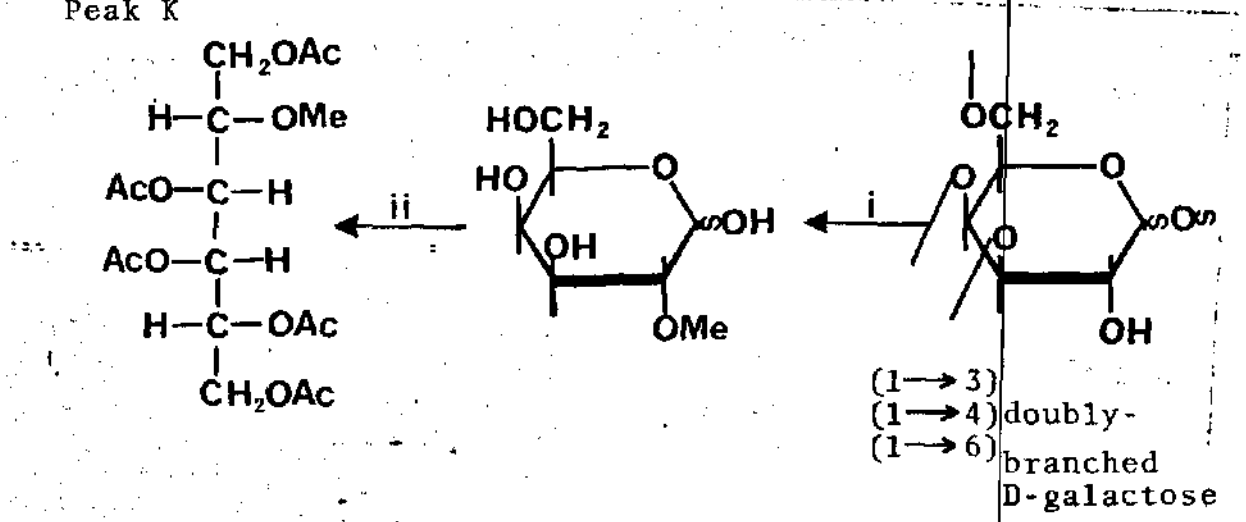
Scheme 25



Scheme 26



Scheme 27



Scheme 28

It is evident that all arabinose residues present in PP II-R are in the form of non-reducing end units and, for the most part, are in the furanose ring form (Peak A); only a few arabinose residues are in the pyranose ring form (Peak B). All rhamnose residues are mid-chain units substituted at O-3 (Peak C). Although most of the glucose (derived from D-glucuronic acid) is in the form of O-4 substituted chain units (Peak G), a fraction of the glucose appeared as non-reducing end units (Peak D), probably derived from 4-O-methylglucuronic acid. Galactose, the most abundant component, is present as end units (Peak E), mid-chain units (Peaks F and H), and branch point units (Peaks I, J, and K).

A comparison of the number of branch units with that of end units and mid-chain units indicates a highly branched structure for PP II (Table 7). As expected, there is an end

unit for every branch point. Because peak K is a double branch point, it gives rise to two end units; thus it is counted twice.

Table 7

Comparison of the number of end units with branch-point units and chain units in PMAA derivatives of hydrolyzed MPP II-R

	mole % from Table 4		
Component	branch unit	chain unit	end unit
A			31.8
B			1.7
C		10.4	
D			2.7
E			9.0
F		1.7	
G		10.2	
H		0.9	
I	1.5		
J	19.6		
K	21.1(10.54x2)		
Total	42.2	23.2	45.2

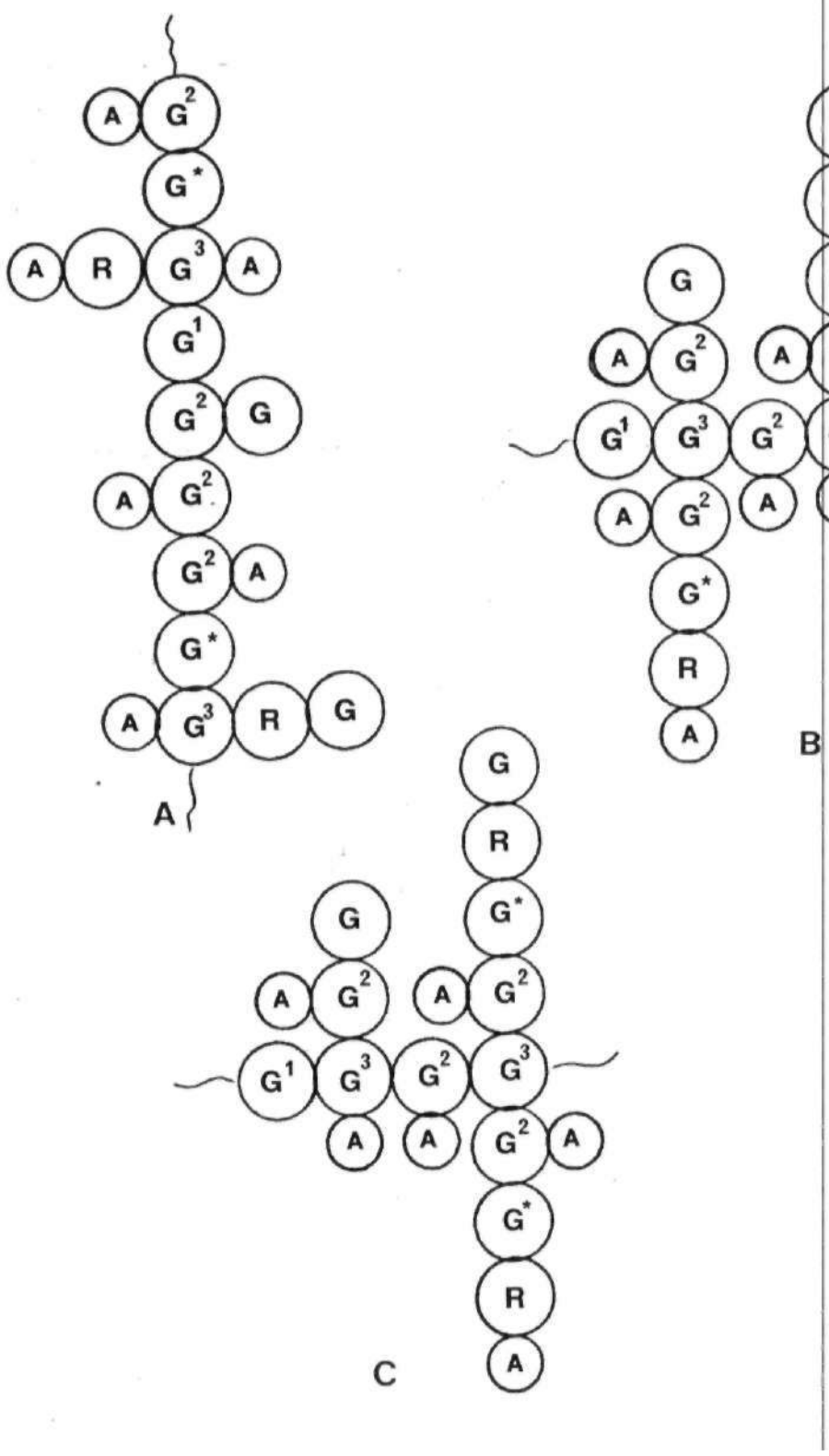
At this point, a statistical fragment which could represent an average repeating unit in PP II (if such a fragment exists) could be proposed. Based on the mole % of fragments identified by methylation analysis, the number of each unit expected in a statistical 18-residue fragment was determined (Table 8).

Table 8

Constituents of a statistical 18-residue fragment of PP II-R

Component	mole %	# in 18-unit fragment
A terminal arabinofuranose	31.8	6.3(-6)
B terminal arabinopyranose	1.7	-
C (1→3)rhamnose	10.4	2.1(-2)
D terminal glucose	2.7	-
E terminal galactose	9.0	1.8(-2)
F (1→3)galactose	1.7	-
G (1→4)glucose	10.2	2.0(=2)
H (1→6)galactose	0.9	-
I (1→3), (1→4)galactose	1.5	-
J (1→3), (1→6)galactose	19.6	3.9(-4)
K (1→3), (1→4), (1→6)galactose	10.6	2.1(-2)

Three ways of arranging the pieces to give possible structures are shown in Figure 27. Structures proposed at this point were based solely on statistical data from methylation analysis of the native polysaccharide because the sequence of the sugar units was as yet unknown.


A
B
C

terminal arabinose = (A)

terminal galactose = (G)

(1⟶ 3)rhamnose = (R)

(1⟶ 4)glucose = (G^*)

(1⟶ 3)
(1⟶ 6)galactose = (G^2)

(1⟶ 3)
(1⟶ 4)
(1⟶ 6)galactose = (G^3)

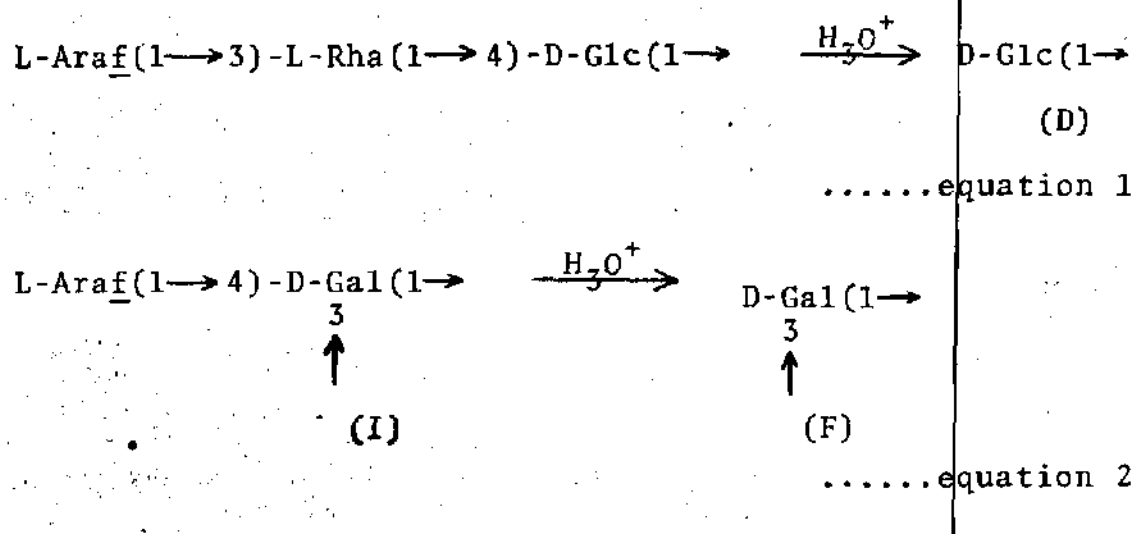
(1⟶ 3)galactose
(1⟶ 6)galactose = (G^1)

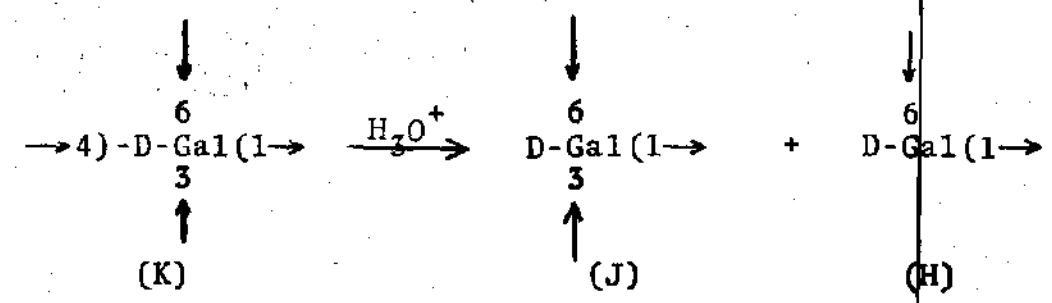
Evidence from partial hydrolysis was required to proceed with the elucidation of the structure of PP II-R. However, structure A (Fig. 27) could be ruled out. A polysaccharide with a structure of this type, i.e., a straight chain with very short branches, would give highly viscous solutions in water, even at low concentrations; but solutions of PP II-R have very low viscosities, suggesting a highly branched compact structure.

Because PP II-R had such a large percentage of branch points and terminal arabinofuranose units, it was postulated that mild hydrolysis would remove the easily hydrolyzed

furanoside end units without depolymerization, thus simplifying the structure.

Mild hydrolysis did simplyfy the structure somewhat, although not as much as had been expected. The methylated product of hydrolysis, MPP II-RA, indicated a decrease in the amount of terminal arabinose units (Table 4, peaks A and B), the disappearance of all O-3,O-4 disubstituted galactose (peak I, branch point), a decrease in the amount of O-3,O-4, O-6 trisubstituted galactose (peak K, double branch point), and a decrease in the amount of O-3 substituted rhamnose (peak C). Slight decreases were also apparent in the amounts of terminal galactose (peak E) and O-4 substituted glucose (peak G). Increases in the amount of terminal glucose (peak D), O-6 substituted galactose (peak H), and O-3,O-6 disubstituted galactose (peak J, branch point) were observed. Based on these changes in the relative amounts of the components in MPP II-RA, a number of postulates were made (Scheme 29).





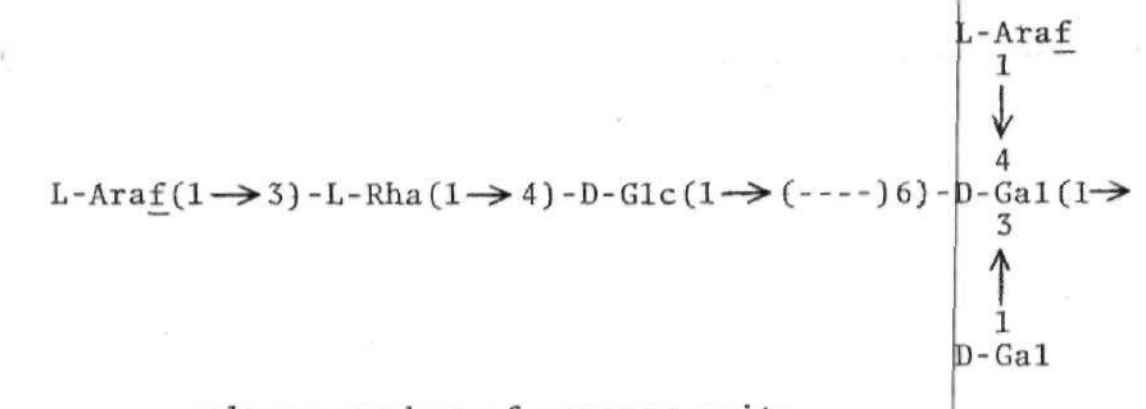
.....equation 3

Scheme 29

Because terminal glucose increased by about the same amount that O-3 substituted rhamnose decreased, it was hypothesized that these two sugar residues were directly linked; and because rhamnose residues were not end units, it was hypothesized that they were linked to terminal arabinose or galactose units, these being the only end units lost during mild hydrolysis.

The increase in terminal glucose units along with the decrease in O-3 substituted rhamnose units and some terminal arabinose or terminal galactose units may be accounted for by equation 1. Equation 2 may account for the small increase in O-3 substituted galactose residues. Equation 3 may account for the decrease in doubly branched units (peak K) and the increases in both singly branched units (peak J) and the O-6 substituted galactose residues (peak H). An increase in O-6 substituted galactose residues could also have resulted from hydrolysis of the O-3 bond of some O-3,O-4 disubstituted branch point units (peak J).

Because the only components identified from the mild hydrolyzate were arabinose, rhamnose, a small amount of galactose, and a disaccharide (D1, galactose:arabinose 1:1), a fragment may be proposed which could account for most of these observations and rationalizations (Fig. 28).



----- = unknown number of monomer units

Fig. 28

Fragments from more drastic partial hydrolysis provided the information required to deduce the sequence of sugars in PP II-R. Methylation analysis of the fragment F3 indicated that it was a disaccharide consisting of a terminal glucose residue (peak D) linked to O-6 of galactose (peak H), thus confirming that the glucuronic acid residues in PP II are linked to O-6 of galactosyl residues (Fig. 29).

Methylation analysis of the isolated aldobiouronic acids confirmed that they were indeed glucuronic acid(1→6) galactose, for only two components were observed after reduction and methylation analysis, viz., terminal glucose

and O-6 substituted galactitol.

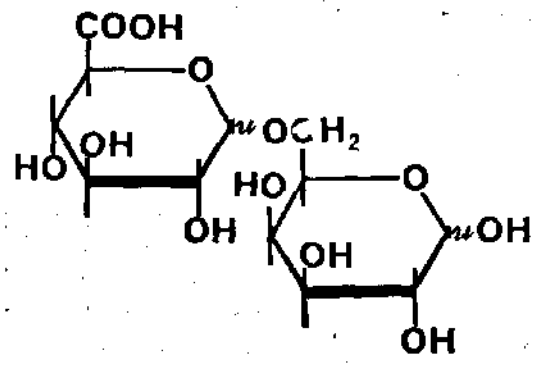


Fig. 29

Initially, it was puzzling that two different fragments (UA1 and UA2) identical by methylation analysis could be isolated. However, at the initial stages of this investigation a small amount of an unidentified component (2.8 mole %), was evident in the glc of PAAN derivatives of neutral hydrolyzates of PP II-R (Fig. 7), which was absent from the neutral hydrolyzates of PP II (Fig. 6). Obviously this must be a uronic acid component, which is not converted to glucose when reduced. Because 4-O-methylglucuronic acid is quite common in polysaccharides containing glucuronic acid, we assume that UA2 is 4-O-methylglucuronic acid(1→6)galactose (Fig. 30). Thus UA2 would give upon reduction and methylation the same product as UA1 (Scheme 30), but would give different products upon reduction and hydrolysis, accounting for the minor peak in Figure 7. Methylation analysis of PP II-R

indicated the presence of 2.7 mole % terminal glucose (peak D) probably derived from 4-O-methylglucuronic acid residues which occur at non-reducing termini in the native polysaccharide.

