STRUCTURAL POLYSACCHARIDE CHEMISTRY WITH ANALYTICAL APPLICATIONS TO LEMON GUM

by

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ABSTRACT

Chairman: Professor G.G.S. Dutton

A gas chromatographic method has been developed whereby the following compounds may be determined quantitatively and simultaneously: ethylene glycol, glycerol, erythritol, threitol, arabinose, xylose, mannose, galactose and glucose. These products correspond to the constituent parts of periodate oxidized, borohydride reduced polysaccharides. Quantitative analysis of the fractions obtained on total and partial hydrolysis of such polyalcohols provides a new and powerful analytical tool in polysaccharide chemistry. Optimum conditions for the Smith degradation of a polysaccharide have been illustrated by a detailed analysis of lemon gum.

A new method for the reduction of uronic acids in poly- and oligosaccharides has been developed. Reduction can be carried out after only esterification of the uronic acids. Reaction at this stage avoids possible degradation or fractionation during esterification and subsequent saponification of polysaccharide hydroxyl groups. Good levels of reduction [75%] have been achieved with reduction of highly branched lemon gum.

Silylated polyhydroxy compounds and galacturonic acid were examined by proton magnetic resonance spectroscopy. The trimethylsilyl [TMS] protons of such ethers and esters show good separation from one another. Analysis of the trimethylsilyl proton peaks provides information concerning the number and degree of subsitution of hydroxyl and acid functions present in the parent molecule. Signal strength due to the nine protons on each trimethyl silyl group provides a chemical means of signal enhancement. Analysis was carried out on 2 mg. of a minor TMS glucofuranose derivative.

Lemon gum has been analysed by the method of Lindberg et al [81]. Analysis of lemon gum is the first example of the application of partially methylated alditol acetate mass spectroscopy to a plant gum. The results of this analysis are in good agreement with previous analysis of this gum.

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To my wife.

INTRODUCTION

Lemon gum is a highly branched polysaccharide exudate. During the course of structural investigations of this polymer, a detailed structure was published by Jones and Stoddart [1]. Although work continued to confirm the structure proposed, the direction of investigation was altered. Methods were designed to improve the quality of polysaccharide structural determinations while decreasing the quantities of material required.

The isolation of pure polysaccharides has been greatly stimulated by several important improvements in methods and techniques. Investigators can now isolate small quantities of extremely pure polysaccharides using preparative moving boundary electrophoresis, starch block electrophoresis, ultracentrifugation using density gradients, gel permiation chromatography, and various types of ion exchange chromatography. The general drawback of these methods is an inability to isolate large quantities of polymer. This problem can be further complicated by a lack of available sample. Biological materials may contain polysaccharides in low concentrations or as very complex mixtures. Time can be a critical factor since a glyco protein may denature. However, while the techniques of polymer isolation have improved so have techniques for the investigation of small quantities of carbohydrates.

In the ensuing sections new methods detailing improvements in techniques already applied to carbohydrates and analytical procedures not previously described will be presented. Their application to model compounds and lemon gum [as an example of a complex polysaccharide] will be developed.

PERIODATE OXIDATION OF CARBOHYDRATES AND EVALUATION OF REACTION PRODUCTS BY GAS LIQUID CHROMATOGRAPHY

PERIODATE OXIDATION IN STRUCTURAL STUDIES OF POLYSACCHARIDES

Periodate oxidation results in a specific attack on a carbohydrate polymer. Periodic acid, or its salts will oxidize vicinal hydroxyl functions, the cleaved α/β glycols forming a dialdehyde. The existence of three contiguous hydroxyls will result in the formation of a mole of formic acid from the central carbon atom. Periodate oxidation is complicated by steric effects, electronic effects and over oxidation. Since this field has been well outlined in several reviews [2,3] it will be sufficient to say that the dialdehyde that results from an oxidation forms an extremely complex system. Direct hydrolysis of the dialdehyde is virtually impossible to interpret. Severe degradation of the small component units formed in the oxidation occurs. In previous work in this laboratory [4], experiments aimed at preparation of partially methylated tetroses by oxidation between C-2 and C-3 of two hexoses were unsuccessful because of side reactions, lack of hydrolysis and degradation. If this simple system does not yield clear results, the concept of analysis of complex polysaccharides at the polyaldehyde stage becomes impossible.

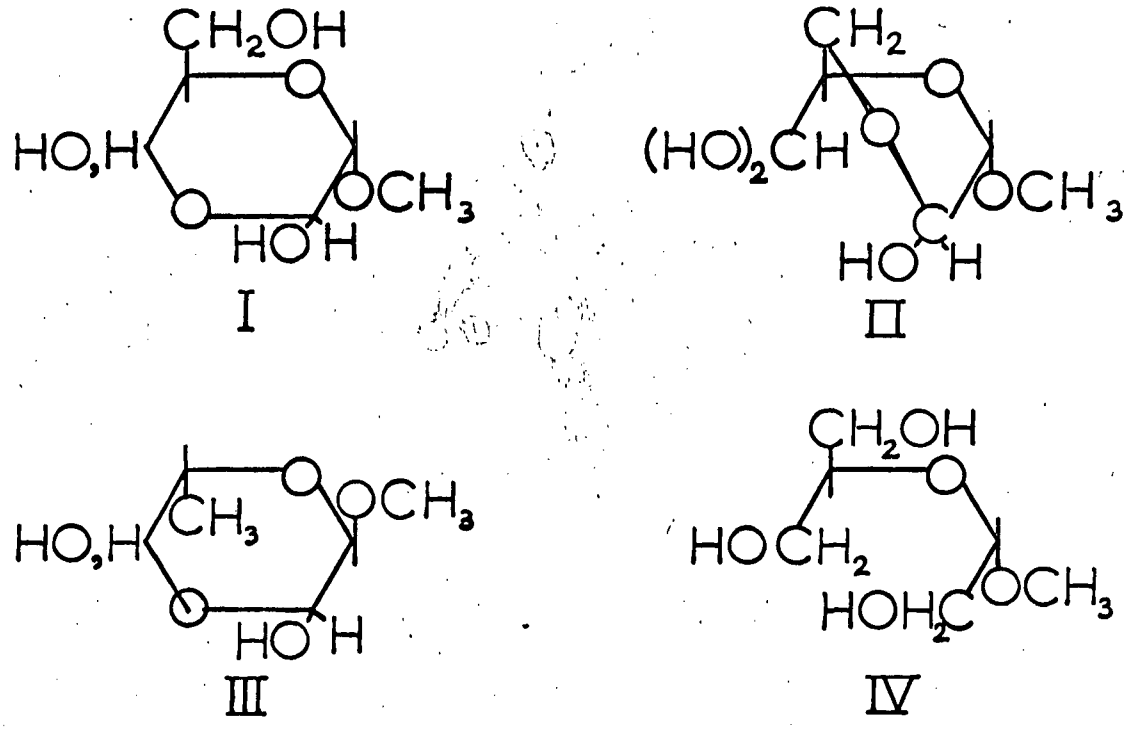
The Barry degradation [5] was developed to analyse the polyaldehyde oxidation product. The polyaldehyde was treated with phenylhydrazine in dilute acetic acid solution. An insoluble product formed, containing approximately one molecule of phenylhydrazine condensed with each dialdehyde group. This complex broke down when heated in an aqueous or ethanolic solution

of phenylhydrazine and acetic acid. The products were phenylosazones and unoxidized parts of the polysaccharide all of which may be identified. There were, however, two areas of difficulty with this procedure. The products of the oxidized portion of the molecule were osazones and hence structural asymmetry of a portion of the molecule was lost. Secondly it was relatively difficult to purify and quantitatively estimate the osazones produced.

A method was developed by Smith [6] to simplify the interpretation of results from periodate oxidation of polysaccharides. The procedure involved reduction with metal borohydrides and subsequent controlled hydrolysis of the polyalcohol which was formed. Dialdehydes formed on periodate oxidation do not exist as free aldehydes but like sugars themselves, undergo cyclization and hydration [7]. Some examples of cyclized model compounds [I, II, III] which have been analysed are shown in Figure 1. Alcohols such as IV are readily hydrolysed with dilute mineral acid at room temperature. Cyclic acetals such as I, II, and III are relatively stable. Smith realized that instability of alcohols such as IV toward dilute acid at room temperature, compared with glycosides, provided a new and more powerful tool for controlled degradation of polysaccharides. When a sugar moeity of a polysaccharide is cleaved by periodate and reduced, the resulting alcoholic derivative, being a true acetal, is sensitive to acid. Sugar units which are not cleaved and are attached glycosidicly to cleaved or unattached units remain intact being relatively stable to acid. Using the marked differences in stability of glycosides over true acetals a wide variety of oligosaccharides have been obtained. The structures of the stable units provide an indication of the polysaccharide subunits.

FIGURE 1

MODEL PERIODATE OXIDATION PRODUCTS BEFORE AND AFTER REDUCTION



Named the Smith degradation this procedure has been reviewed and utilized extensively [8, 9, 10, 11]. Periodate oxidation, reduction and total hydrolysis of a polysaccharide, often incorrectly called a Smith degradation, also provides a considerable source of information regarding fine structure in the polymer.

Two limitations exist which detract from the use of total or partial hydrolysis of the Smith polyalcohol. One of these problems is the inability of standard analytical techniques to determine quantitatively the totally reduced products of the oxidation, that is to say ethylene glycol, glycerol, erythritol and threitol. Ethylene glycol is especially difficult to qualitatively identify on paper chromatograms because of diffusion in chromatographic solvents, streaking and lack of reaction. The second problem is the

wide variety of rates of hydrolysis of different glycosidic linkages and different true acetals. These factors cause difficulties in carrying out partial hydrolysis. The solutions to these problems will be outlined in the following two subsections. They will demonstrate a whole new approach and usefulness of Smith oxidations involving partial and total hydrolysis.