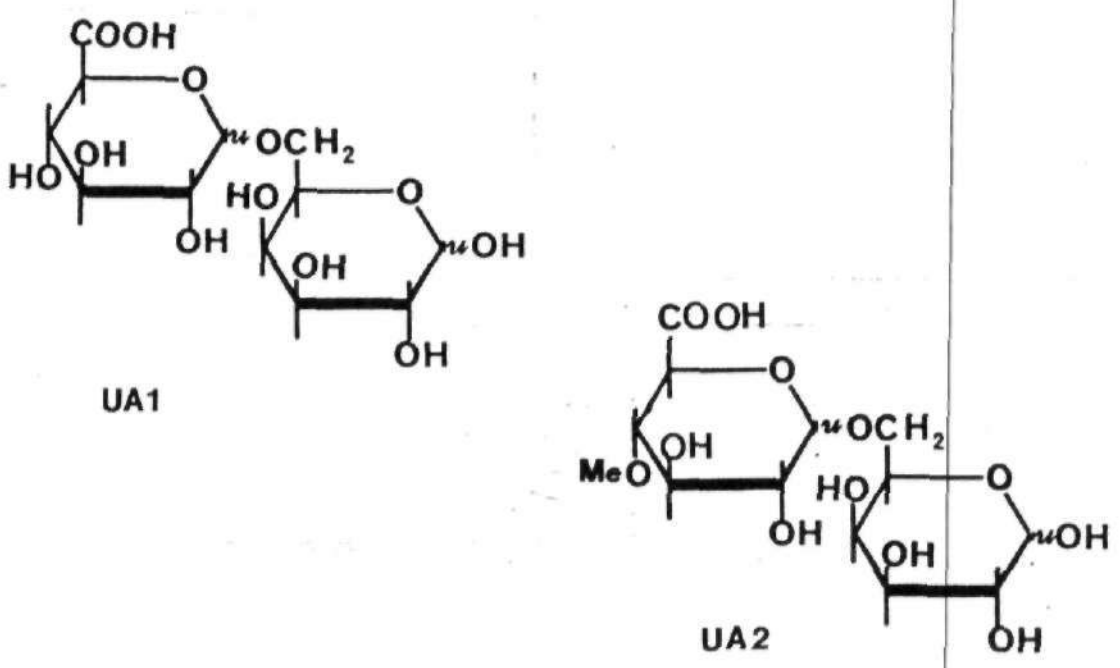
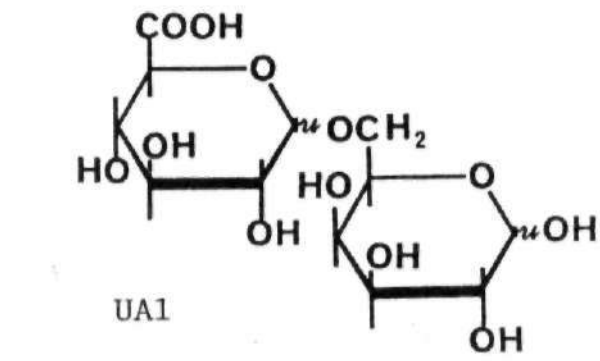


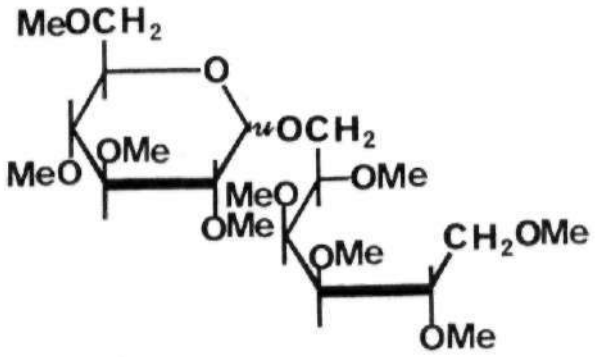
Fig. 30

The fragmentation pattern of terminal glucose has already been discussed (Scheme 11). The methylated galactitol unit had a shorter retention time than tetra-O-methylglucose (OV225), which is consistent with the observation that the more methyl groups on the PMAA derivatives, the more volatile they are. Mass spectral behavior of methyl ethers of alditols has been reviewed by Lonngren and Svensson (25); as is the case with alditol acetates, molecular ions of PMAA derivatives

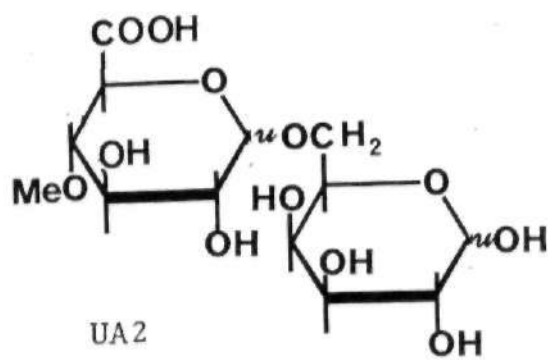


UA1

1. reduction
2. methylation



1. reduction
2. methylation



UA2

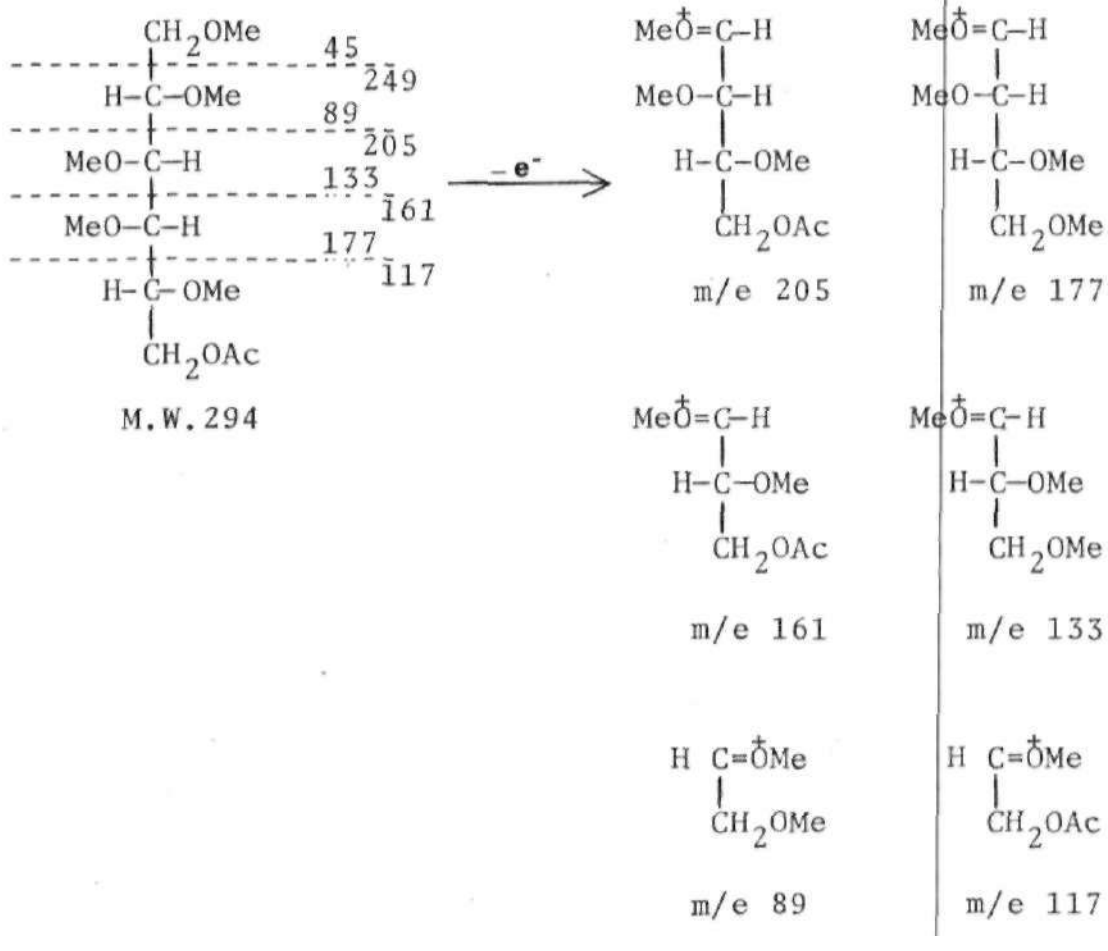
Scheme 30

are not observed and stereoisomers give identical spectra.

Because evidence that the glucuronic acid residue was (1⟶6)linked to a galactose residue was already available, the fragmentation pattern of the galactitol unit was expected to be consistent with that of 6-O-acetyl-1,2,3,4,5-penta-O-methylgalactitol. It was (Scheme 31).

6-O-Acetyl-1,2,3,4,5-penta-O-methylgalactitol

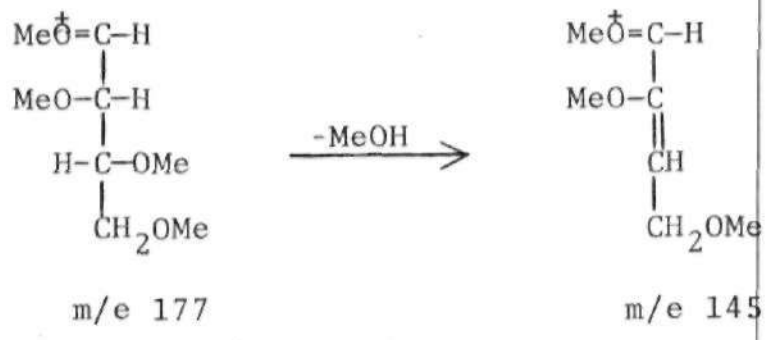
Primary fragmentation



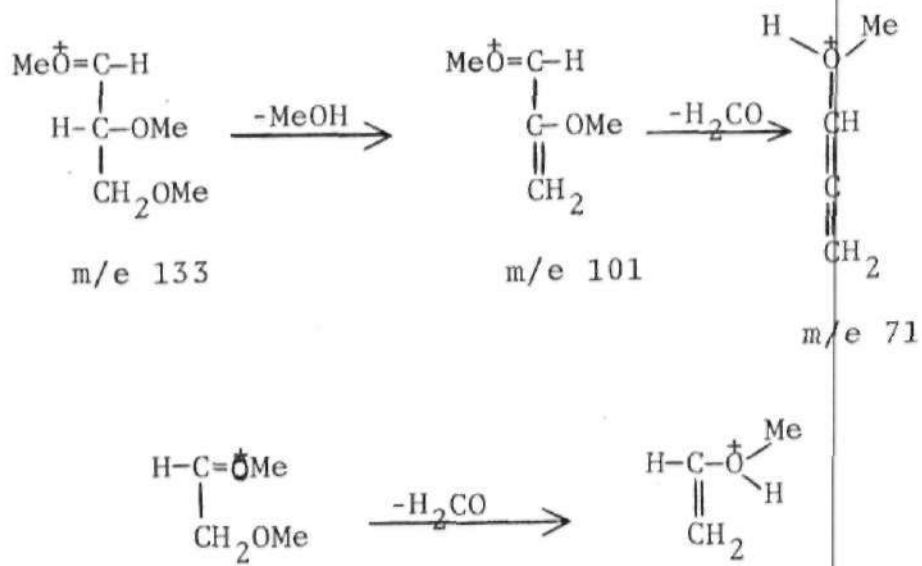
Secondary fragmentation

m/e 205 $\xrightarrow{-MeOH}$ m/e 173

m/e 161 $\xrightarrow{-HOAc}$ m/e 101 $\xrightarrow{-H_2CO}$ m/e 71



m/e 177 m/e 145



m/e 133 m/e 101 m/e 71

m/e 89 m/e 59

Scheme 31

The other fragment, P1, isolated from partial hydrolysis of PP II-R consisted of only glucose and galactose. Paper chromatography indicated that P1 might be a mixture of two oligosaccharides of about the size. Methylation analysis of P1 indicated the presence of five components: terminal glucose (peak D), terminal galactose (peak E), O-3 substituted galactose (peak F), O-6 substituted galactose (peak H), and O-3,O-6 disubstituted galactose (peak J), in an approximate ratio of 2:1:1:4:1 respectively. An equimolar mixture of the following two oligosaccharides is consistent with this data.

D-Gal(1→6)-D-Gal(1→6)-D-Gal(1→3)-D-Gal (a)

D-Glc(1→6)-D-Gal(1→6)-D-Gal
3
↑ (b)
1
D-Glc(1→6)-D-Gal

It would appear that the backbone of the PP II structure is made up of (1→6)- and (1→3)-linked galactose residues.

After having collected this much information about PP II, a structure could be proposed that is consistent with the data (Fig. 31), recognizing ofcourse that PP II is probably both polymolecular and polydisperse as are all

known polysaccharides of this type.

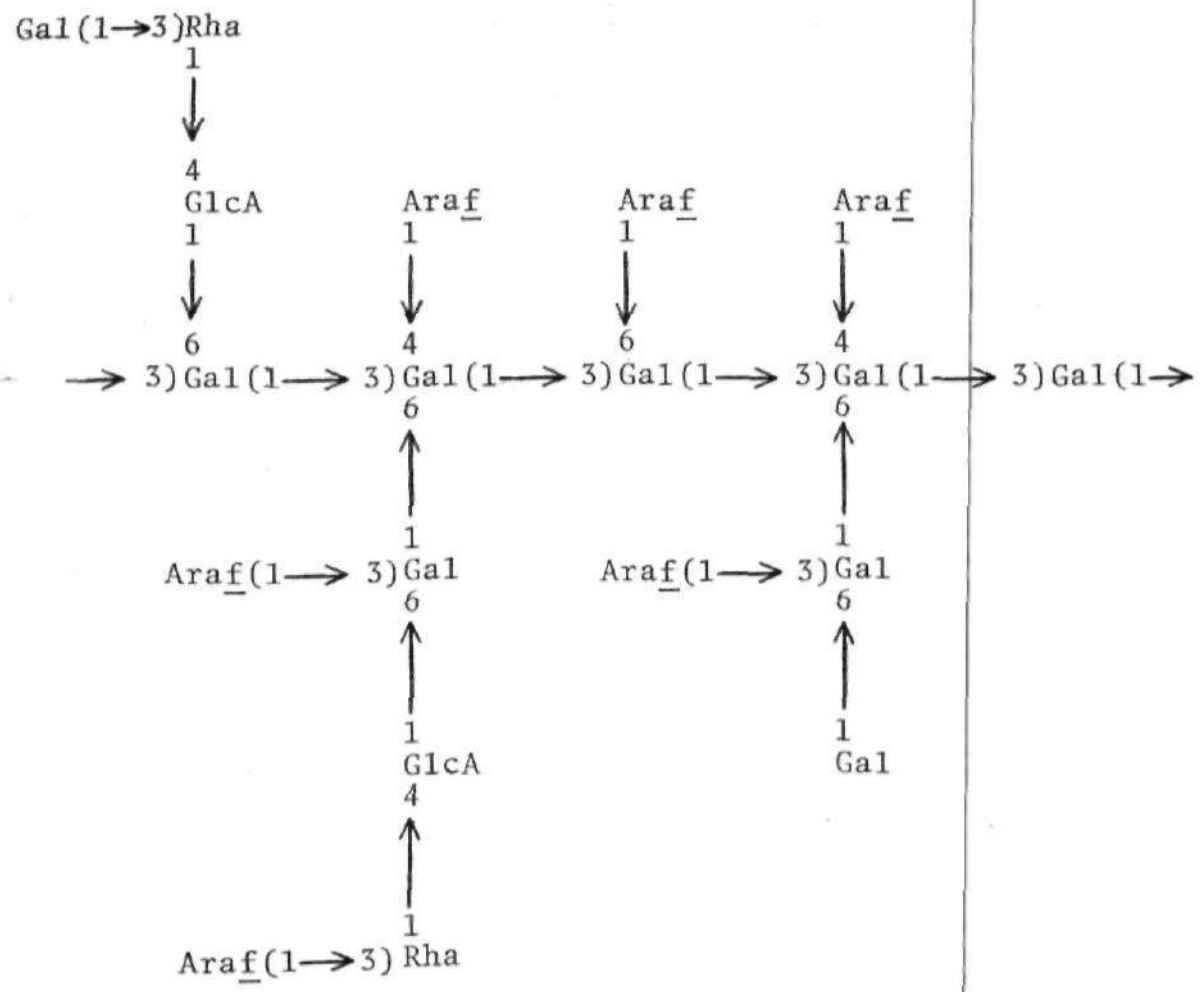


Fig. 31 Proposed structure for a fragment of PP II

At this point, the striking resemblance of the proposed structure of PP II to that of the well known polysaccharide from Acacia sp., gum arabic, became evident (Fig. 32) and the question became "did the crude papain from Sigma Chemical Company contain added gum arabic?".