SIMULTANEOUS ESTIMATION OF POLYHYDRIC ALCOHOLS AND SUGARS

As mentioned before, one method of investigating the structure of a polysaccharide involves periodate oxidation, borohydride reduction and complete hydrolysis [12]. For example, in the case of a linear 1 →4 linked pentosan, the products will be ethylene glycol from the non reducing end and glycerol from the interior units. When any of the interior units carry side chains these units are immune to periodate oxidation and thus subsequent hydrolysis gives monosaccharides as well as polyhydric alcohols. Much useful information can be obtained from a knowledge of the ratio between non reducing end groups, interior units and branch points. Two different analytical methods have normally been used in this connection after separation of the components by paper or thin-layer chromatography. Thus, polyhydric alcohols have been estimated by chromotropic acid [13] and monosaccharides by phenol sulfuric [14] or other colorimetric method. The necessity of using two distinct analytical procedures in addition to the separations involved is tedious and introduces possibilities of error. The present work reports a method using gas-liquid chromatography [G.L.C.] whereby mixtures of polyhydric alcohols and monosaccharides may be analysed simultaneously and accurately.

TABLE I

POSSIBLE REACTION PRODUCTS FROM PERIODATE OXIDIZED POLYSACCHARIDES

Polysaccharide	Possible Products
Arabinoxylan	Ethylene glycol, glycerol, xylose, arabinose
Glucomannan	Glycerol, erythritol, glucose, mannose
Galactoglucomannan	Glycerol, erythritol, threitol, glucose, mannose, galactose
Arabinogalactan	Ethylene glycol, glycerol, threitol, arabinose, galactose

This investigation arose from the need to have a simple method for the analysis of polysaccharides occurring in wood and plant gums. Table I illustrates the possible products from four such typical polysaccharides.

The method involves transformation of the mixture of sugars and polyhydric alcohols resulting from the periodate oxidation of a polysaccharide into derivatives sufficiently volatile for separation by G.L.C. In order to test the viability of the method, several test mixtures were prepared corresponding to the possible products shown in Table I. For each system studied the relative proportions of the components were varied over wide limits thus covering the different situations encountered in various naturally occurring polysaccharides. The components were separated as their trimethylsilyl [TMS] derivatives [15] on a column employing SF-96 as the liquid phase.

The separation of the products arising from an arabinogalactan is shown in Figure 2 and the data obtainable from an arabinoxylan are given in Table II with the separation shown in Figure 3. Similarly the simulated glucomannan is represented by Table III and Figure 4. In a like manner the simulated galactoglucomannan system is shown in Table IV and Figure 5. These results need little explanation except in the last case. According to the current view [16] of the structure of a wood galactoglucomannan one would not expect galactose to survive the periodate oxidation. However, galactose was included as a check on hexose analysis.

The model experiments reported here were an opportunity to test the validity of the proposed method and therefore the most general cases were studied. It is clearly seen from the figures that the separations are excellent and the agreement between calculated and found values is good. The worst separation in the present study is that between threitol and erythritol in the galactoglucomannan system. But even here it is possible to calculate the relative amounts [17] in fair agreement with theory [Table V]. This problem will only commonly arise where a polysaccharide contains galactose units linked 1 - 4 together with glucose and/or mannose similarly linked.

Examination of Figure 6 will show that the pentoses have a lower retention time [R_T] than the hexoses. This means that the same column and procedure may be used to separate all of the polyhydric alcohols and sugars mentioned in Table I. More importantly it means that this same system may be used to separate and estimate all five of the sugars commonly occurring in wood polysaccharides. This separation has now been described by others whose results appeared while our work was in progress [18-21]. Particularly

FIGURE 2: SEPARATION OF PRODUCTS AS TRIMETHYLSILYL DERIVATIVES FROM AN ARABINOGALACTAN

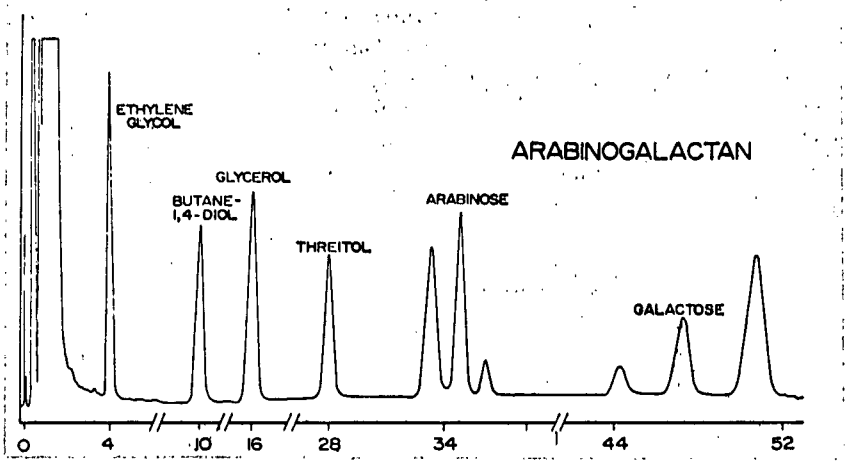


FIGURE 3: SEPARATION OF PRODUCTS AS TRIMETHYLSILYL DERIVATIVES FROM AN ARABINOXYLAN

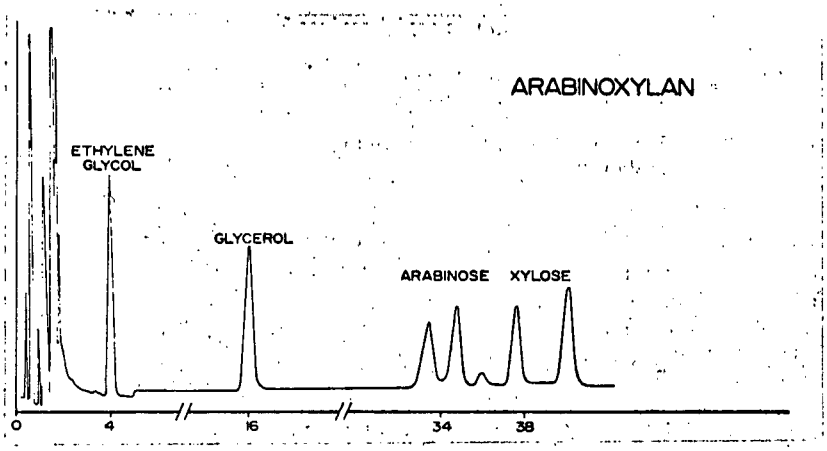


TABLE II

ANALYSIS OF REACTION PRODUCTS FROM AN ARABINOXYLAN

	% *composition by weight*			
	Ethylene glycol	*Glycerol*	*Arabinose*	*Xylose*
Found	15.6	18.8	29.3	36.0
Calculated	15.8	19.0	29.9	35.5
Found	30.5	38.1	29.2	3.15
Calculated	30.8	37.7	28.9	3.44
Found	42.9	5.20	3.31	48.5
Calculated	42.8	5.14	4.07	47.8

FIGURE 4: SEPARATION OF PRODUCTS AS TRIMETHYLSILYL DERIVATIVES FROM A GLUCOMANNAN

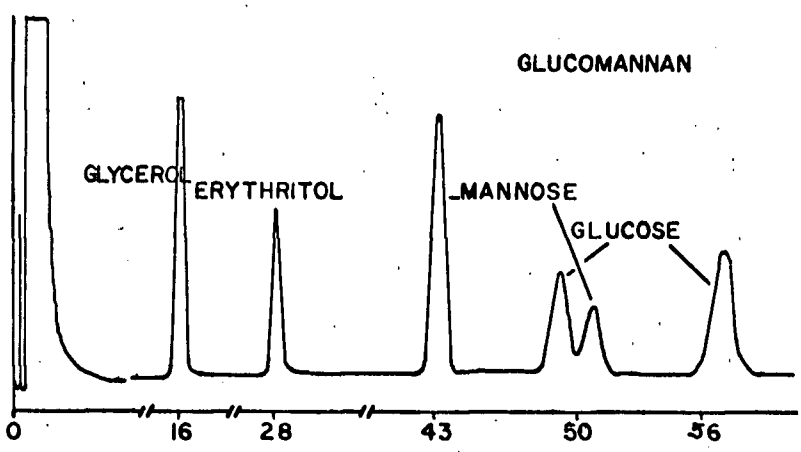


FIGURE 5: SEPARATION OF PRODUCTS AS TRIMETHYLSILYL DERIVATIVES FROM GALACTOGLUCOMANNAN

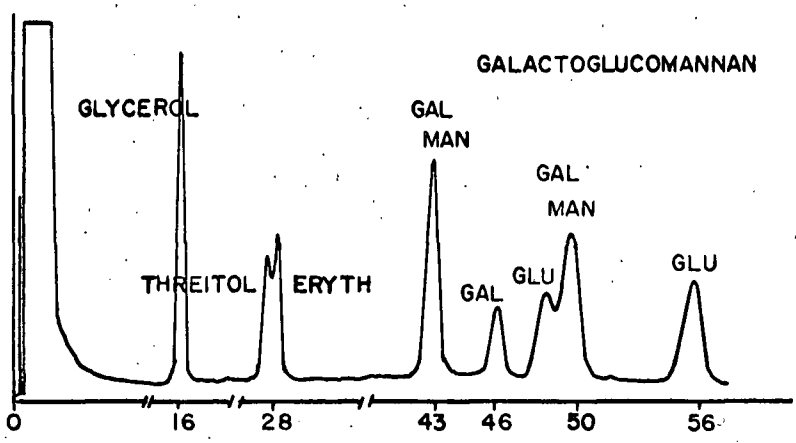


FIGURE 6: SEPARATION OF PRODUCTS AS TRIMETHYLSILYL DERIVATIVES FROM CRUDE HEMICELLULOSE

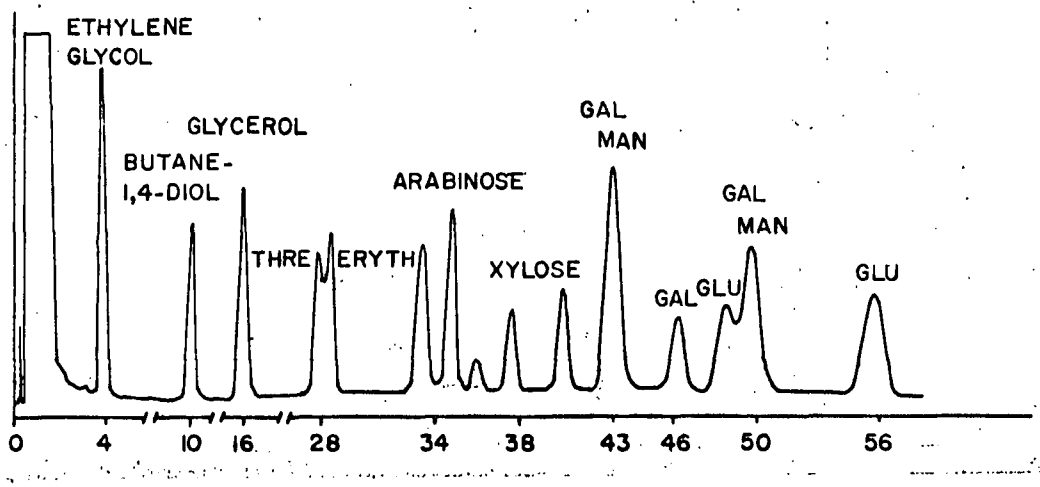


TABLE III

ANALYSIS OF REACTION PRODUCTS OF A GLUCOMANNAN

	% composition by weight			
	Glycerol	*Erythritol*	*Mannose*	*Glucose*
Found	19.2	9.20	35.5	36.4
Calculated	19.2	8.70	36.0	36.4
Found	31.4	1.48	65.1	2.22
Calculated	33.2	1.46	62.8	3.11
Found	6.78	29.6	6.20	57.5
Calculated	6.34	28.6	5.85	59.2

TABLE IV

ANALYSIS OF REACTION PRODUCTS OF A GALACTOGLUCOMANNAN

	% composition by weight			
	Glycerol	*Galactose*	*Mannose*	*Glucose*
Found	17.6	25.7	29.38	28.2
Calculated	15.3	27.8	28.4	28.5
Found	4.12	53.2	39.1	3.71
Calculated	4.02	54.8	37.4	3.74
Found	4.08	2.85	38.6	54.7
Calculated	3.94	3.62	36.8	55.7
Found	—	47.5	2.61	50.2
Calculated	—	47.2	4.81	48.4

TABLE V

ANALYSIS OF THREITOL AND ERYTHRITOL MIXTURES

	% composition by weight	
	Threitol	*Erythritol*
Found	54.5	45.5
Calculated	51.5	48.5
Found	83.2	16.8
Calculated	84.1	15.9
Found	20.8	79.2
Calculated	17.5	82.5

TABLE VI

ANALYSIS OF SIMULATED CRUDE HEMICELLULOSE HYDROLYSATES

	% composition by weight				
	Arabinose	*Xylose*	*Galactose*	*Mannose*	*Glucose*
Found	17.9	23.1	20.4	16.95	22.0
Calculated	18.4	21.6	21.1	17.25	21.7
Found	17.75	22.6	21.6	34.0	4.07
Calculated	18.2	21.7	21.3	34.4	4.37
Found	3.15	39.8	3.03	16.62	39.0
Calculated	3.28	38.7	3.81	15.3	39.0
Found	33.8	4.07	38.5	3.64	20.0
Calculated	33.6	3.85	38.9	3.94	19.92

notable is the excellent Swedish paper to which further reference will be made [22]. The results presented in Table VI are further confirmation of the accuracy of the method used in this laboratory.

The results quoted in Tables II - VI were obtained using an instrument with a thermal conductivity detector and systems containing free sugars. Each of these factors requires brief comment. Firstly, different substances cause such a detector to respond to a varying degree, thus for identical molar amounts of different compounds the areas under the peaks are not the same. It is therefore necessary to determine the Molar Response Factor [M.R.F.] for each compound to be determined. This is done relative to an internal standard, which in the present work was butane -1,4-diol. This compound is readily available, easily purified and has a convenient retention time, as Figure 6 shows. The view has recently been expressed that an inert compound is perferable [23] and terphenyl has been suggested. Although this compound has certain advantages, solubility in carbohydrate hydrolysis mixtures can limit its use as a quantitative internal standard.

When working with compounds of similar molecular complexity it may be acceptable to assume that all the response factors are identical. However, when the compounds examined range from C_2 to C_6 this assumption is incorrect [24], as shown in Table VII. These values are presented here to show the variation which may be expected. It is our experience that the exact M.R.F. applicable to any analysis depends on the precise experimental conditions used. The value is influenced by such factors as the rate of temperature programming. A contrary view has been expressed [25] but at this time it would seem advisable for each worker to determine suitable response factors using exactly

the same experimental conditions as in the analytical determinations. It would be most unwise to assume without verification that the figures quoted in Table VII are valid for other systems.

TABLE VII

MOLAR RESPONSE FACTORS OF POLYOLS AND SUGARS

Compound	Molar Response Factor
Ethylene glycol	0.832 ± 0.033
Butane -1,4-diol	1.00 ± 0.000
Glycerol	1.26 ± 0.014
Threitol	1.78 ± 0.088
Erythritol	1.72 ± 0.061
Arabinose	1.74 ± 0.061
Xylose	1.77 ± 0.064
Galactose	2.08 ± 0.126
Glucose	2.02 ± 0.091
Mannose	2.02 ± 0.059

Secondly, in solution any one sugar exists as an equilibrium mixture of the anomers of the furanose and pyranose forms. When certain peaks overlap it will be necessary to use a peak corresponding to one anomeric form as a measure of the total amount of that particular sugar. This fact has been clearly discussed in the Swedish paper cited previously [22]. Since the relative equilibrium concentrations of the different forms are highly dependent on the solvent used [26] it follows that the figures quoted in that paper [22] are only valid for that particular system. For accurate results it is thus necessary for each worker to adopt a standardized procedure for the treatment

of polysaccharide hydrolysates. The composition of the sugar peaks obtained in the present work is given in Table VIII and shown in Figure 6. The equilibrium composition of the solutions is shown in Table IX.

TABLE VIII

COMPOSITION OF PEAKS IN GAS PHASE CHROMATOGRAM OF A MIXTURE OF ARABINOSE, XYLOSE, GALACTOSE, GLUCOSE AND MANNOSE

Major Component	Minor Component[s]
Arabinose 1, 2, 3	Xylose 1 + 2
Xylose 3 + 4	--
Mannose 1	Galactose 1
Galactose 2	--
Galactose 3	Mannose 2 Glucose 1
Glucose 2	--

TABLE IX

NUMBER OF SUGAR ANOMERS DETECTED BY GAS-LIQUID CHROMATOGRAPHY AND THE PERCENTAGE COMPOSITION OF EQUILIBRIUM SOLUTIONS

Sugar	No. of Anomers	Percentage Composition[a]
Arabinose	3	
Xylose	4	1 + 2 3.58 ± 0.28
		3 + 4 96.4 ± 0.28
Galactose	3	1 8.19 ± 0.80
		2 29.3 ± 1.27
		3 62.6 ± 1.02
Mannose	2	1 74.5 ± 1.21
		2 25.5 ± 1.21
Glucose	2[b]	1 38.3 ± 0.33
		2 61.7 ± 0.36

a Anomers numbered in order of elution.
b It is possible that glucose shows a third anomer present in very small concentration.

The analysis of a periodate oxidized glucomannan by gas-liquid chromatography of the derived erythritol and glycerol acetates was reported in 1960 by Bishop and Cooper [27]. More recently Zinbo and Timell [28] have used the silyl derivatives for the analysis of a xylan. In neither case has the fate of glycol aldehyde, obtainable from carbons one and two, been studied, although the former authors noted the existence of an unidentified peak with a greater retention time than glycerol triacetate and suggested this might be due to glycol aldehyde diacetate.

In an attempt to discover what happens to the glycol aldehyde a solution of this compound in pyridine was silylated and examined using the same chromatographic conditions as before. There resulted a single peak indistinguishable from glycerol and attributed to the dimer [29]. When an aqueous solution of glycol aldehyde was concentrated to dryness and silylated, two peaks were obtained. One of these had the same retention time as glycerol and the other a shorter [presumably the monomer]. If an aqueous solution of glycol aldehyde was first reduced with sodium borohydride only one peak corresponding to ethylene glycol was obtained. This suggested one way of dealing with the problem. When, however, synthetic mixtures of glycol aldehyde and the neutral products listed in Table I were analysed, several new and unexpected peaks were obtained. These peaks were assumed to be due to acetals formed between glycol aldehyde and the polyhydric alcohols [29]. These observations did not accord with those made on periodate oxidized amylose, where the amount of glycol aldehyde should equal the sum of glycerol and erythritol. Furthermore, the gas chromatographic analysis of the products from mesquite gum gave results in good agreement with those obtained by paper chromatographic separations [30].