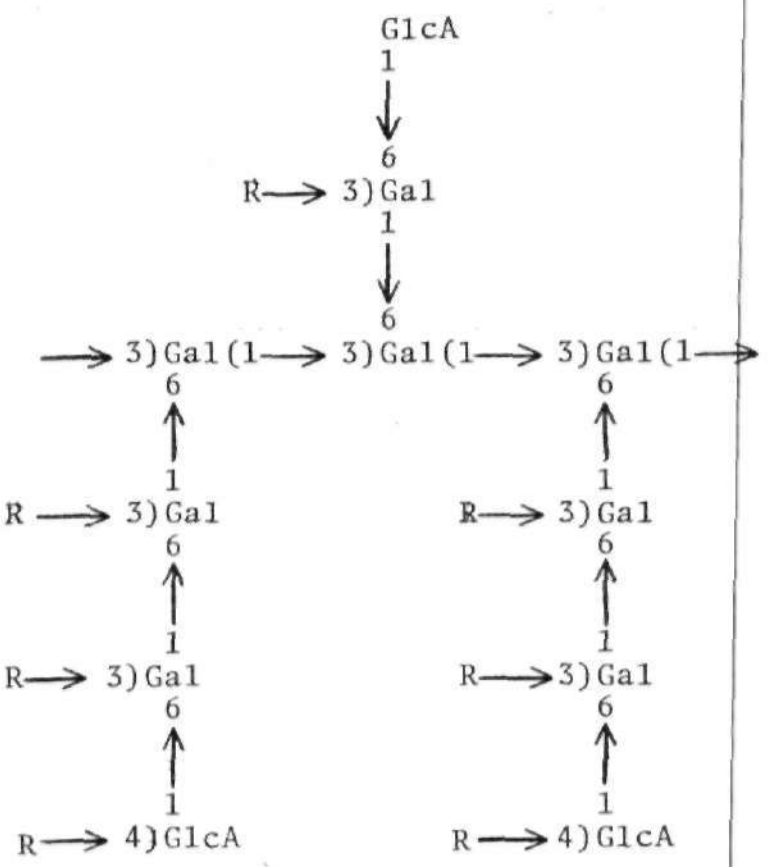


Fig. 32 Possible core structure of gum arabic (23) where R=L-Rha(1⟶ , L-Araf(1⟶ , D-Gal(1 ⟶3)-L-Ara(1⟶ , or L-Arap(1⟶ 3)-L-Araf(1⟶ .

Papaya latex is unstable because of bacterial contamination and the presence of enzyme activities. Because crude papain has several commercial applications, the most important being the chill proofing of beer and tenderization of meat, means have been sought to stabilize papaya latex for shipment. Removal of water from the latex decreases its papain activity. A significant improvement in the processing

of papaya latex which gives a purer, more stable, more soluble "papain was described by R.L. Boudart of the Republic of Zaire in a patent application of 8 November 1968 (2). Spray drying of the papaya latex is the central focus of the Boudart process.

Although no details of the Boudart process are available, none of the available references (2) mention the addition of any gum arabic before spray drying, and the technical service people at Sigma Chemical Co. are unaware of any addition of gum arabic, it is probable that gum arabic was added to the papaya latex before spray drying.

For sevaral reasons, gum arabic is the most important and widely used encapsulating agent for spray drying. The usual process of spray drying involves first the preparation of an emulsion of the water-insoluble material in a gum arabic solution. When the water is removed, the gum arabic surrounds the droplet of hydrophobic material, preventing evaporation and oxidation. Yet gum arabic is quite soluble, so the encapsulated material is readily released when the spray-dried powder is put into water. Gum arabic permits spray-drying at higher temperatures than is possible when some substitutes are used; this allows the more rapid removal of water and exposure of the material (papain) to heat for a minimum period of time (26).

This dissertation describes the structural analysis

of the most highly purified gum arabic, probably a single molecular species, reported to date. Although the species from which this preparation was obtained is unknown, the the structure of gum arabic, which has been described as "extensively examined" but "still not entirely clear" (27), is now much clearer.

APPENDIX

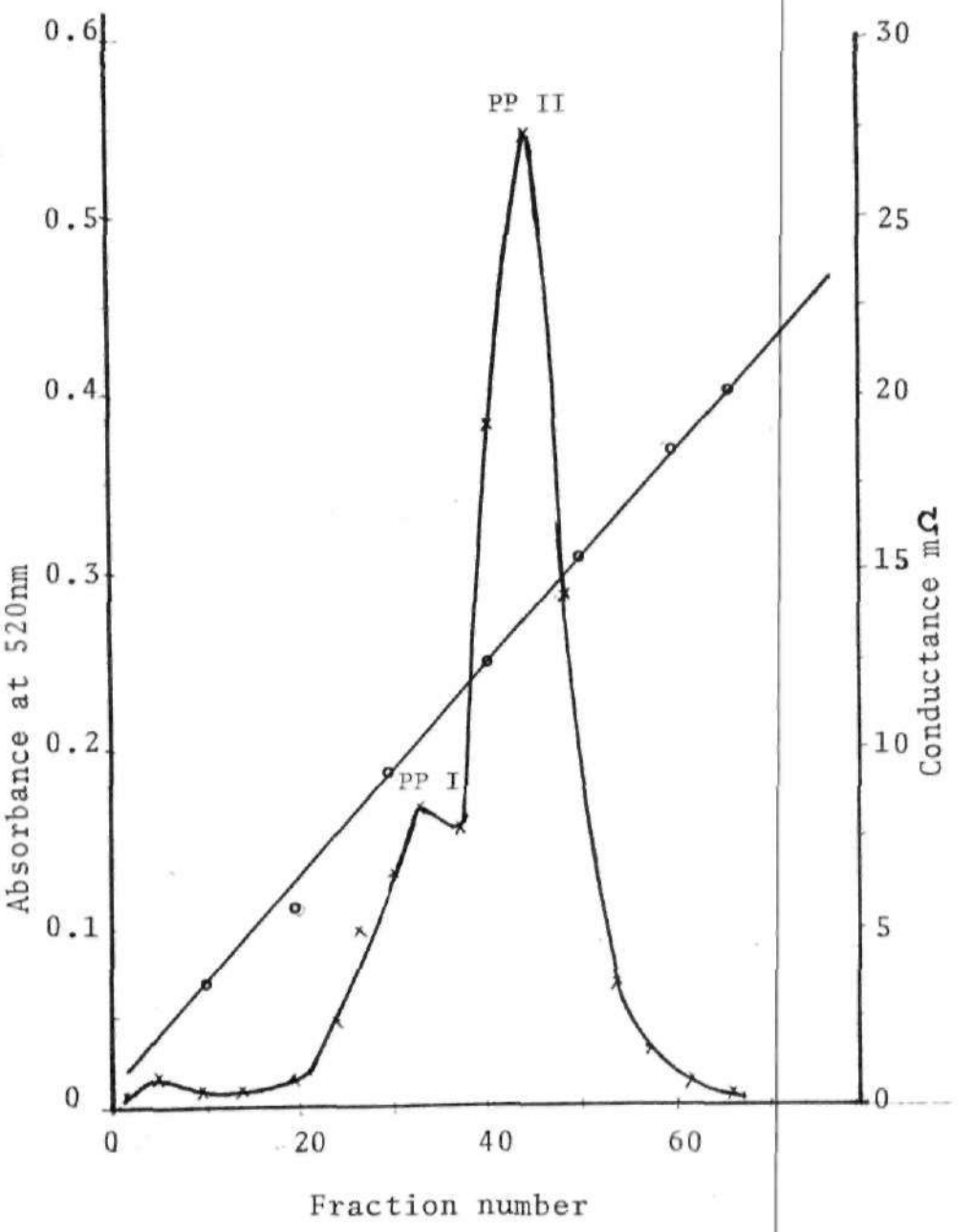


Fig. 1 Fractionation of papain polysaccharides on DEAE-cellulose

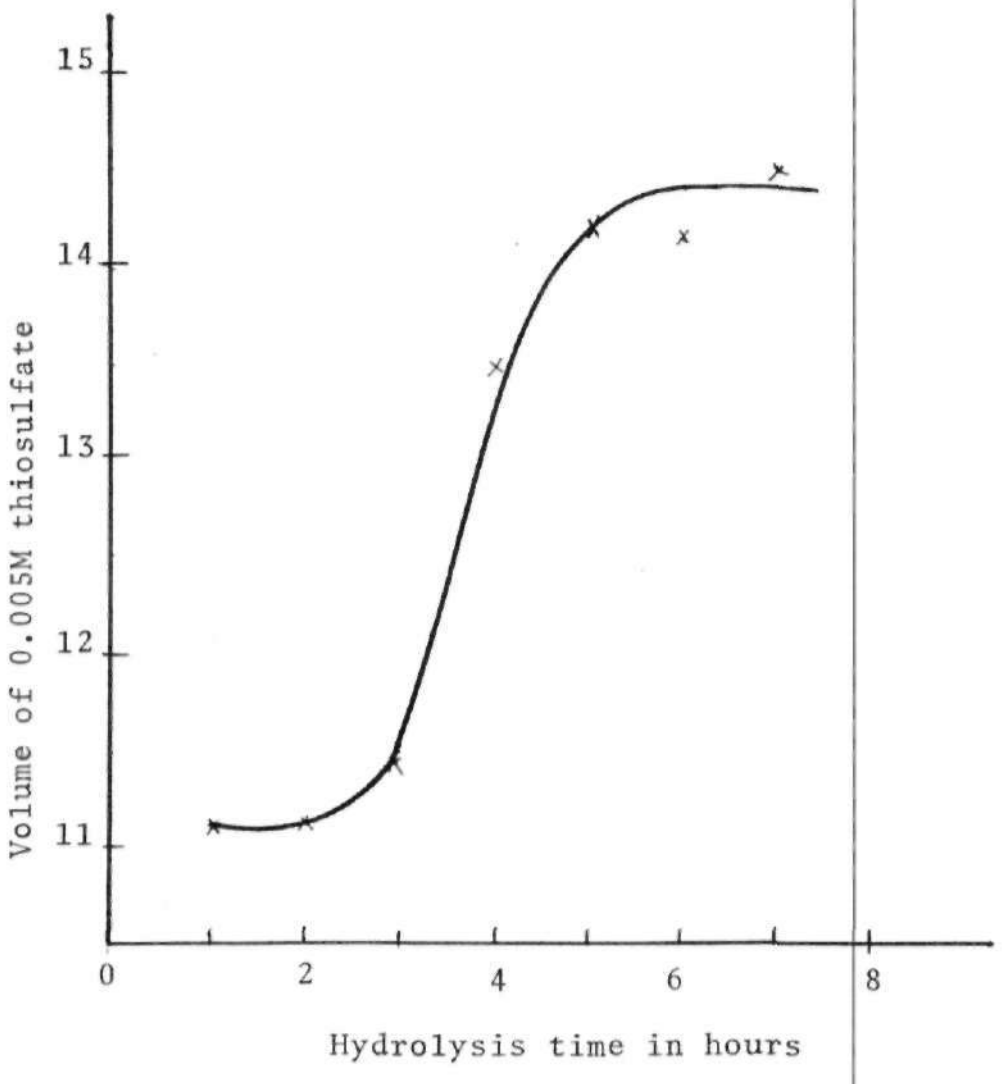


Fig. 2 Optimum hydrolysis time for PP II

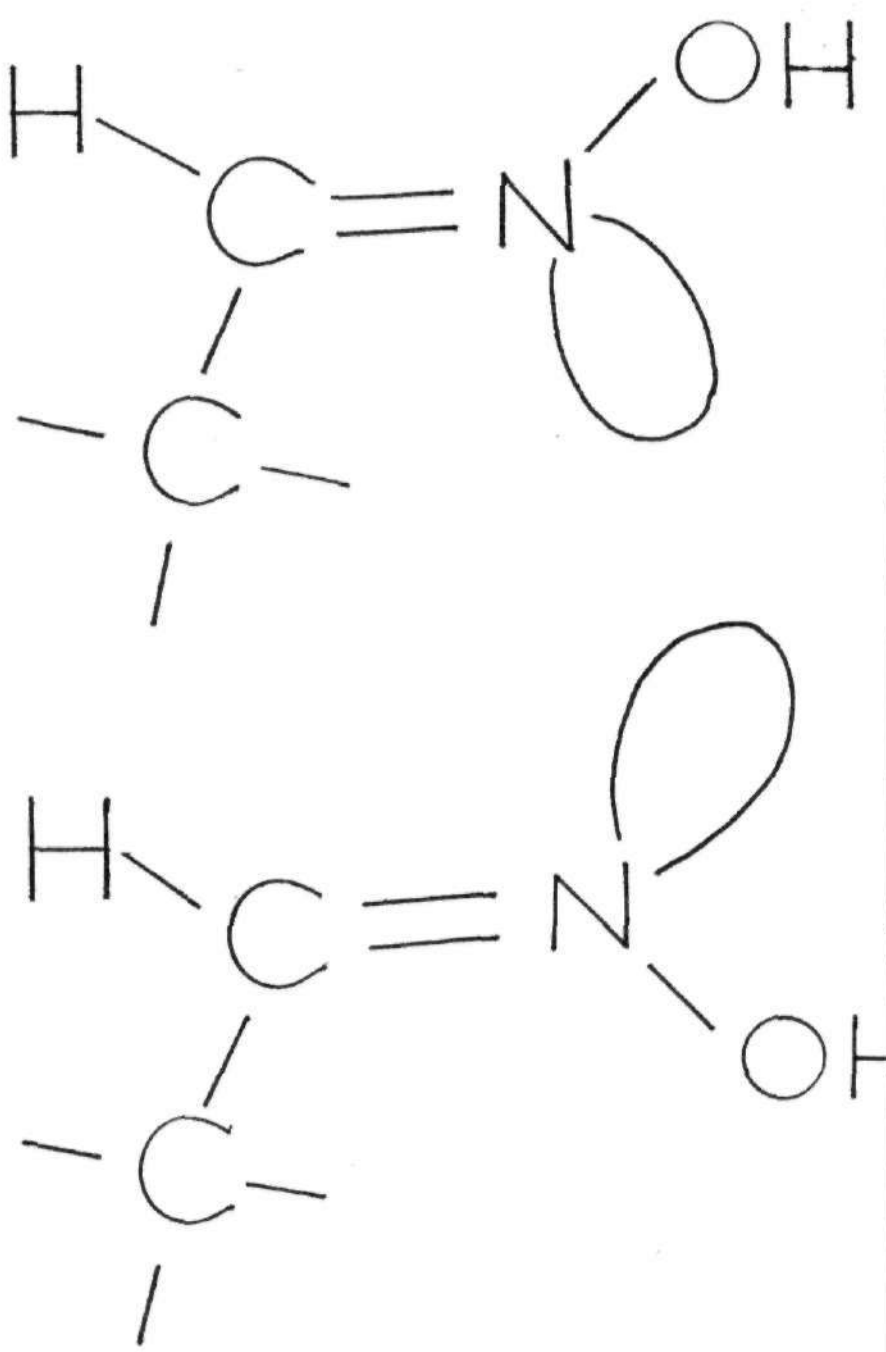


Fig. 3 Two oxime derivatives of one sugar

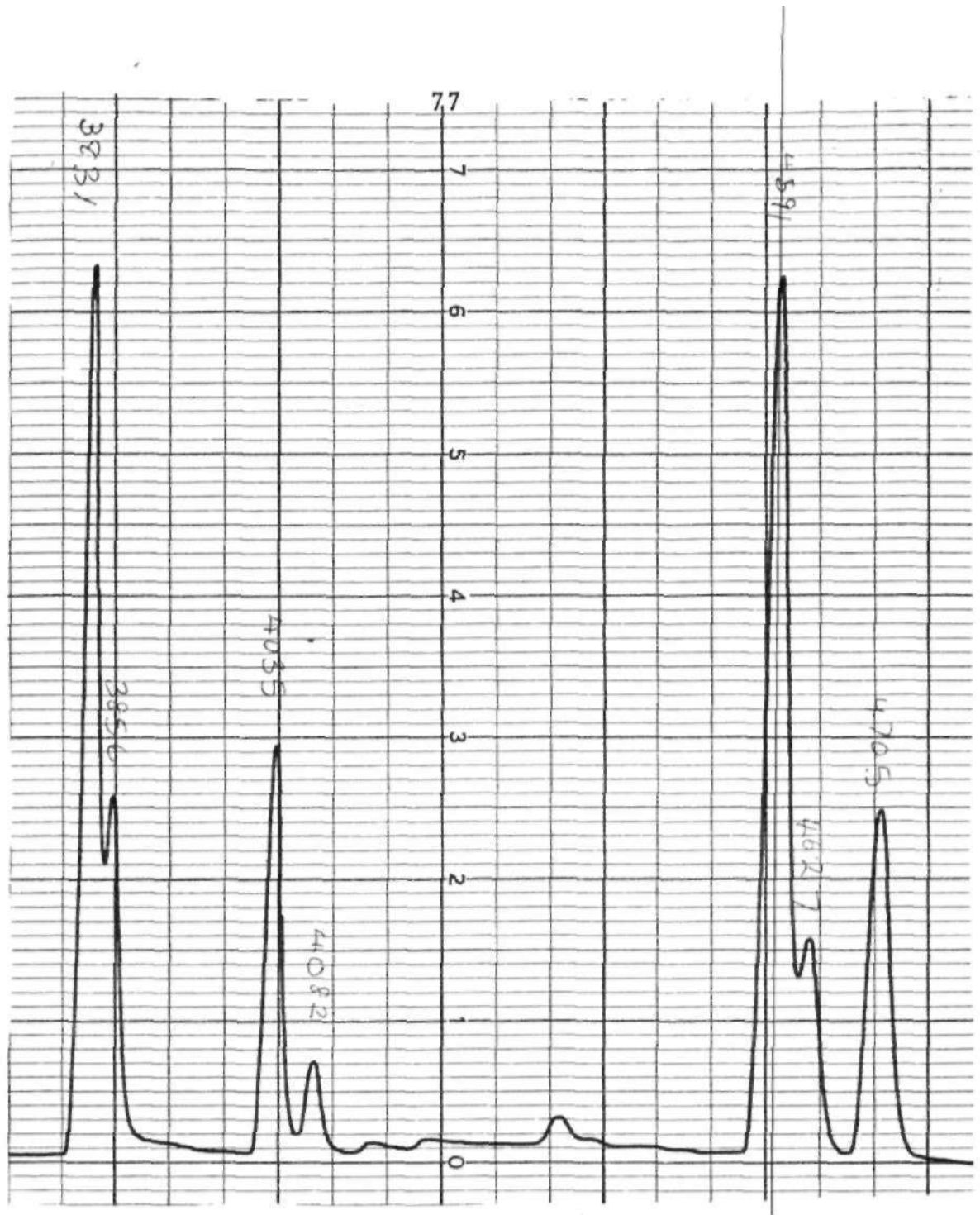


Fig. 4 Glc of TMS-oxime derivatives of hydrolyzed PP II-R on a 3% SE 52 column at 100° -250° (linear temperature program 2°/min)