The difference in treatment of the model systems and the polysaccharides was an acid hydrolysis step. When the model systems containing glycol aldehyde were heated at 100° for 24 hours with 1N sulfuric acid and then analysed, no unidentifiable peaks were obtained. There was no change in the ratio of the sugars present. The hydrolysates were dark brown and contained a brown precipitate. There was a small increase in the glycerol concentration and a small decrease in ethylene glycol. The former is no doubt due to traces of glycol aldehyde dimer since in a separate experiment in which glycol aldehyde alone was treated with acid a small peak occurred in the glycerol region. The apparent loss of ethylene glycol cannot at present be explained since it was verified that this compound was not lost on ion exchange resins nor on concentration of its aqueous solutions.

It is concluded that the glycol aldehyde is almost entirely destroyed in the total hydrolysis of the polyalcohol and is thus not a serious factor in such analyses. Where a highly accurate value for glycerol is necessary this may be obtained by reduction of the hydrolysate with borohydride before analysis to convert any remaining glycol aldehyde dimer to ethylene glycol.

The first applications of gas-liquid chromatography to polysaccharide chemistry were reported for methylated sugars [31] because of their volatility. This technique is now widely applied and has also been used in conjunction with periodate degradation to facilitate the resolution of a complex mixture of isomeric sugars [32-34]. With the introduction of trimethylsilylation by Sweeley et al. [15] this technique has been extended to the separation of sugars [18-22] and glycosylalditols obtained by Smith degradation [34 and references therein]. Although the work reported here arose out of our interests

in wood polysaccharides and plant gums it is clear that the procedure is a general one and may be used for many other types of polysaccharides. It has already been applied with success to a study of sapote gum [35] and mesquite gum [30]. Work has been carried out to extend the method to systems containing deoxy and amino sugars [36] thus permitting the study of a wider variety of polysaccharides.

CONTROL DURING THE SMITH DEGRADATION

In addition to using gas-liquid chromatography for the examination of total hydrolysis of a Smith polyalcohol, this analytical method has provided a new and most powerful tool in the structural elucidation of polysaccharides by Smith degradation. As mentioned before, Smith degradations involve utilization of differences in the ease of hydrolysis of various acetal structures generated during the periodate oxidation and subsequent reduction. Unfortunately the Smith degradation is not as simple as would first appear. The problem arises from the different rates at which different glycosides and acetals hydrolyse. Two extreme examples may be used to illustrate this point. Pyranosidic linkages are considerably more stable than corresponding furanosidic linkages. It has been possible to utilize this property to isolate L-arabinose from mesquite gum [37]. L-arabinose in polysaccharides often occurs as furanoside and hence may be readily hydrolysed by 0.01N sulfuric acid at 95°C. Hydrolysis of mesquite gum for 36 hours under these conditions produced a residual polysaccharide containing no L-arabinose. At the other extreme, 4-O-methyl-D-glucuronic acid when attached glycosidically to other carbohydrate residues is extremely difficult to hydrolyse. Under conditions of hydrolysis sufficiently drastic to hydrolyse all other pyranosidic linkages, two thirds

of the 2-O-[4-O-methyl-D-glucopyranosyluronic acid]- α -D-xylose of a softwood xylan will remain unhydrolysed [38]. This resistance to hydrolysis can probably be extended to the acetal resulting from cleavage between carbons two and three of the uronic acid.

The ability to choose correct hydrolysis conditions, acid strength and duration, without an adequate analytical technique for a Smith polyalcohol[A] is severely hampered. One must contend with glycosidic linkages which are easily hydrolysed as well as stable acetals. No set conditions appear to be employed in preparing Smith degraded residual polysaccharides. An oat β -D-glucan, oxidized and reduced, was hydrolysed with 0.5N hydrochloric acid for 8 hours at room temperature [6]. Since there are no uronic acids in the glucan the acid treatment might seem rather severe. Brome grass hemicellulose on the other hand is known to contain uronic acid. Its Smith polyalcohol was hydrolysed with 0.1N hydrochloric acid at room temperature for 6 hours [6]. Although the isolation of small glycosylalditols was the object of the latter experiment, these conditions might not be expected to completely hydrolyse all acetals generated by the oxidation and reduction. A further example where possibly too much acid was used was in the hydrolysis reported for lemon gum [1]. Experimental data shows that optimum acid concentration is 0.5N rather than 1.0N. As well, optimum duration of acetal hydrolysis with minimum loss of arabinose is 16 hours rather than the reported 48 hours.

A normal acetal resulting from a periodate oxidized and borohydride reduced monosaccharide glycoside can be hydrolysed completely by 0.1N sulfuric

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[A] Smith polyalcohol will imply a periodate oxidized and sodium borohydride reduced polysaccharide.

acid at room temperature in eight hours. These conditions indicate the minimum acid strength required for analysis of Smith polyalcohols. Glycosidic linkages will be preserved. This property will allow isolation of intact glycosylalditols released during hydrolysis while retaining as much as possible of the unoxidized polysaccharide. The degree of polymerization [D.P.] will remain high preserving important structural features for subsequent examination.

Linkages other than the above may not be hydrolysed under the specified conditions. Methyl 4-O-methyl- α -D-glucuronate methyl ester was oxidized with periodate and reduced with borohydride. The resulting polyol, although easier to hydrolyse than the related neutral parent glycoside, required hydrolysis with 0.5N sulfuric acid for 16 hours at room temperature. This is more than sufficient acid strength to hydrolyse a considerable proportion of L-arabinofuranosyl linkages. Some pyranosidic linkages will be broken as well. A problem exists therefore in the structural analysis of some polymers. Mild acid hydrolysis will not cleave all oxidized and reduced uronic acids. Subunits which would be potential sites of periodate oxidation or methylation will still be blocked. Stronger acid on the other hand will cleave glycosidic bonds which have formed a constituent part of the overall structure of the polysaccharides. There are two potential solutions available for this problem. The first involves use of optimum hydrolysis conditions. These conditions involve control of hydrolysis parameters to achieve maximum acetal cleavage with minimal glycosidic hydrolysis. The second solution requires the reduction of uronic acids in the polymer. This reduction eliminates those acetal structures which were difficult to hydrolyse. Thus as much as possible of the intact original structure will be retained by mild hydrolytic conditions.

The analysis of polyols and sugars outlined in the first portion of this thesis provides the first practical method for carrying out detailed analysis of any Smith polymer for optimum hydrolysis conditions. All parameters may be varied; time, temperature, acid strength. Since only small amounts of Smith polysaccharide are required for hydrolysis, in normal practice only 3 milligrams or less, the limit is set by the ability to handle precipitation of the residual polysaccharide. Precipitating the residual polysaccharide as hydrolysis proceeds, produces a fraction containing only high molecular weight products. This fraction may then be dialysed and freeze dried if desired. The resultant residual polysaccharide can be subjected to total hydrolysis, thus avoiding complications which result from glycol aldehyde acetal formation. This technique has been successfully applied to lemon gum. Much milder conditions than those previously used are necessary for maximum yield of residual polysaccharide with a minimum amount of unnecessary degradation.

THE OPTIMUM HYDROLYSIS OF OXIDIZED LEMON GUM

Determination of optimum hydrolysis conditions for periodate oxidized and borohydride reduced lemon gum involves examination of parameters affecting the degree of hydrolysis. Lemon gum polyalcohol was prepared as outlined in the experimental section. Small samples of the polyalcohol were dissolved in water. The normality was adjusted to a series from 0.05N to 1.0N by the addition of concentrated acid. Each sample was allowed to hydrolyse for twenty-four hours at room temperature. The samples were neutralized and

residual polysaccharide was isolated. Analyses were carried out to determine remaining unhydrolysed acetal linkages. The sample hydrolysed with 0.5N sulfuric acid showed almost total hydrolysis of linkages which could be residual acetal links.[1] Figure 7 shows the reduction in glycerol as acid strength was increased from 0.05N to 0.5N.

Removal of all glycerol can be seen to require a 0.5N acid strength. A lower acid strength would have required a prolonged hydrolysis. The acid strength having been chosen it was necessary to determine optimum hydrolysis time. Optimum hydrolysis time will minimize loss of L-arabinose with respect to elimination of glycerol. Lemon gum polyalcohol was dissolved in 0.5N sulfuric acid and samples were taken at intervals to follow removal of components with respect to time. To a certain extent this is a difficult thing to accurately assess. Fragments may be lost during hydrolysis which contain unoxidized carbohydrates. These fragments result from two forms of hydrolysis. Hydrolysis occurring at acetal linkages may liberate Smith fragments, for example, arabinosyl glycerol or galactosyl glycerol. In addition there will be hydrolysis which will break normal glycosidic bonds such as arabinofuranose linked units. The assay of how well acetals have been cleaved will be indicated by residual glycerol since only glycosidic glycerol should be stable.

---

[1] New gas-liquid chromatography columns were prepared in an identical manner to those used initially. Peaks eluted prior to glycerol were obscured by a pyridine tailing peak. The peak resulted from some form of adsorption or reaction between copper and pyridine. It is at this time still not clear what difference exists between the two tubing samples. Results with the second tubing were only slightly affected since the major peak produced by the polyol on hydrolysis and attributable to an acetal was the glycerol peak. It was possible to eliminate the tailing peak by switching to stainless steel columns. Since only ethylene glycol was affected extensive use was still made of copper tubing columns. It has been subsequently discovered that copper tubing may be made passive by silvering the inner side with a mirror silvering solution.