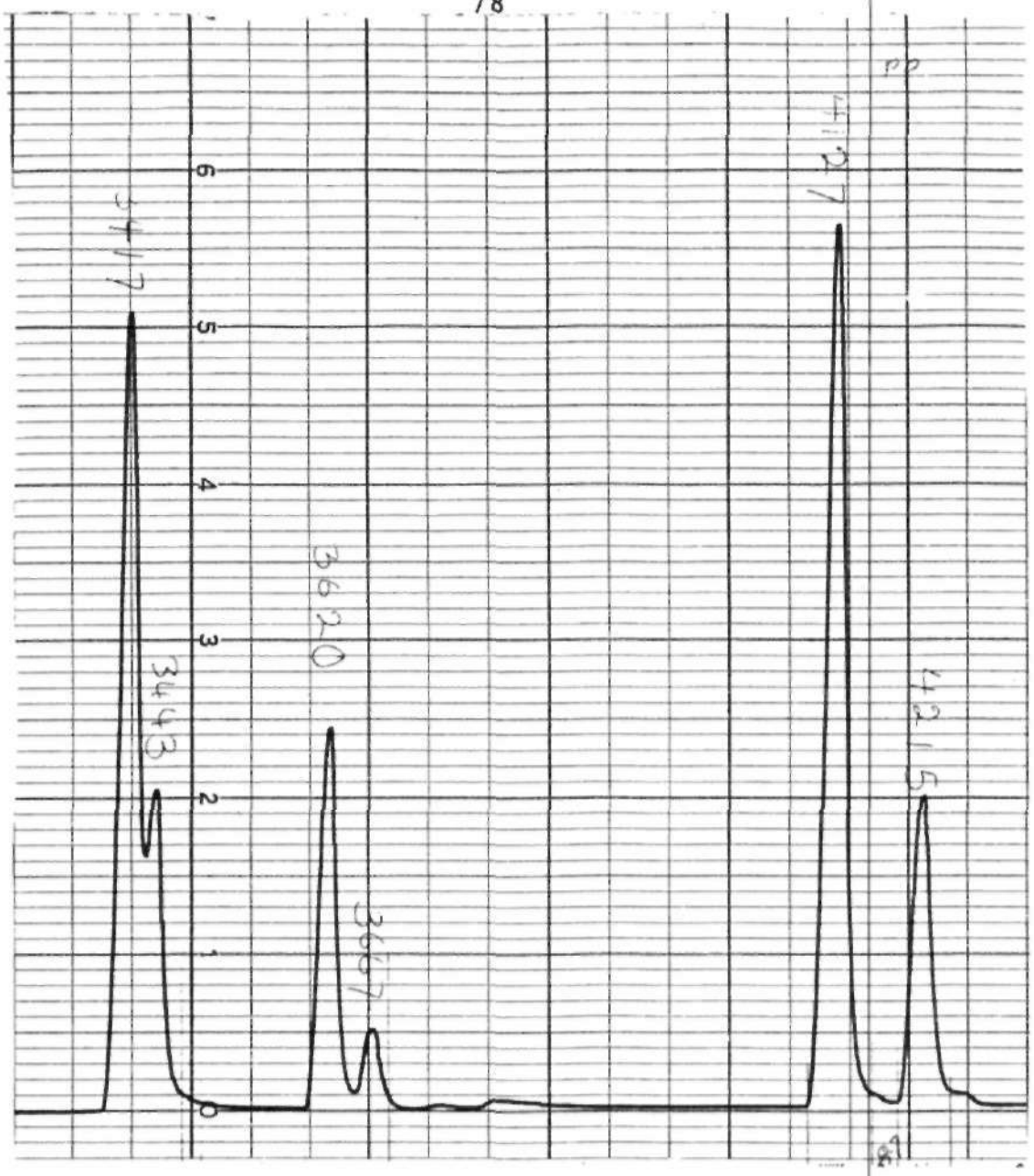


Fig. 5 Glc of TMS-oxime derivatives of hydrolyzed PP II on a 3% SE 52 column at 100°-250° (linear temperature program 2°/min)

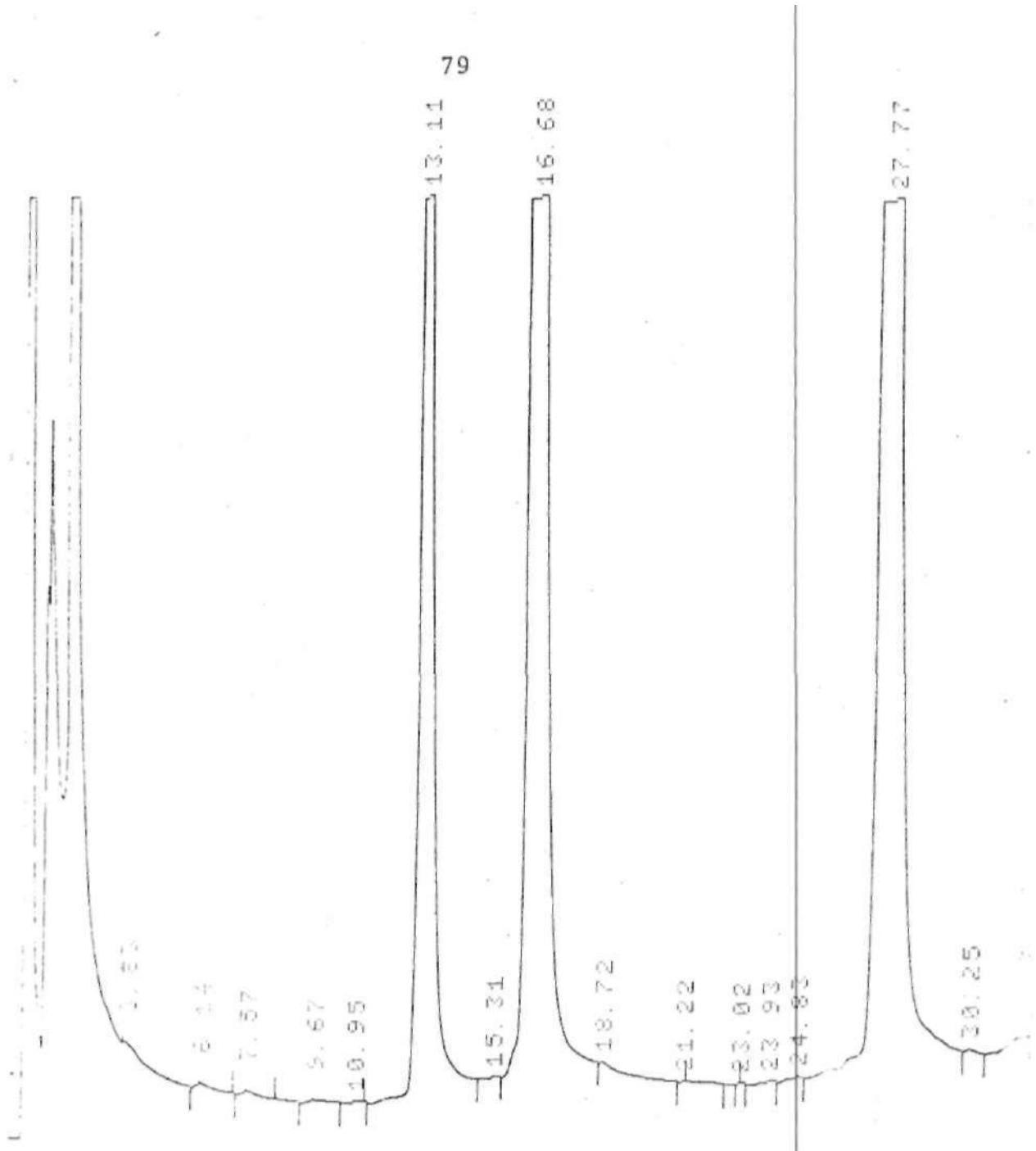


Fig. 6 Glc of PAAN derivatives of hydrolyzed PP II on a 3% OV225 column at 190°-215° (linear temperature program 2°/min)

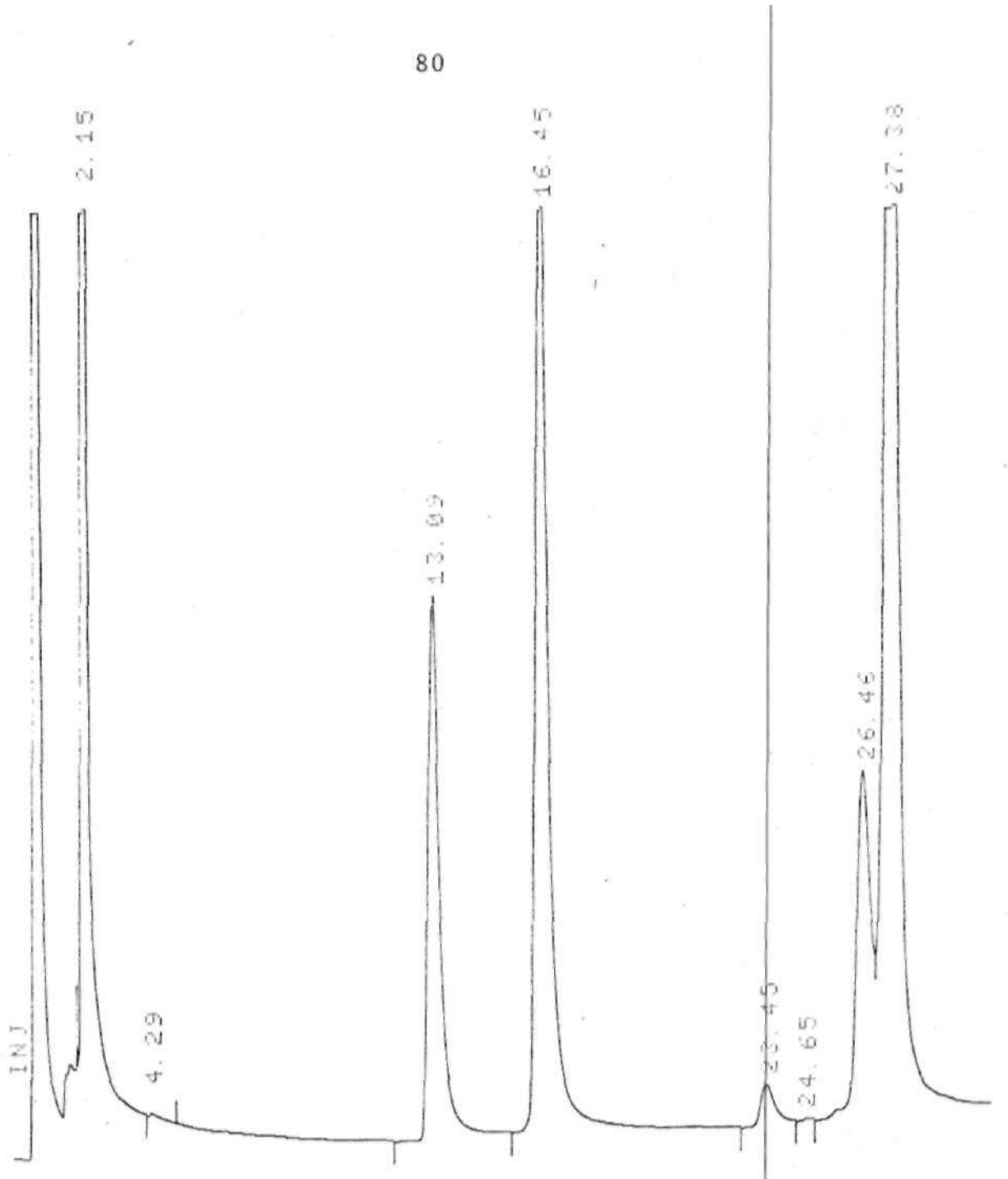
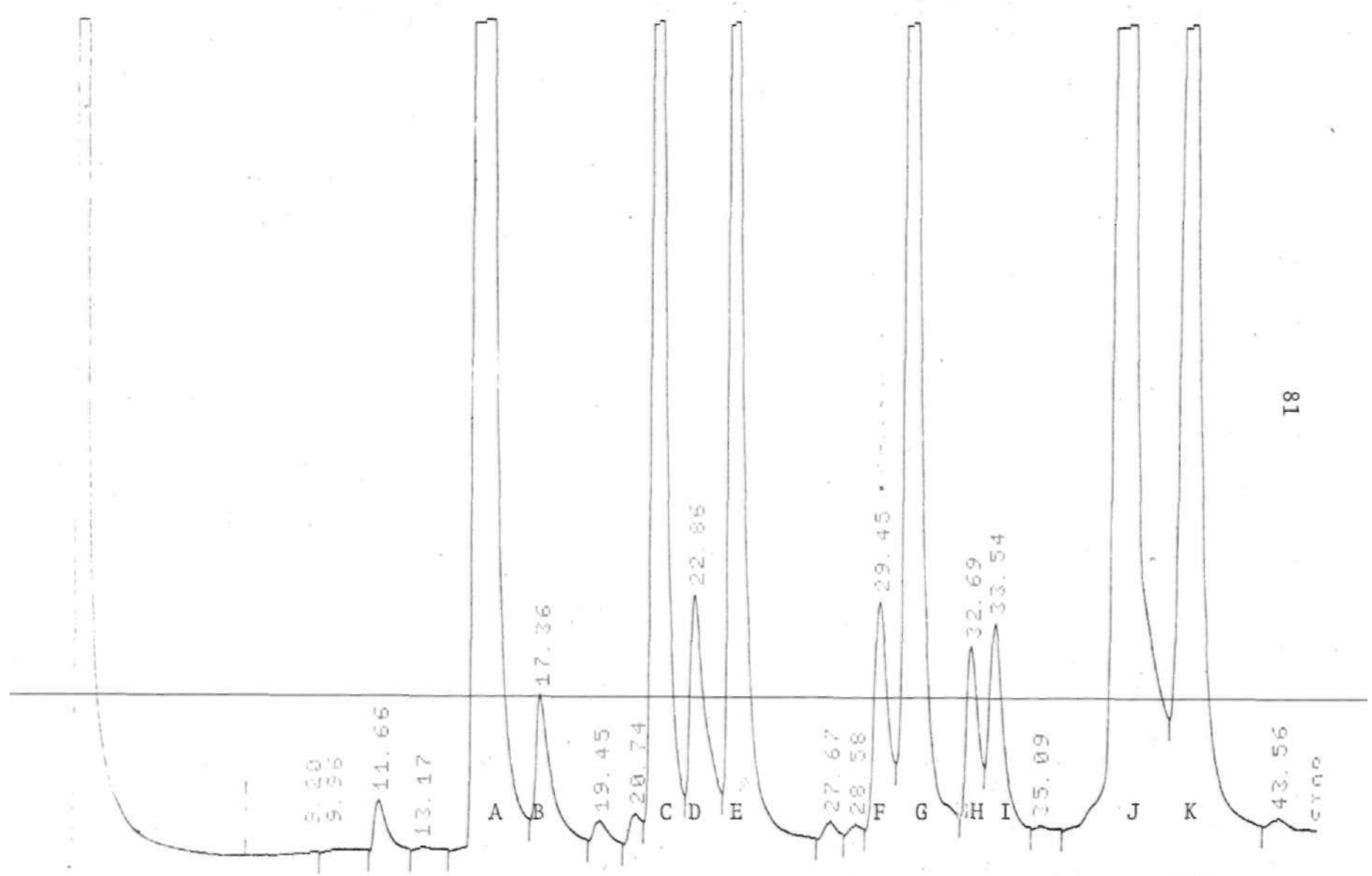