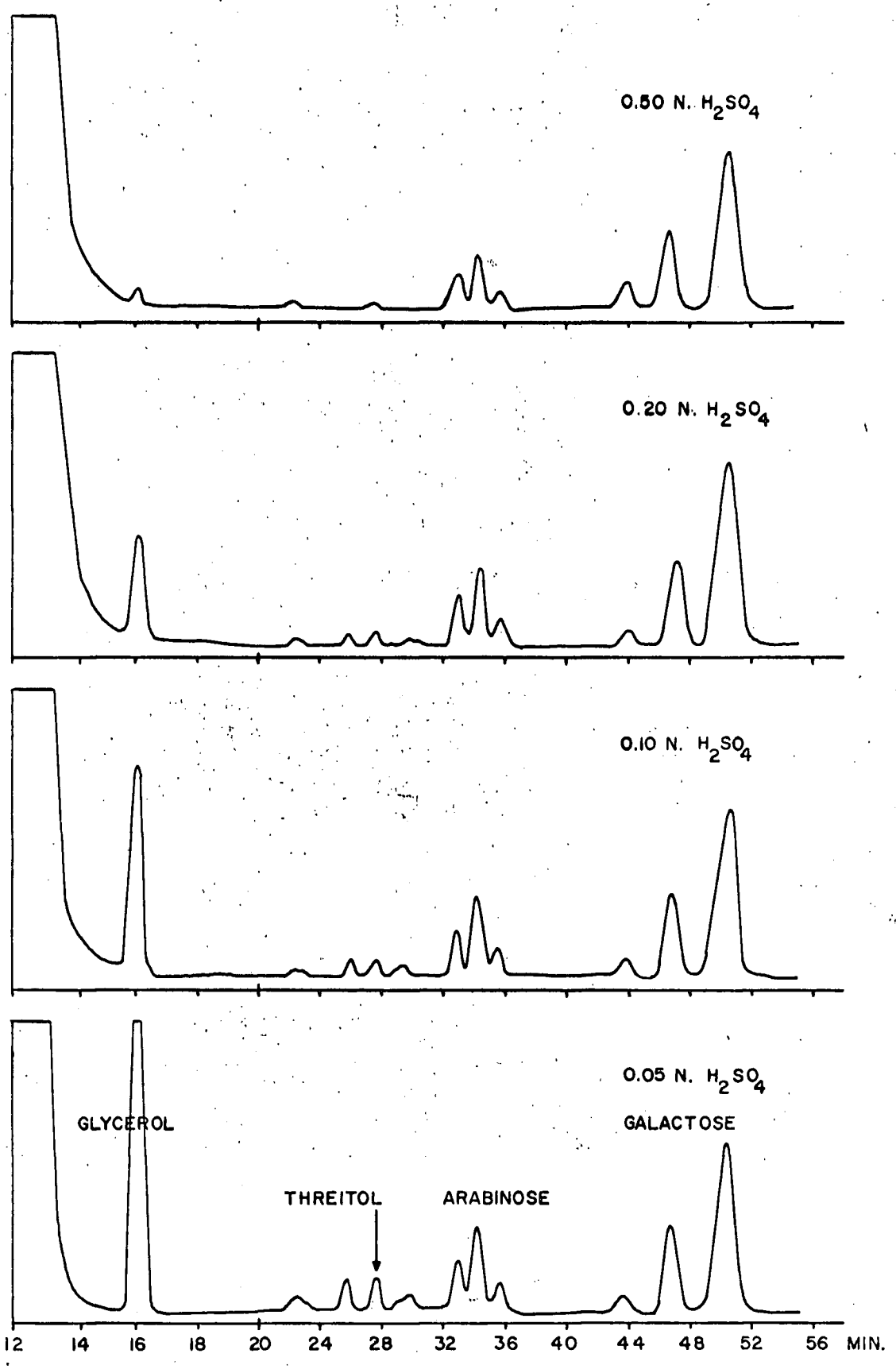


FIGURE 7: GAS CHROMATOGRAMS OF TRIMETHYLSILYL DERIVATIVES FROM LEMON GUM SMITH POLYALCOHOL HYDROLYSED WITH VARIOUS SULFURIC ACID STRENGTHS FOR 24 HOURS AT 20°C

Additional end groups will be formed if large fragments are released by the action of acetal and glycoside hydrolysis. Under these conditions an increase in stable glycerol will occur. There exists however a large difference in rate of hydrolysis between acetal and glycosidicly bound glycerol. By plotting the degree of hydrolysis against time, it is possible to follow the decrease in components as hydrolysis proceeds. Examination of the plot will indicate the optimum hydrolysis time for the polymer. Figure 8 shows gas chromatograms indicating progressive loss of glycerol. Figures 9 and 10 show loss of components plotted with respect to a response for galactose. There was, however, loss of galactose. This fact was clearly seen on hydrolysis of the soluble fraction from the precipitation solvent. The graphs indicate the time for optimum yield of residual polysaccharide with a minimum of contaminating acetal structures.

During the course of these hydrolysis studies it was confirmed that residual glycerol present in the precipitated polysaccharide was chemically bound and not mechanically entrained or hydrogen bonded. This confirmation was determined in the following manner. The polysaccharide obtained after 32 hours of hydrolysis was dissolved in water and dialysed against running water for 24 hours. The polymer isolated by freeze drying was shown to contain the same amount of residual glycerol. Clearly the glycerol was covalently bonded or it would have passed freely from the dialysis tubing.

Analysis of the graphs which plot decrease in components, indicated that removal of all but a residual amount of glycerol required 8 - 16 hours. Hydrolysis for that period formed a polysaccharide containing minimal glycerol. These conditions are considerably milder than those reported by Jones and

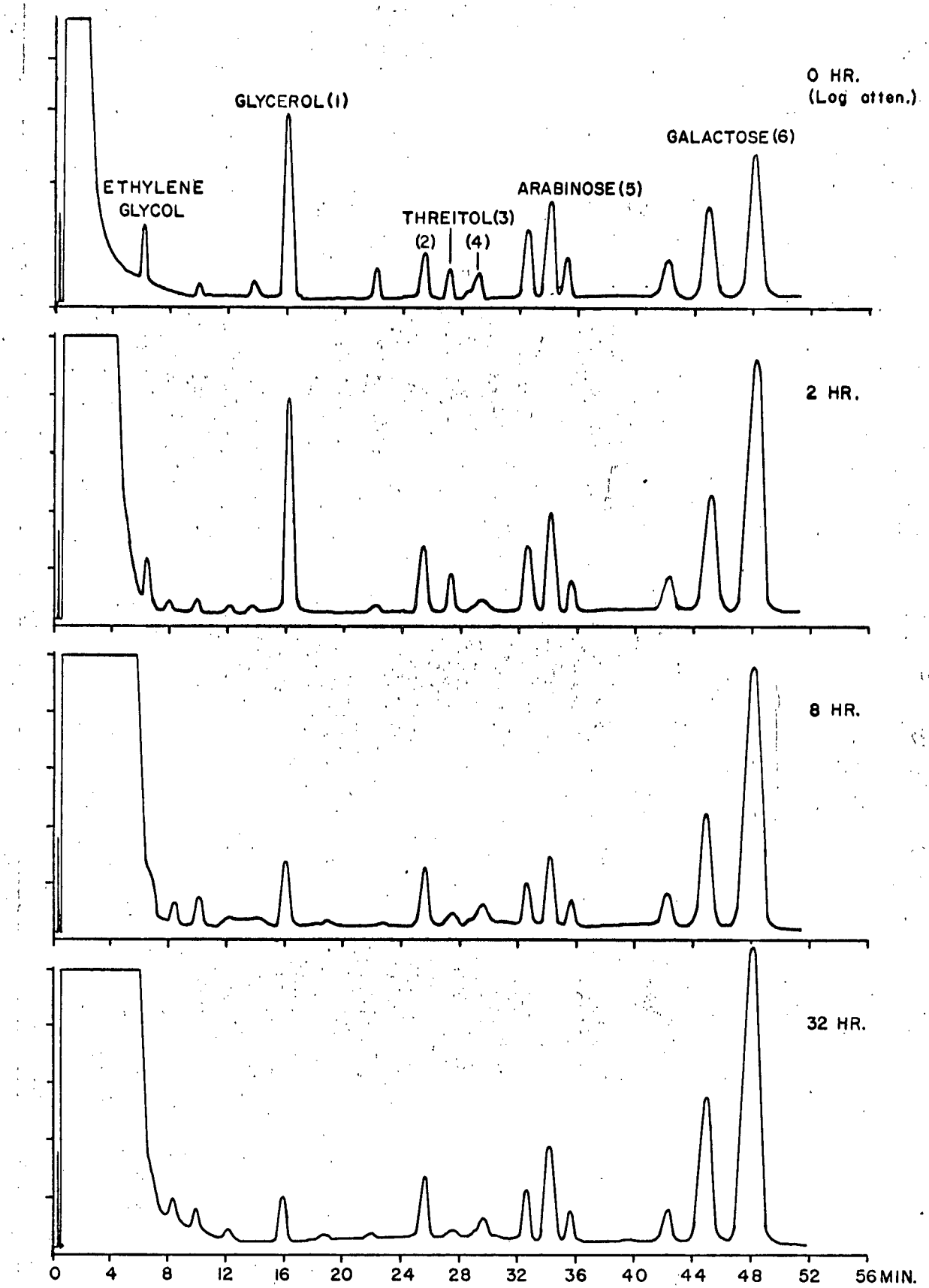


FIGURE 8: GAS CHROMATOGRAMS OF TRIMETHYLSILYL DERIVATIVES FROM LEMON GUM SMITH POLYALCOHOL HYDROLYSED FOR DIFFERENT PERIODS OF TIME WITH 0.5N SULFURIC ACID AT 20°C