Fig. 7 Glc of PAAN derivatives of hydrolyzed PP II-R on a 3% OV225 column at 190°-215° (linear temperature program 2°/min)


9.40
9.96
11.66
13.17
A
17.36
B
19.45
20.74
C
22.86
D
E
27.67
28.58
F
29.45
G
32.69
H
33.54
I
35.09
J
K
43.56

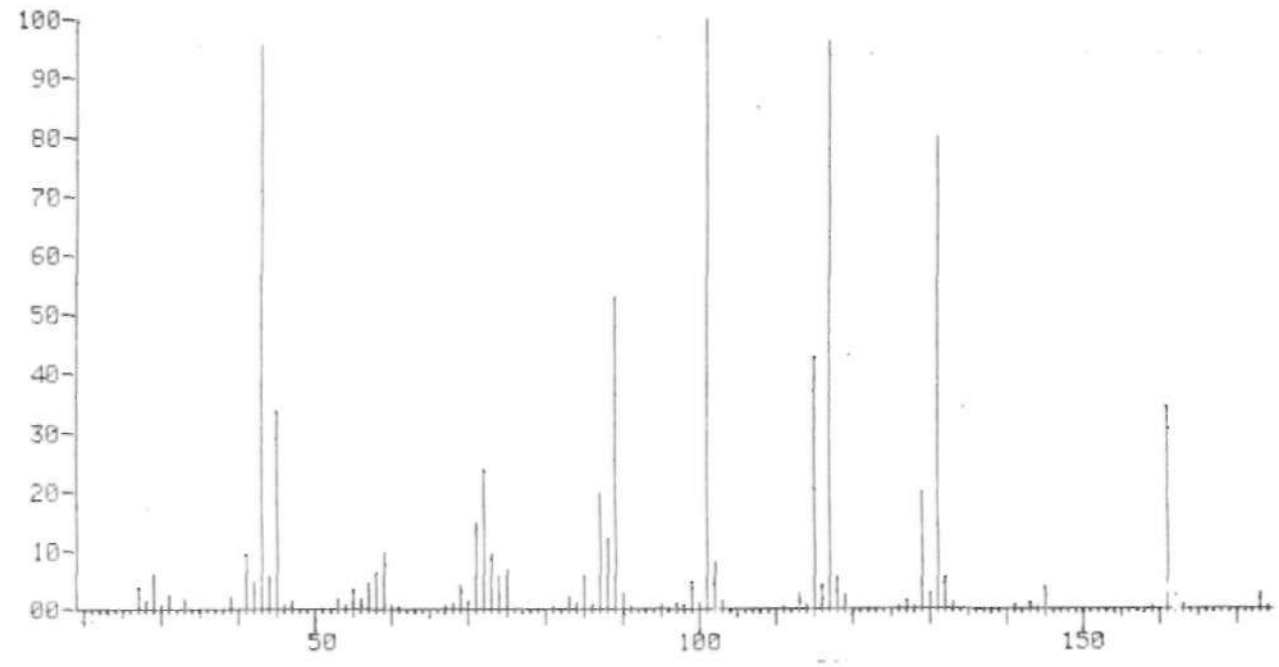


Fig. 9 1,4-Di-O-acetyl-2,3,5-tri-O-methylarabinitol (Peak A)

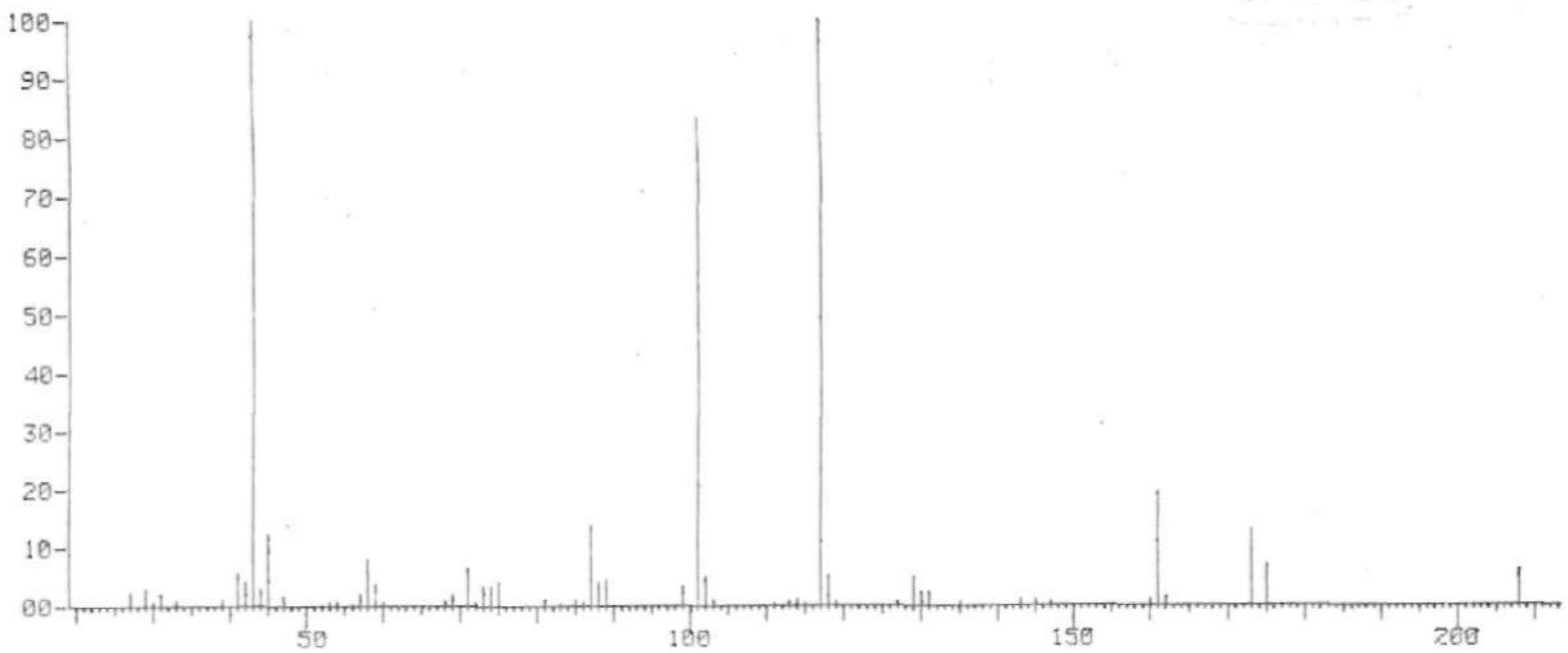


Fig. 10 1,5-Di-O-acetyl-2,3,4-tri-O-methylarabinitol (Peak B)

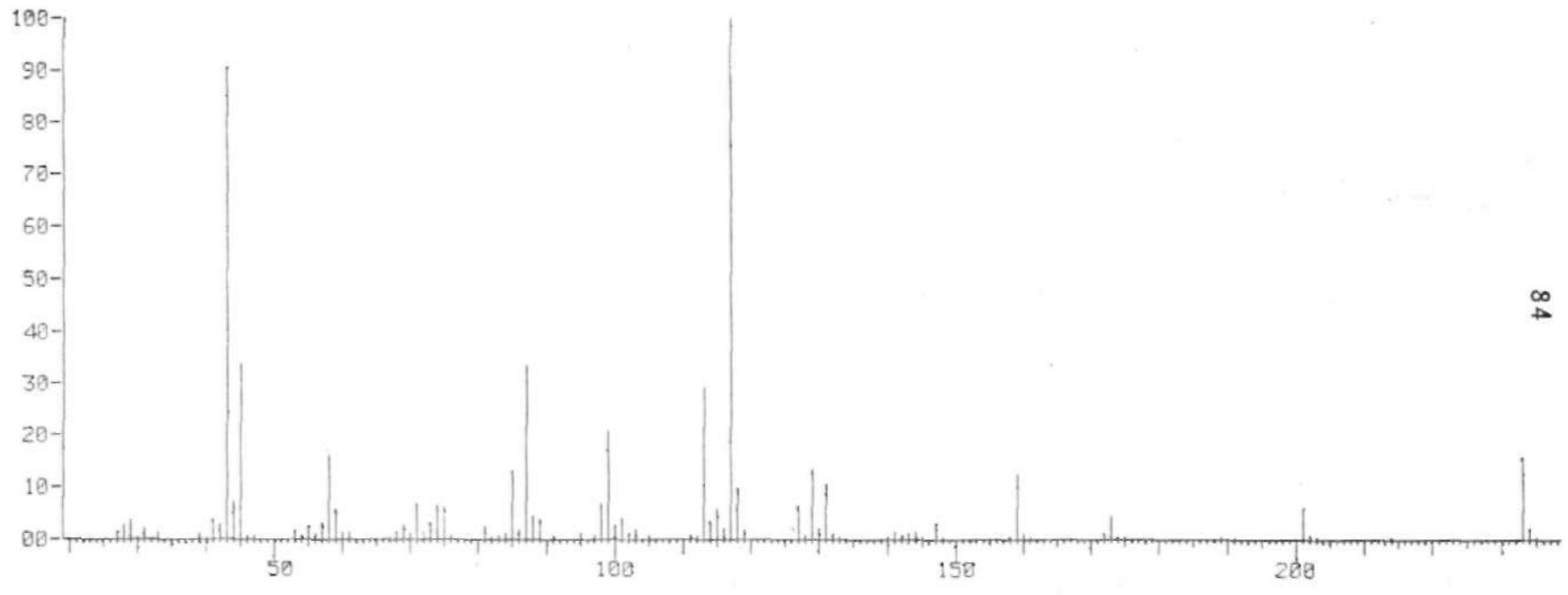


Fig. 11 1,3,5-Tri-O-acetyl-2,4-di-O-methylrhamnitol (Peak C)

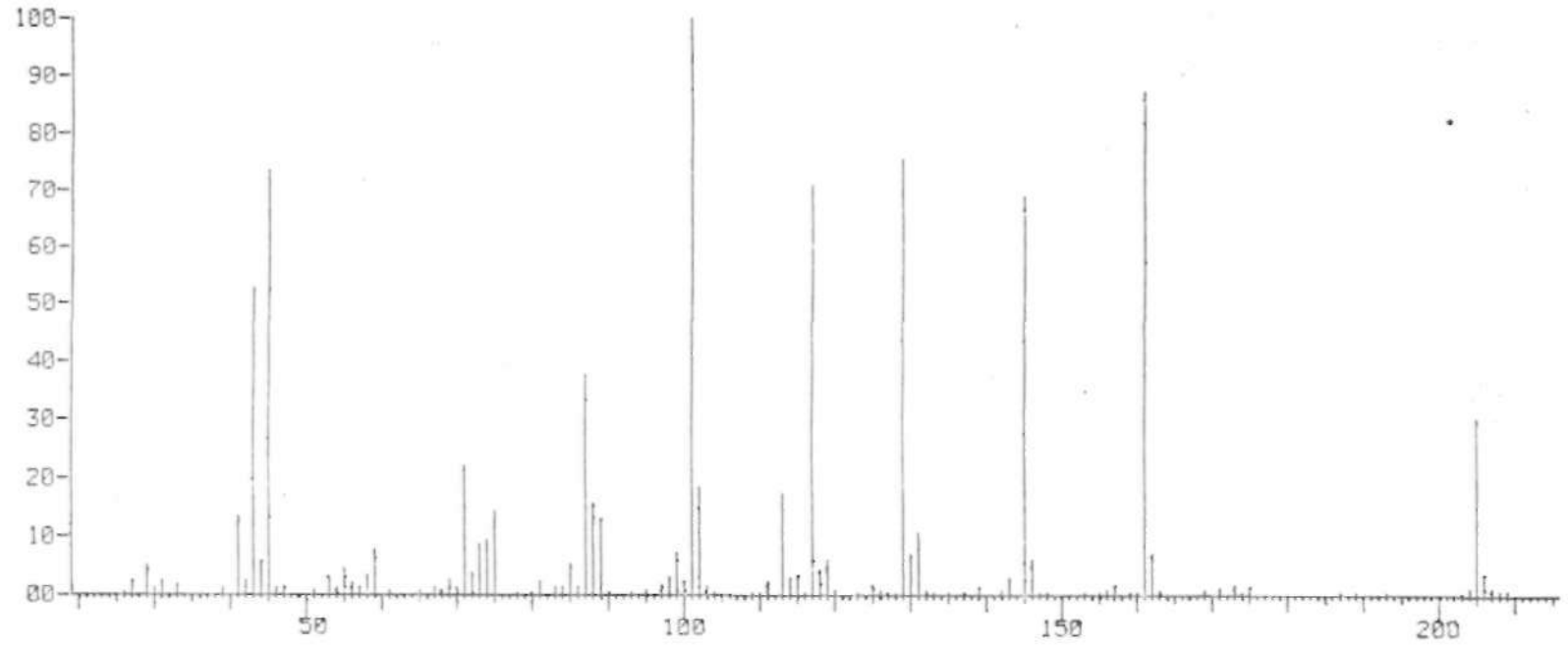


Fig. 12 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (Peak D)

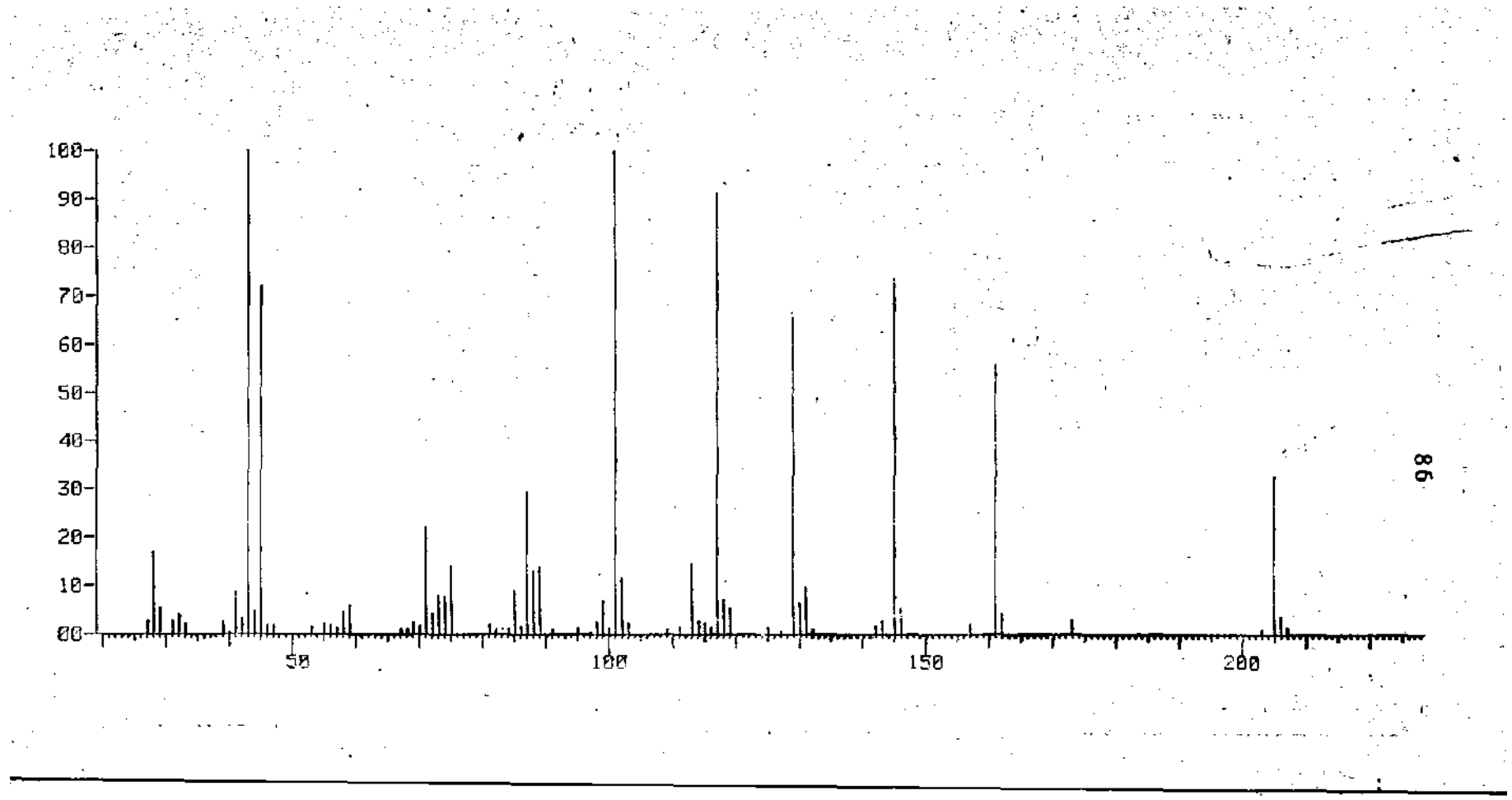


Fig. 13 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol (Peak E)

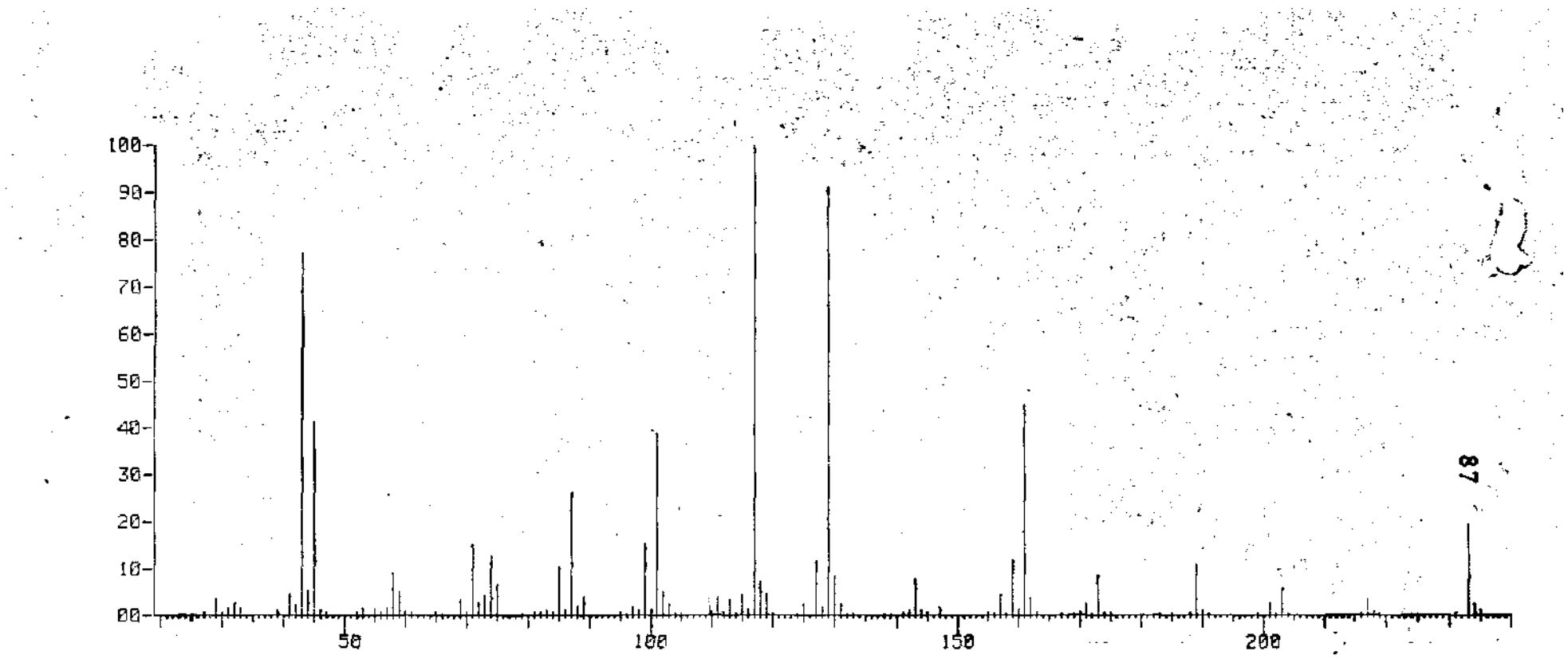


Fig. 14 1,3,5-Tri-O-acetyl-2,4,6-tri-O-methylgalactitol (Peak F)

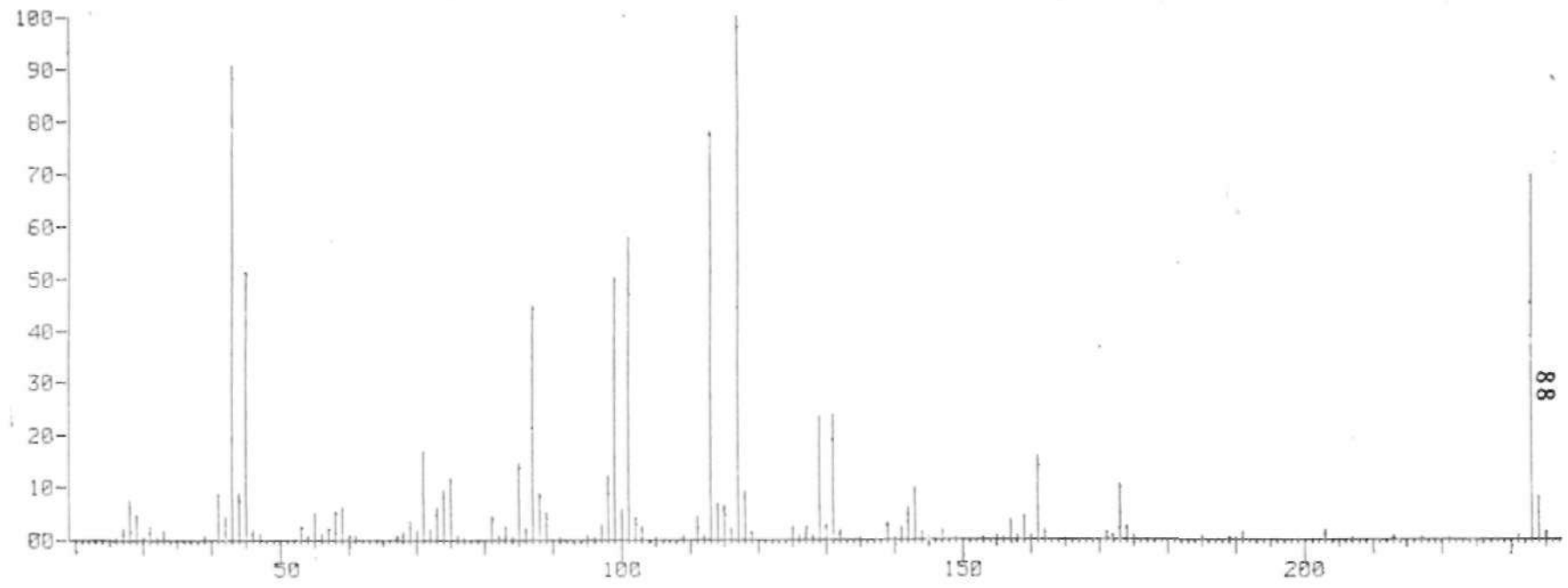


Fig. 15 1,4,5-Tri-O-acetyl-2,3,6-tri-O-methylglucitol (Peak G)

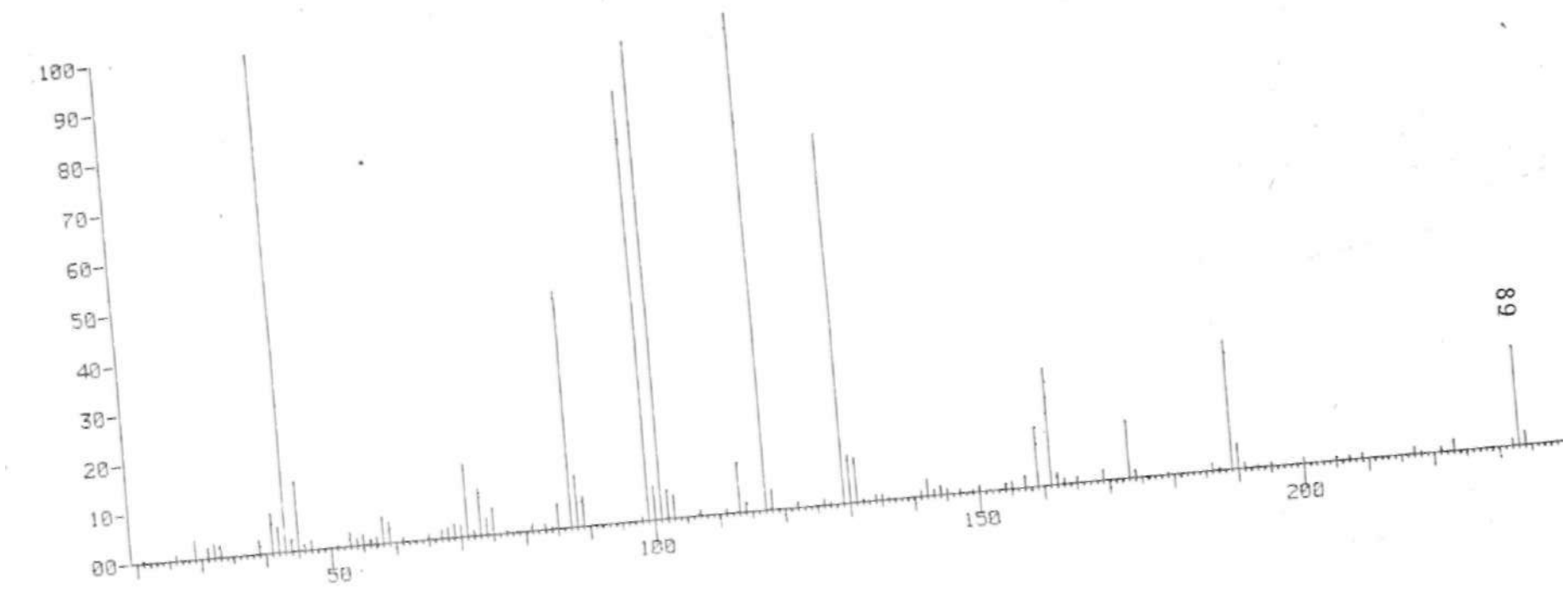
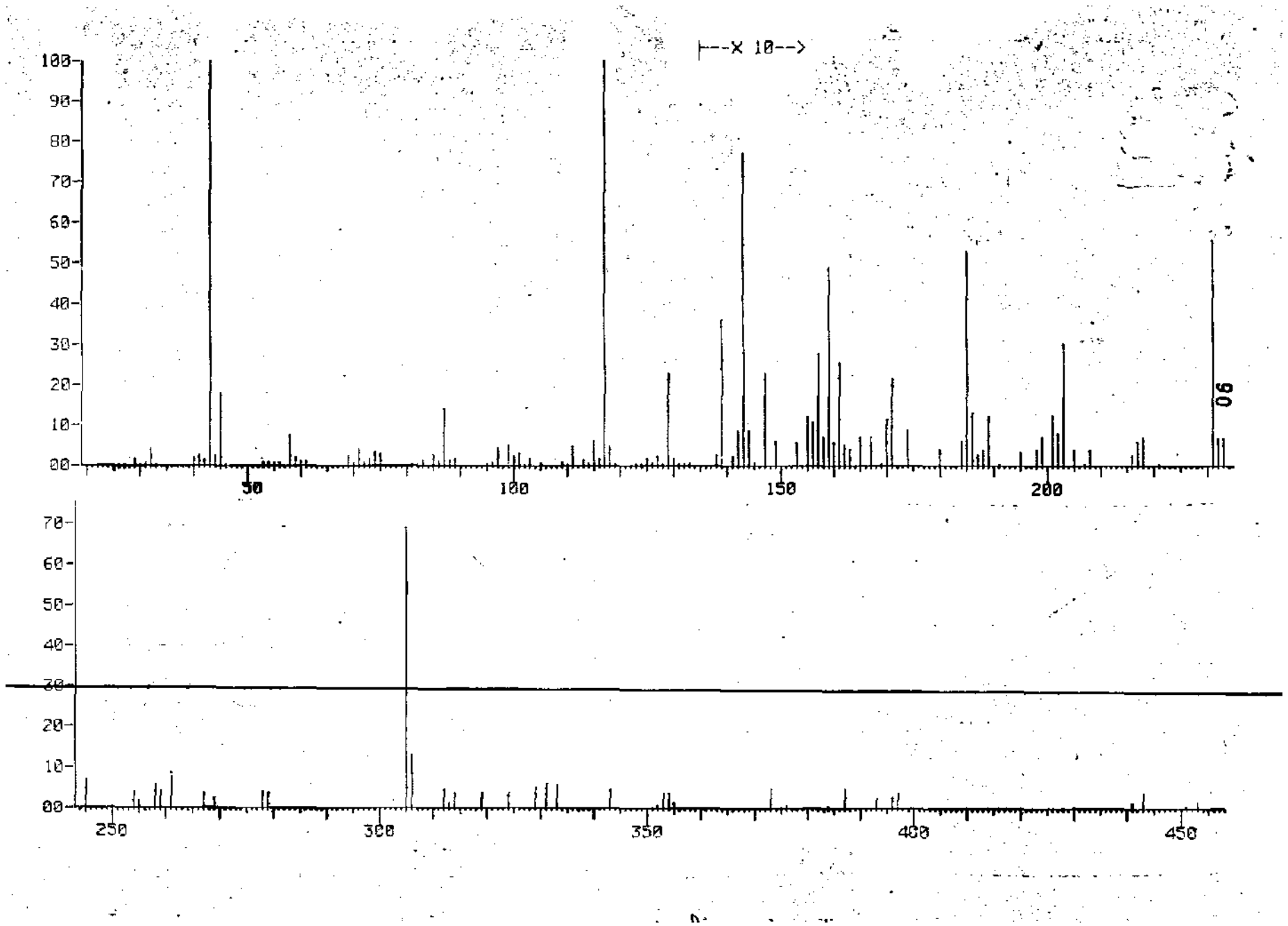


Fig. 16 1,5,6-Tri-O-acetyl-2,3,4-tri-O-methylgalactitol (Peak H)


100
90
80
70
60
50
40
30
20
10
00
⊢--X 10-->
50
100
150
200
06
70
60
50
40
30
20
10
00
250
300
350
400
450

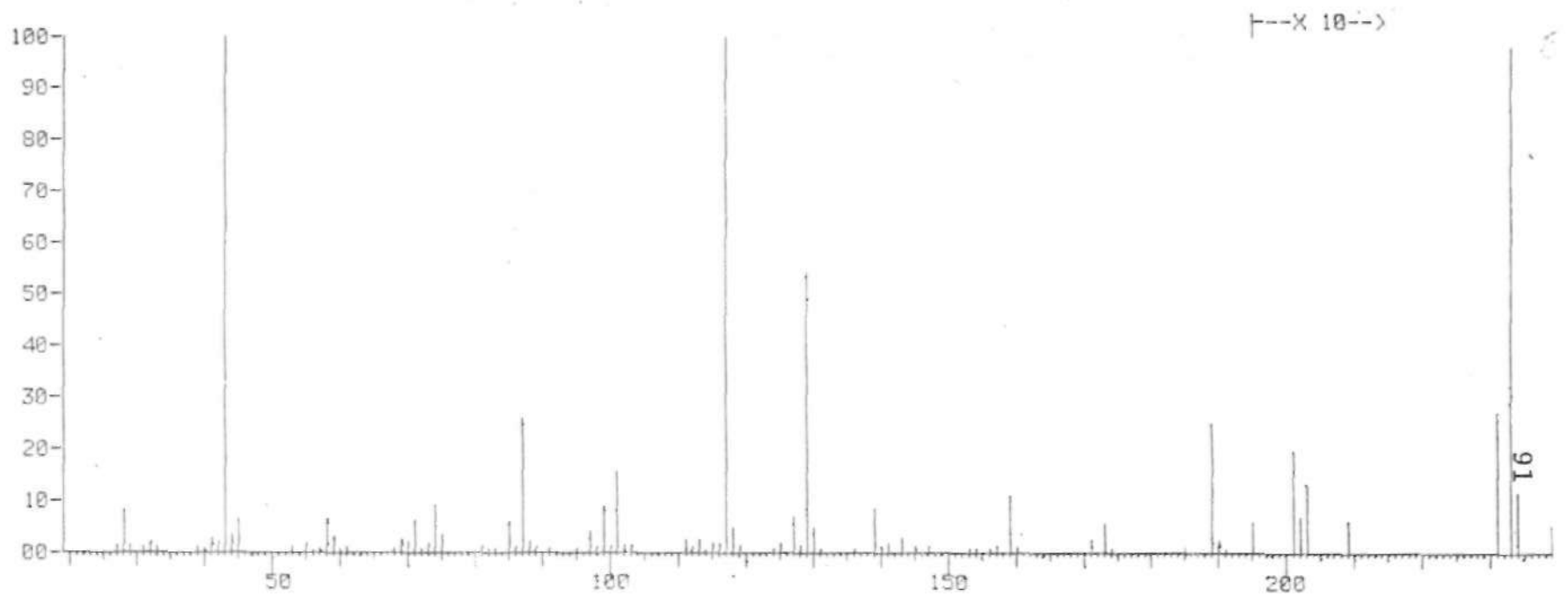


Fig. 18 1,3,5,6-Tetra-O-acetyl-2,4-di-O-methylgalactitol (Peak J)