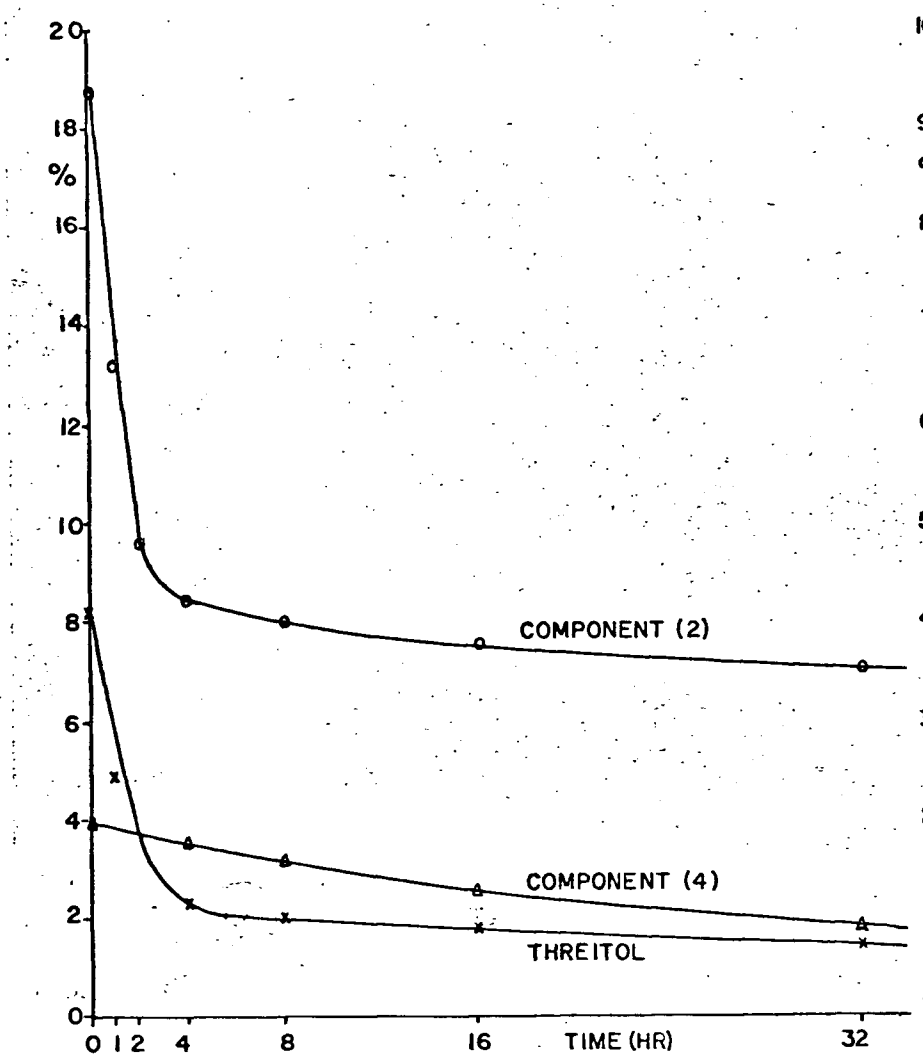


FIGURE 9: RELATIVE G.L.C. PEAK AREAS FOR COMPONENTS 2,3,4 FROM LEMON GUM SMITH POLYALCOHOL

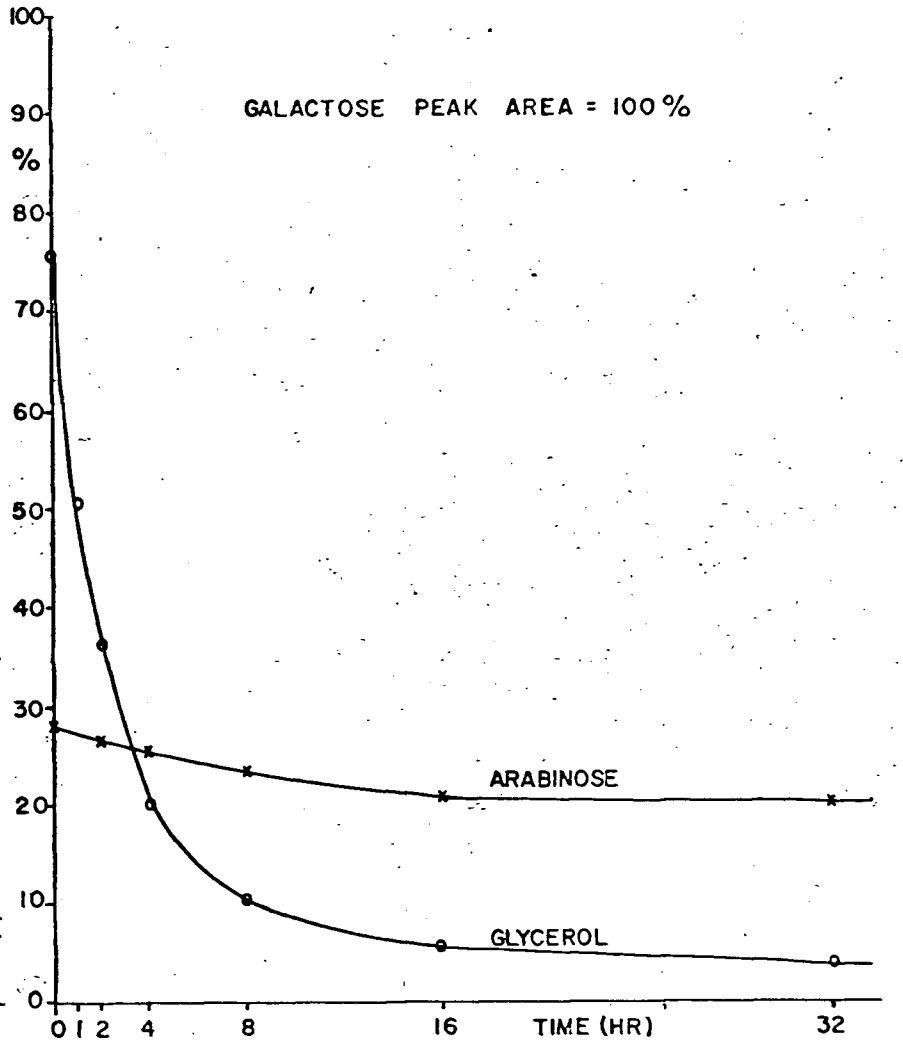
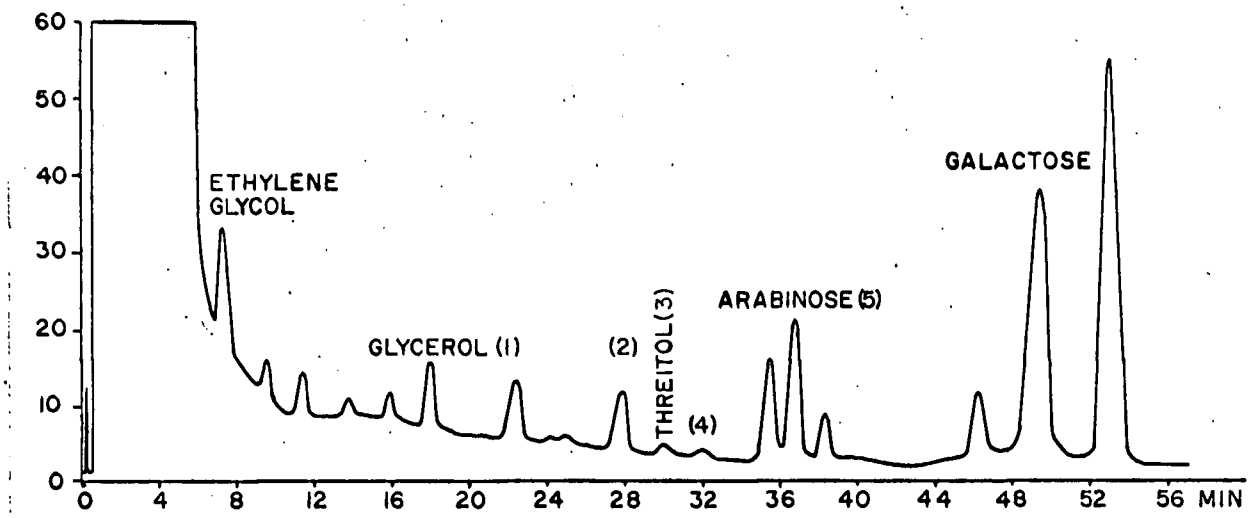


FIGURE 10: RELATIVE G.L.C. PEAK AREAS FOR COMPONENTS 1 [GLYCEROL] AND 5 [ARABINOSE] FROM LEMON GUM SMITH POLYALCOHOL

Stoddart [1]. Their hydrolysis of borohydride reduced - periodate oxidized lemon gum involved treatment at room temperature with one normal sulfuric acid for forty-eight hours.