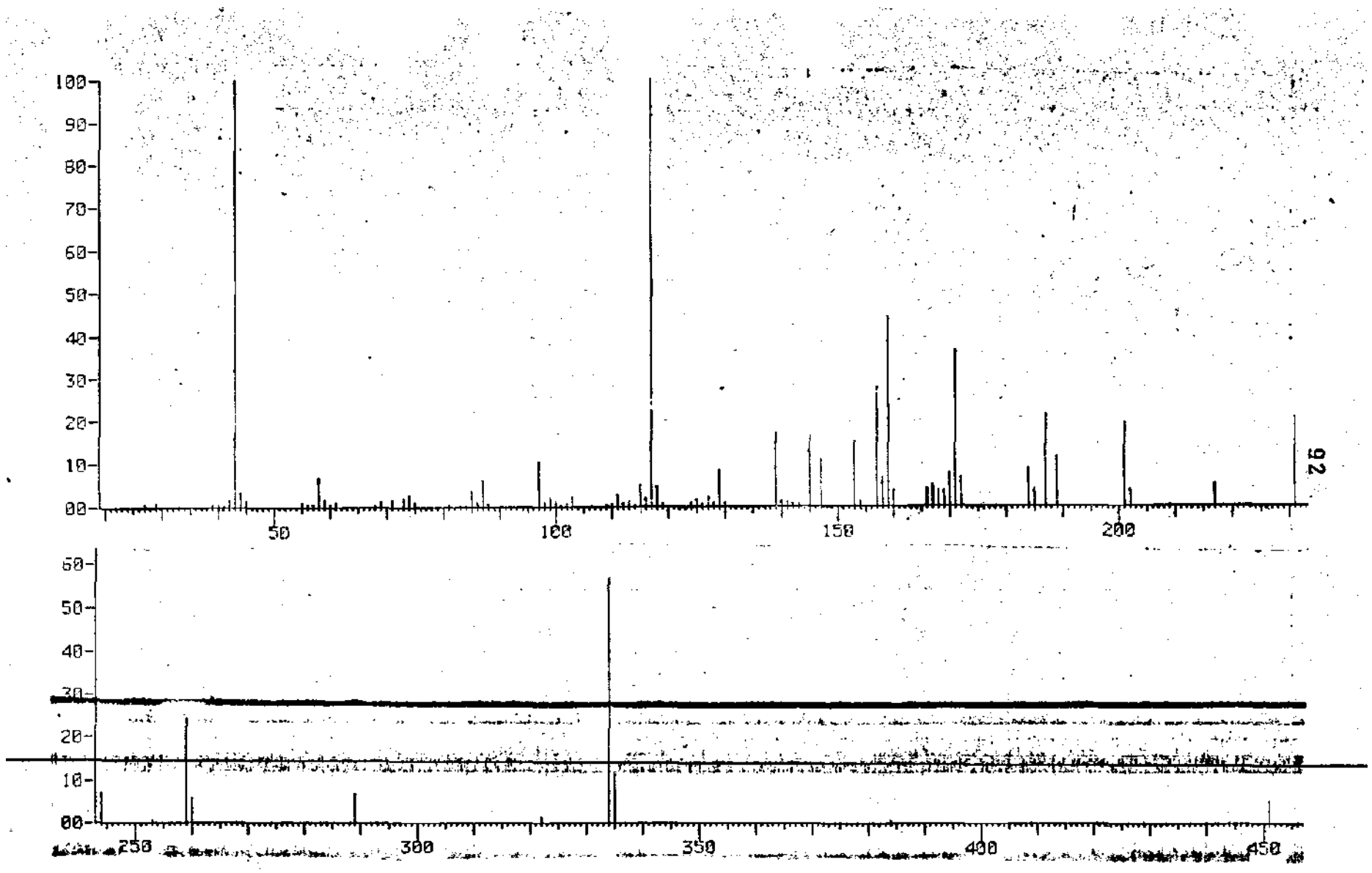


Fig. 19 1,3,4,5,6-Penta-O-acetyl-2-O-methylgalactitol (Peak K)

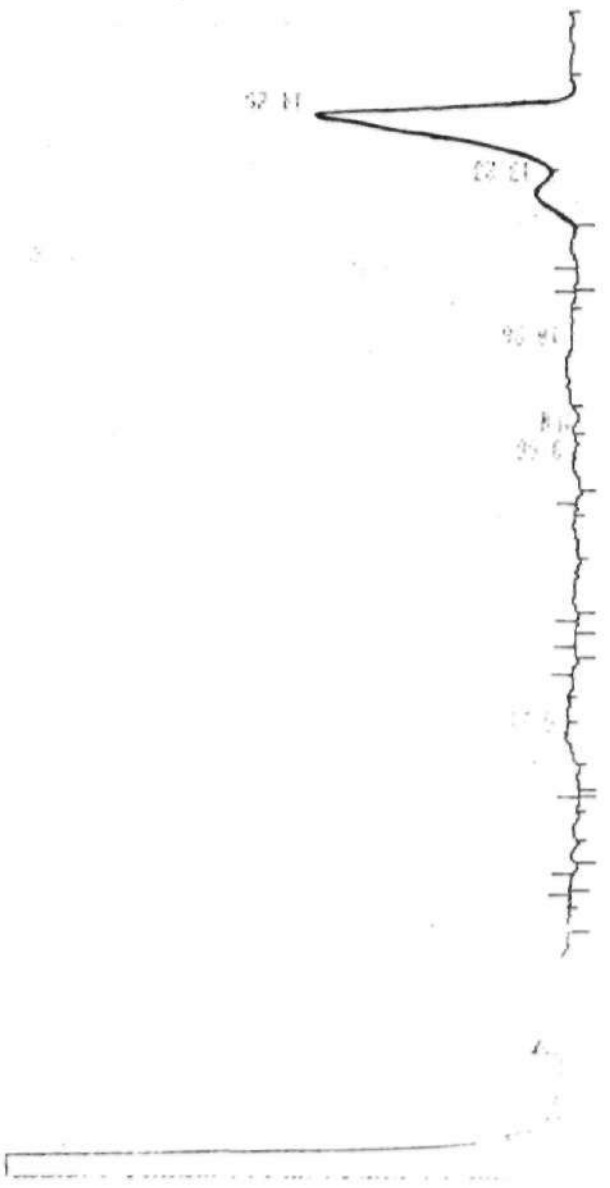


Fig. 20 Glc of PAAN derivatives of P1
on 3% OV225 at 190°-215°(2°/min)

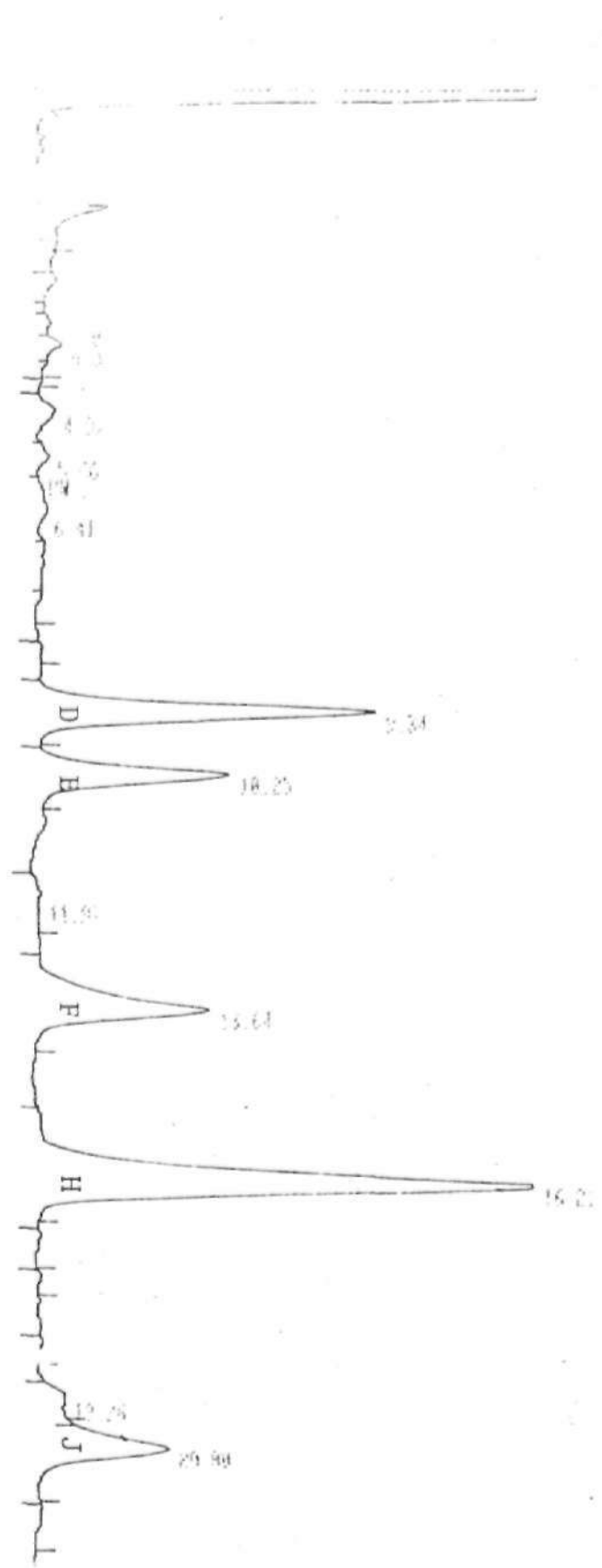


Fig. 20a GLC of PMAA derivatives of MP1 on 3% OV225 at 165°-215° (2°/min)

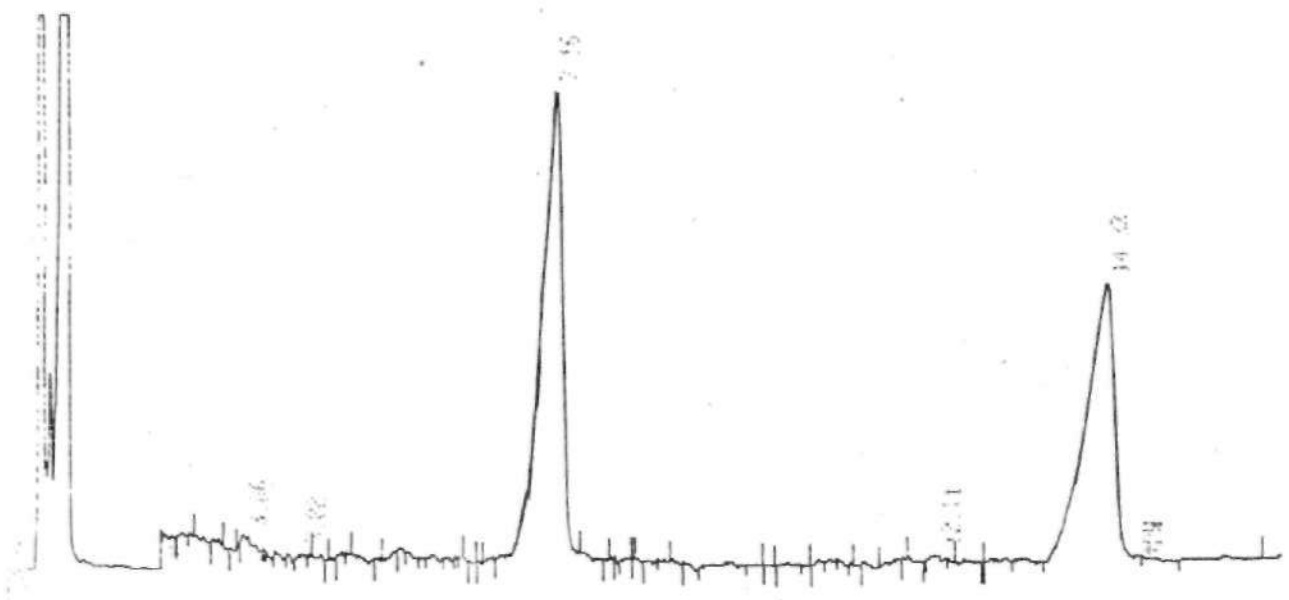


Fig. 21 Glc of PAAN derivatives of D1
on 3% OV225 at 190^{o}-215^{o}(2^{o}/min)

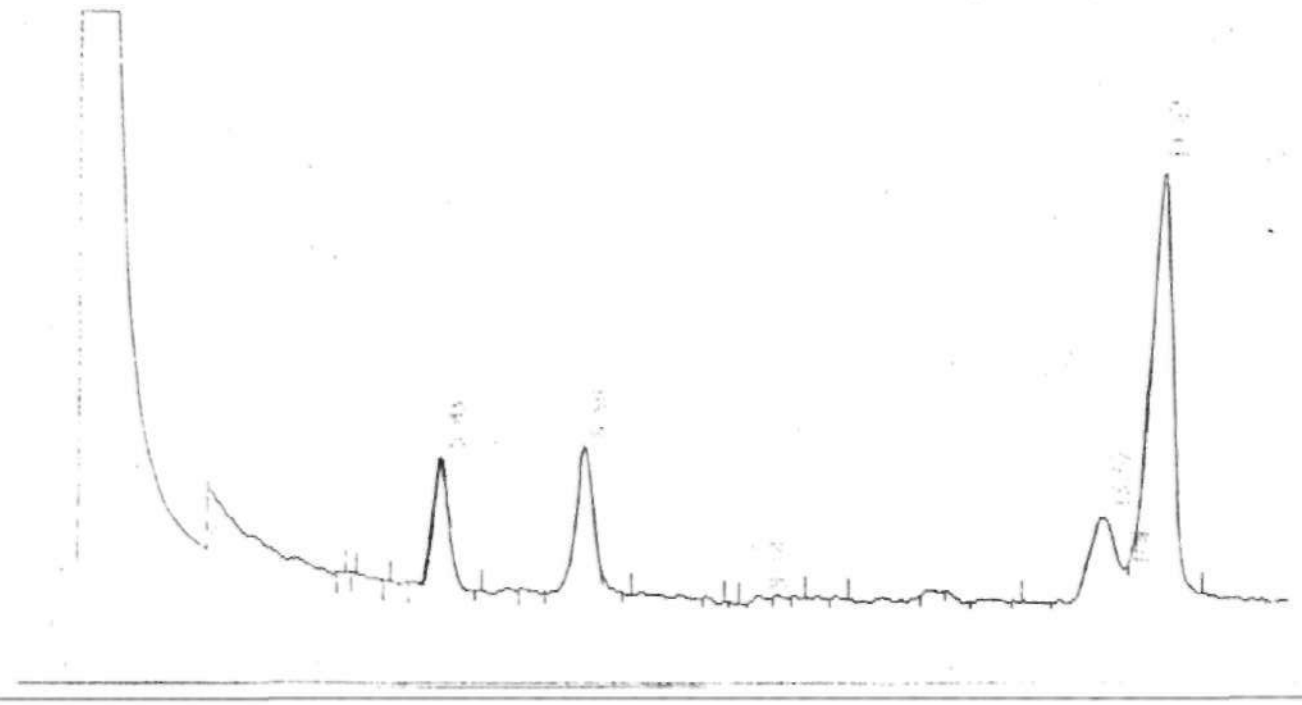


Fig. 22 Glc of PAAN derivatives of PP II-RA
on 3% OV225 at 190°-215°(2°/min)

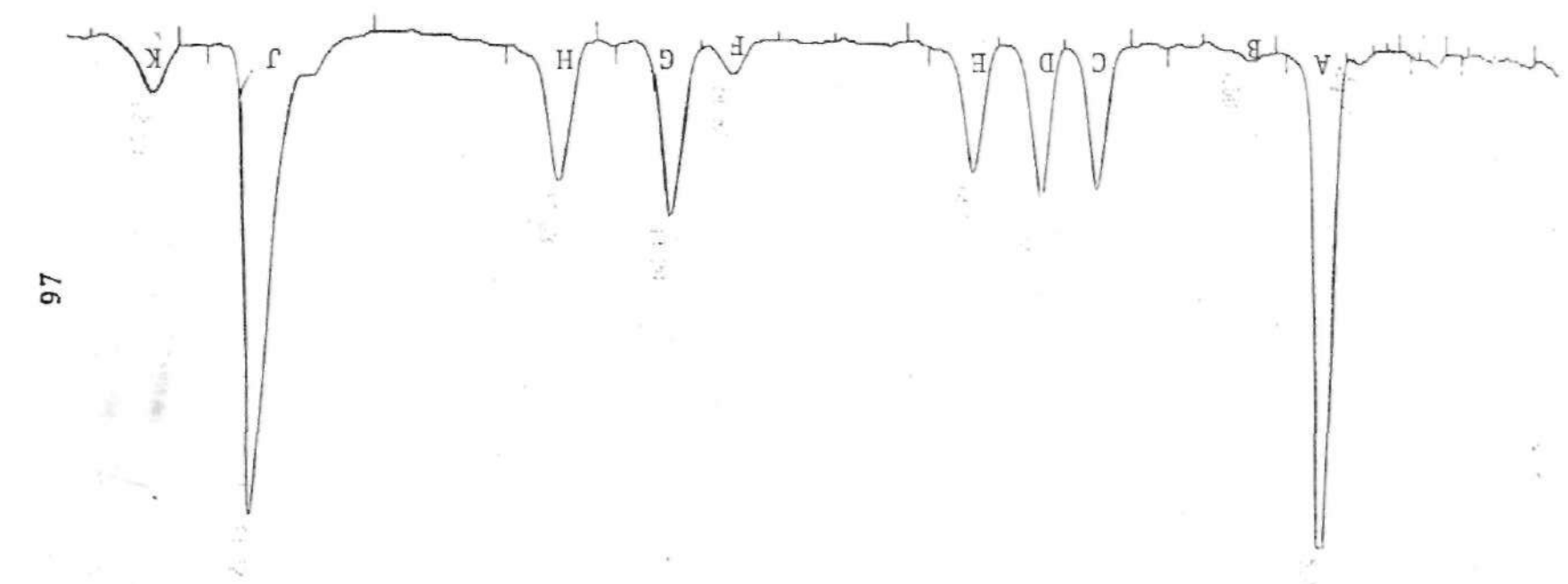


Fig. 23 Glc of PMAA derivatives of MPP II-RA
on 3% OV225 at 165°-215°(2°/min)

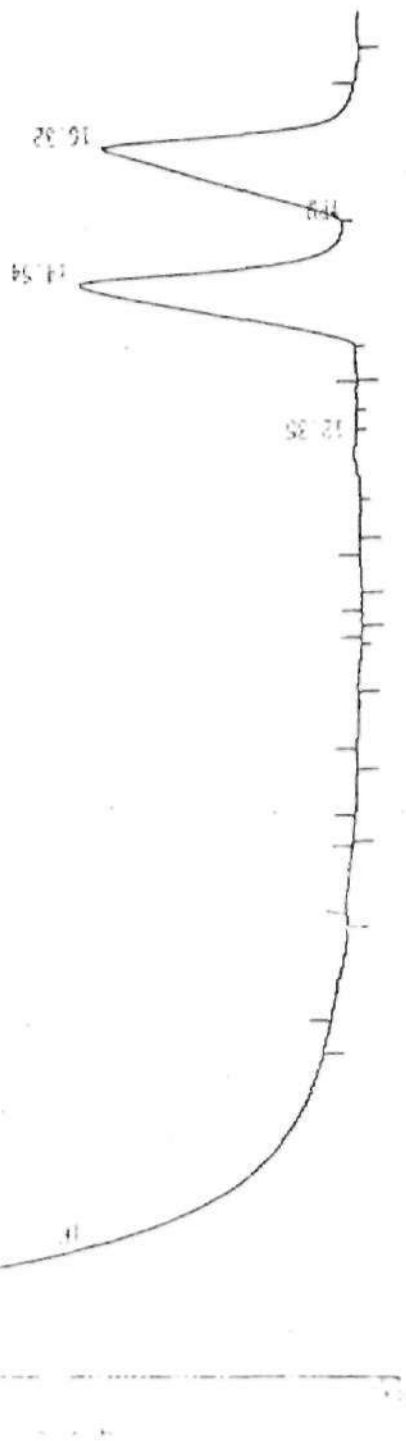


Fig. 24 Glc of PAAN derivatives of UA1
on 3% OV225 at 190°-215°(2°/min)

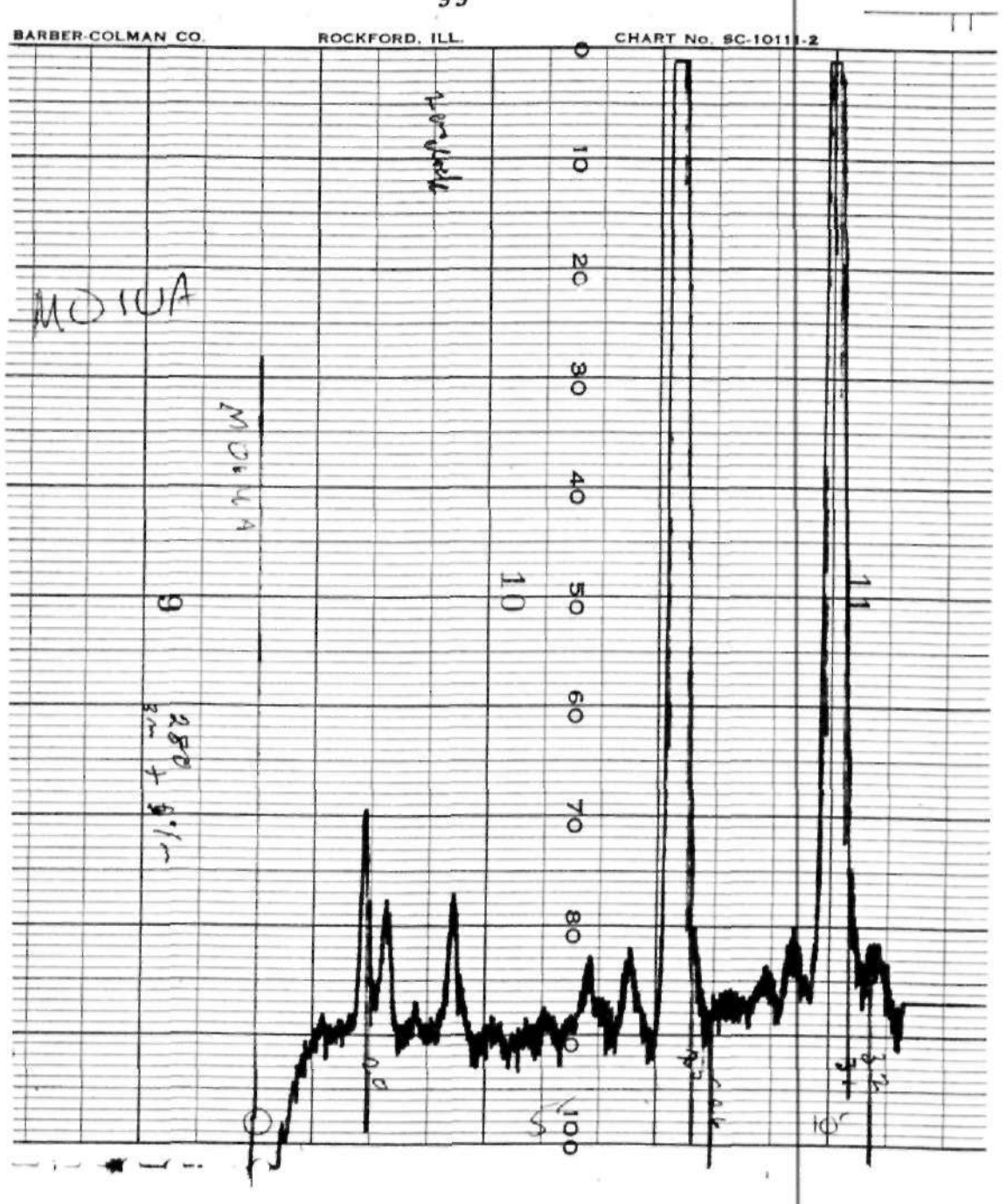


Fig. 25a Glc of PMAA derivatives of MUA1
on 3% OV225 at 180°-215°(6°/min)

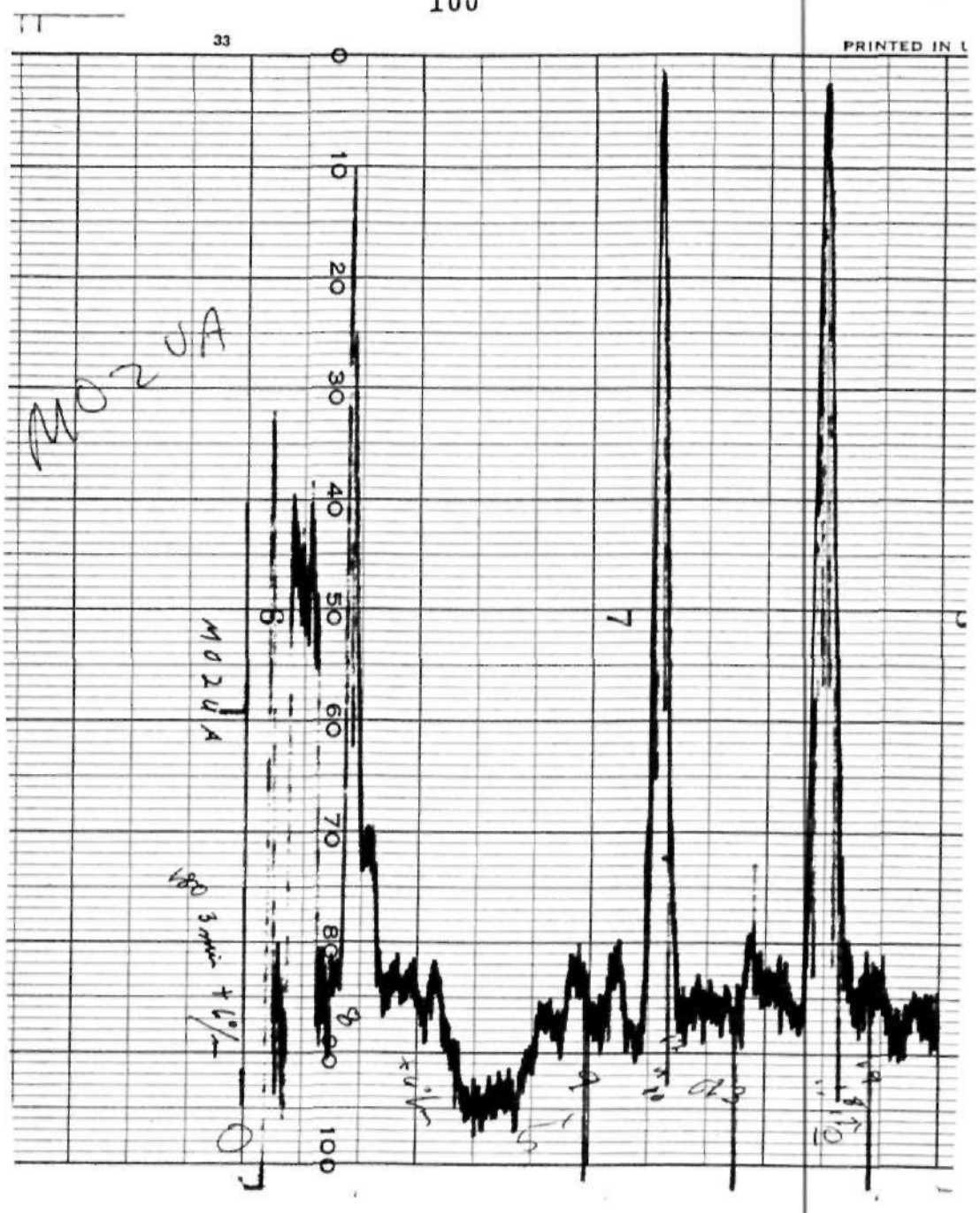


Fig. 25b Glc of PMAA derivatives of MUA2 on 3% OV225 at 180°-215°(6°/min)

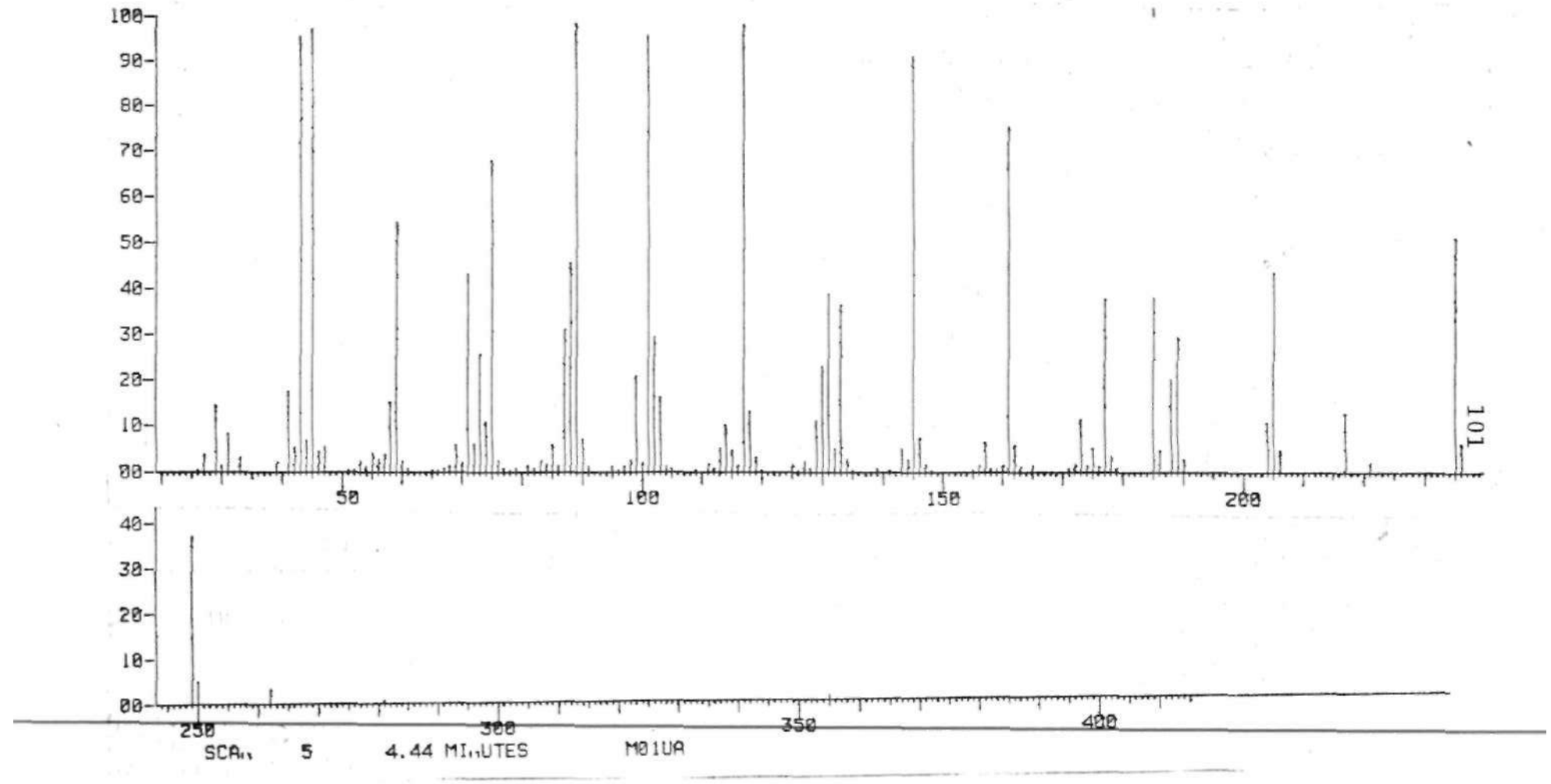


Fig. 26a 6-O-Acetyl-1,2,3,4,5-penta-O-methylgalactitol
(MUA1 and MUA2)

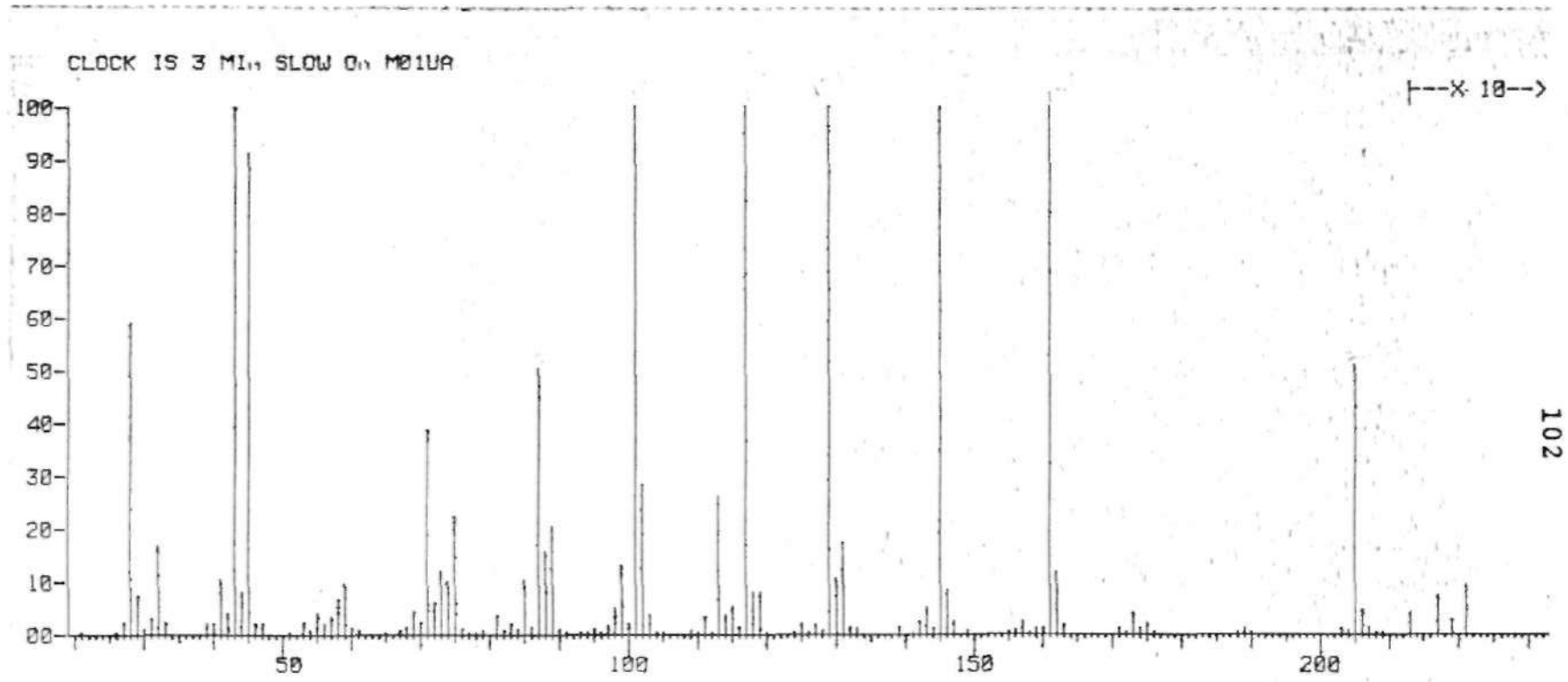


Fig. 26b 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methylglucitol
(MUA1 and MUA2)

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