FIGURE 11

GAS CHROMATOGRAM OF LEMON GUM SMITH POLYALCOHOL HYDROLYSED FOR 32 HOURS WITH 0.5N SULFURIC ACID AT 20°C AFTER DIALYSIS



Evaluation of data presented indicates that even at the milder conditions used in our work degradation of the polysaccharide has occurred. Only trace quantities of galactose were present in the supernatant confirming that the galactan portion of the polysaccharide had been subjected to only mild degradation. If this were not the case one would have expected significantly larger losses of free galactose from the polymer. The galactose which was lost has been in the form of low molecular weight Smith fragments such as galactosyl glycerol. Arabinose was found to a considerable extent in the supernatant. Since arabinose is furanosidicly linked, one would conclude that it had been produced from the polymer by two mechanisms. Some free

arabinose resulted from direct hydrolysis of araban portions of lemon gum. The second portion of arabinose resulted from hydrolysis of Smith fragments since subunits such as arabinofuranosyl glycerol are susceptible to mild acid hydrolysis.

TABLE X

ANALYSIS OF LEMON GUM SMITH POLYALCOHOL AND ITS HYDROLYSIS AND OXIDATION PRODUCTS

	Glycerol	Mole % Arabinose	Galactose
Lemon gum Smith polyalcohol	27.1	14.0	8.8
Lemon gum SI [16 hr. - 0.5N H_2SO_4 - 20°C]	2.8	14.6	82.7
Lemon gum Smith polyalcohol supernatant after 32 hrs. [TOTAL HYDROLYSIS]	21.1	21.8	57.1
Lemon gum Smith polyalcohol supernatant after 32 hrs. [NO FURTHER HYDROLYSIS]	75.3	20.8	4.0
Lemon gum SI polyalcohol	32.8	11.1	49.2
Lemon gum SII [16 hrs. - 0.5N H_2SO_4 - 20°C]	3.2	13.7	83.1

A sample of lemon gum was oxidized, reduced and hydrolysed under optimum conditions to produce lemon gum SI. The composition of the polymer was as indicated above in Table X. Lemon gum SI was oxidized, reduced and hydrolysed in a similar manner to that used in its own preparation. This product was designated lemon gum SII. Analysis of the polyalcohol SII indicated a lower percentage of arabinose than had been observed by Jones and Stoddart [1]. The

retention of some arabinose is to be expected from reported substitution patterns. The large difference observed may be related to increased acid strength during their hydrolysis. More side chains will have been removed from the galactan framework exposing additional sites for oxidation by periodate. With more acetals being formed during the oxidation and reduction the polymer's galactan framework will undergo greater depolymerization.

Additional losses were encountered during both the initial and second oxidation-reduction operations. Each of the two operations resulted in approximately forty percent weight losses. These decreases cannot be attributed to oxidation weight loss due to formic acid or other minor constituents. They must reflect a degree of autohydrolysis and base catalysed depolymerization occurring during the periodate oxidation and borohydride reduction. Polyaldehydes are known to be susceptible to basic hydrolysis and depolymerization [39]. The polyalcohol can be degraded by two acid sources. Acetic acid was added to destroy the excess borohydride while oxidized uronic acids were present in the polymer during reduction and dialysis.

SMITH FRAGMENTS

The optimum yield of residual polysaccharide after Smith oxidation will not necessarily produce an optimum yield of Smith fragments. Degradation of substituent units during hydrolysis is reflected in the appearance of free sugars. For example, any Smith fragment containing arabinofuranosyl linkages will undergo hydrolysis under mildly acidic conditions.

Examination of the alcohol-water soluble fraction from mild acid hydrolysis to lemon gum SI gave results presented in Table X. The alcohol-water soluble fraction obtained on hydrolysis to lemon gum SI contained large quantities of glycerol. This fragment arises from cleavage of arabinofuranose, arabinopyranose [blocked at position four] and galactopyranose [blocked at position two and/or six]. It is known from our own and previous work that galactose in lemon gum is not substituted at C-2 hydroxyl.

The amount of glycerol present in intact lemon gum SI alcohol-water solubles may be divided into two distinct classes. The largest portion of glycerol results from multiple oxidations. For example, when side chains terminated in 4-O[4-O-methyl- α-D-glucopyranosyluronic acid]-L-arabinose are oxidized, both sugars will be cleaved and free glycerol [from arabinose] will be released on mild hydrolysis. The second source of free glycerol on mild hydrolysis arises from glycosidic hydrolysis of labile furanosidic linkages.

Two complications affect the yield of glycerol. Stable uronic acid derived subunits may effect liberation of glycerol while still forming part of the soluble fraction. Secondly, glycolaldehyde formed by periodate cleavage can readily complex with free hydroxyls to form an acetal blocking group.

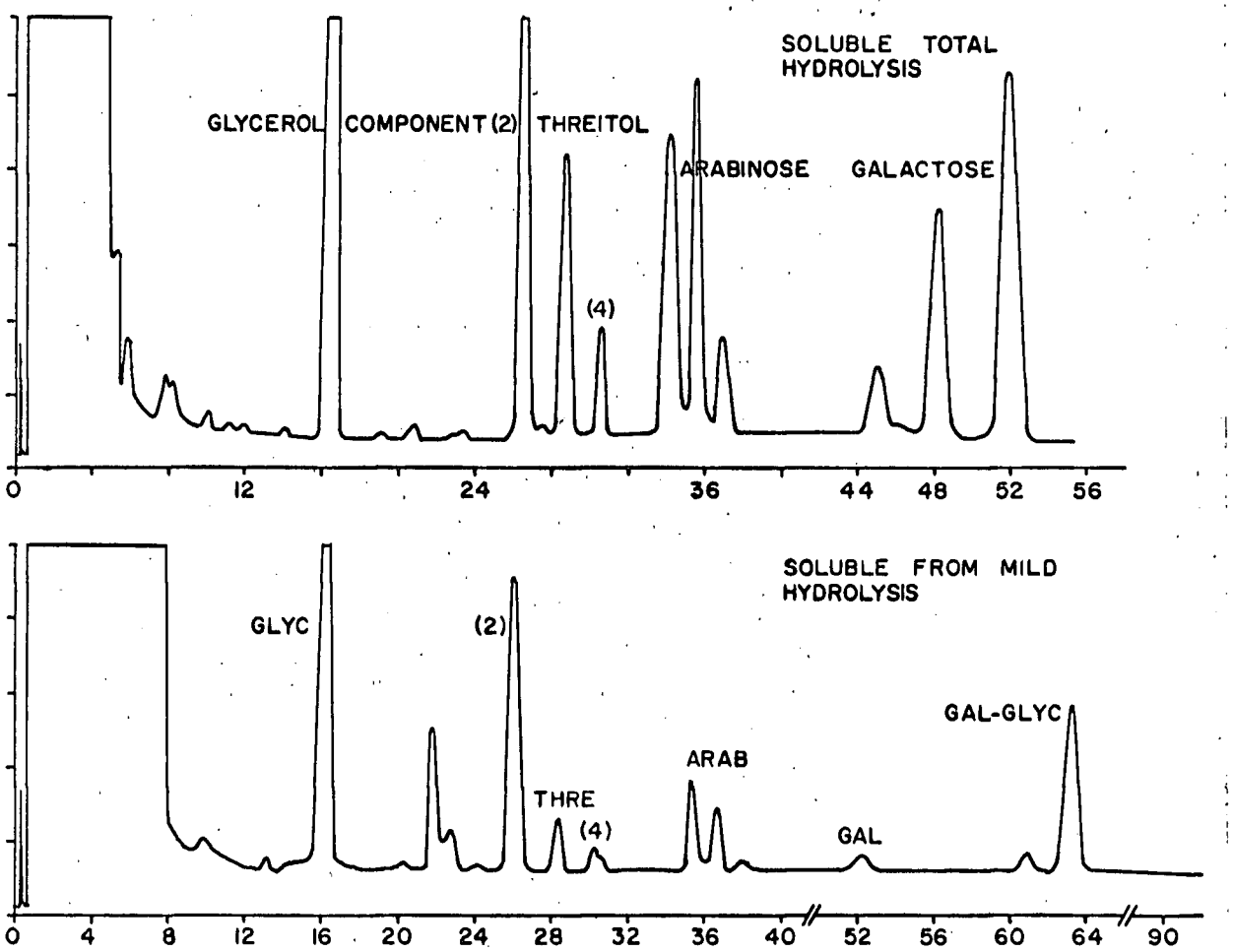
The complexity of this problem was mentioned earlier in discussion of the need for total hydrolysis during analysis of periodate oxidized polymers. Substitution such as this results in chromatographic complexity. These acetals, as new components, have unique retention times and molar responses. A possible but untried solution to this problem may be to hydrolyse in the presence of a large amount of vicinal diol [i.e. butane -2,3-diol]. It is possible that added diol will act as a scavenger for available glycol aldehyde.

Glycerol also exists in the alcohol-water soluble fraction from lemon gum SI in the form of glycosidicly bound glycerol. It is this material which may be regarded as structurally significant. Two conclusions may be drawn from studies of this material. A portion of the soluble fraction was dissolved in acetone-methanol [1:1]. This soluble material contained low molecular weight monosaccharide fragments in a free and glycol aldehyde combined form as well as small amounts of arabinose, galactose and a single glycerol containing Smith fragment. The Smith fragment was a galactopyranosyl glycerol. No other Smith fragments could be positively identified among the other trace components.

The remainder of the original SI soluble fraction [insoluble in acetone-methanol] was shown by GLC to contain no low molecular weight Smith fragments. It contained small amounts of low molecular weight arabinose and galactose oligosaccharides but the bulk of the sample was higher molecular weight oligosaccharides terminated by a glycerol moeity. This was clearly seen when samples of acetone-methanol insolubles were hydrolysed and checked for reducing power.

The presence of high molecular weight galactan oligosaccharides terminated by glycerol provides further proof that the internal galactan framework of lemon gum contains some galactose units substituted at positions six and one only.

FIGURE 12: GAS CHROMATOGRAM OF THE SUPERNATANT FROM THE ISOLATION OF LEMON GUM SI BEFORE AND AFTER TOTAL HYDROLYSIS [at 52 min. the temperature was raised at 3°/min. to a final temperature of 250°C]



REDUCTION OF URONIC ACIDS

As indicated earlier uronic acids were a potential source of error in evaluation of polysaccharide structure by mild acid hydrolysis of the Smith polyalcohol. There exists a solution to the problem of resistance to hydrolysis of uronic acids or acid containing subunits. Reduction of the uronic acids prior to oxidation will result in an acetal which is easily hydrolysed.

Two basic approaches exist for the reduction of an acidic polysaccharide. They involve reduction of either the free uronic acid or an ester of the acid. The most common reduction of a polysaccharide is performed on the fully methylated product. This is readily accomplished with lithium aluminum hydride [LAH] in tetrahydrofuran [THF] or similar dry ether-type solvents. It does not however provide a route to free unsubstituted polysaccharides.

One use that has been made of an ether blocking group is contained in a paper by Rees and Samuel [40]. Alginic acid was esterified with diazomethane, trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane [15] and then treated with LAH in cold THF. The product separated slowly as reduction proceeded. Unfortunately LAH is a poor reagent to use with sugar derivatives containing or forming unblocked hydroxyl groups. LAH forms strong aluminate complexes with these free hydroxyls. It is often necessary to esterify the product to free the substrate from residual reducing agent.

Some workers have used diborane as their reducing agent. It was noted by Smith and Stephen [41] that metal hydrides failed to reduce acylated acidic polysaccharides. Ester groups were attacked first and as a result acidic polysaccharide was precipitated from the reaction mixture. Brown [42] however has shown that diborane reduces carboxyl groups in preference to ester groups. Smith and Stephen [41] first showed that diborane could be an effective reducing agent for acylated acidic polysaccharides. They also noted that the propionate derivative of alginate was more soluble in diglyme and hence reduced more easily.

Other workers have used this procedure to reduce acidic polysaccharides. Hirst et al [43] reduced alginic acid by Smith's method of <u>in situ</u> generation of diborane. This reagent reduced 90% of the uronic acids and produced 5.2%

residual n-propoxy group. The degree of reduction was slightly higher than that which Smith and Stephen found. Ross and Thompson [44] treated an acetylated 4-O-methyl-glucuronoxylan with externally generated diborane, reducing 90% of the carboxyl groups.

Manning and Green [45] compared the reduction of alginic acid by different methods. One of these methods was the use of diborane generated externally on di-O-propionyl alginic acid. A large excess of diborane [6.5 moles per mole of carboxyl group] was used. The same polymer was also reduced by *in situ* generation of diborane from the addition of boron trifluoride to sodium borohydride. Thus any difference between these two reduced polymers could be attributed to the effect of sodium borohydride and boron trifluoride in the polymer solution. This use of externally produced diborane showed for the first time that an ester could be reduced to an ether by diborane alone. They explained this reduction by a modification of the mechanism suggested by Pettit and Kasturi [46] to account for the reduction of esters to ethers with diborane and boron trifluoride. The proposed mechanism explained why more n-propionyl esters were reduced to n-propyl ethers with diborane generated *in situ* than externally. As the sodium borohydride concentration was reduced, boron trifluoride was felt to catalyse the following reaction:

FIGURE 13: THE REDUCTION OF ESTERS TO ETHERS WITH AN EXCESS OF DIBORANE
[adapted from Pettit and Kasturi [46]]

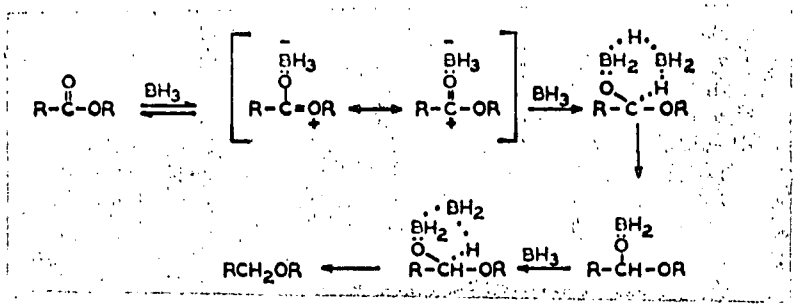


TABLE XII

ANALYSIS OF THE POLYSACCHARIDES OBTAINED FROM ALGINIC ACID BY DIFFERENT REDUCTION PROCEDURES

Reduction Procedure	Polymer	Functional Group					
		Anhydro-uronic acid [%][a]	n-Propionyl[b] ester [%]	Total propoxy-group [%][c]	Alkali-labile n-propoxy-group [%]	Esterified uronic acids[b]	Degree of polymerisation[d] [DP_N]
Methyl di-O-propionyl alginate with $LiBH_4$	B1	6.3	0.00	0.00[e]	0.00	Negative	103
D1 with $LiBH_4$	B2	10.4	0.00	3.9	0.25	Negative	83
Di-O-propionyl alginic acid with B_2H_6	D1	11.0	7.6	3.5	2.3	Negative	111
Di-O-propionyl alginic acid with $NaBH_4 + BF_3$	D2	16.3	3.2	7.5	2.7	Negative	66

[a] Determined by CO_2 evolution. [b] Determined by the hydroxylamine-ferric perchlorate colour test.

[c] Determined by a Zeisel method. [d] Determined by osmometry of the triacetate in 1,1,2-trichloroethane.

[e] Contained 0.40% methoxy-group from diazomethane methylation of di-O-propionyl alginic acid.

Less uronic acid is reduced when diborane is generated *in situ*. Since boron trifluoride catalyses the reduction of n-propionyl esters to ethers, it may also catalyse reductive cleavage of n-propionyl esters. An increased rate of reductive cleavage would have caused the polymer to precipitate sooner thereby lowering the reactivity of the carboxyl group. Sodium ions present in the *in situ* diborane generation may form sodium carboxylate which is not reduced by diborane.

Manning and Green [45] suggested that the differences in D.P. were a result of the strong Lewis acid, boron trifluoride. The degree of depolymerization was dependent on how fast and in what quantity boron trifluoride was added.

The use of diborane for the reduction of acidic polysaccharides prior to periodate oxidation has several serious drawbacks. Even when diborane was generated externally the D.P. of di-*O*-propionyl alginic acid was reduced from 158 to 111, a drop of 30 percent. Although data are not available, a corresponding drop in D.P. might be expected to occur on reduction of other acidic polysaccharides, neutral linkages being more susceptable to acidic hydrolysis. The introduction of ether blocking groups is the most serious drawback to utilization of this method of reduction. In the best diborane case there was 3.5% propoxy ether substitution. This represents in its lowest case an 18% conversion of original n-propionyl ester to n-propyl ether. Alginic acid is a linear 1 $\longrightarrow$ 4 linked polyuronide. Its di-*O*-propionyl derivative will have two out of ten esters reduced to ethers. For every five uronic acid groups oxidizable by periodate in the original polymer only slightly greater than three of the five will be oxidizable after reduction with diborane. In

addition esterification must be employed to produce a soluble polymer and saponification is necessary for removal of the ester groups. Some fractionation may occur on esterification, altering slightly the composition of the polymer. Saponification may produce a small amount of alkaline degradation.

It is important to stress again that diborane suffers severely as a potential reagent for preparing neutral polysaccharide for periodate oxidation. The most serious drawback is the introduction of ether linked n-propyl groups.

The second method employed commonly for conversion of acidic into neutral polysaccharides involves reduction of uronic acid esters with a metal hydride. Two main esters are used, methyl and hydroxyethyl.

Methyl esters are generally formed in one of two ways. A polysaccharide can be esterified by dispersion in methanol containing concentrated sulfuric acid or hydrogen chloride. Bishop and Zitko [47] in an analysis of sunflower pectic acid esterified the galacturonan in methanol containing sufficient sulfuric acid to make the concentration 2N. The mixture was kept at 0-2°C for nineteen days before isolation of the esterified product. This procedure resulted in a significant reduction in D.P. of the polysaccharide. A procedure such as this would be extremely detrimental to a polymer containing labile furanosidic linkages. The second method for forming the methyl ester of an acidic polysaccharide involves the use of diazomethane in an ethereal suspension of the polymer. Direct esterification with diazomethane is not a practical route in preparation of a neutral polysaccharide. Methylation will occur at a number of alcoholic functions in the polymer, interfering with subsequent periodate oxidation of the reduced polysaccharide. Esterification with diazomethane is best carried out on the acetylated or propionylated polysaccharide. The esterified polymers have very limited numbers of free hydroxyl groups capable of methylation.

Hydroxyethylation is an alternate method of esterifying uronic acids. This reaction involves esterification of the uronic acid with ethylene oxide which reacts directly with the polysaccharide acid in aqueous solution. There is reported to be no evidence for hydroxyalkylation of alcohol groups by ethylene oxide. An investigation on a model compound would however be a useful undertaking since isolation of polyethylene glycol esters has been reported in the literature [48].

METAL HYDRIDE REDUCING AGENTS

Sodium borohydride has been reported to give good reductions of ethylene oxide esters [49]. Attempts to utilize this reagent have generally been unsuccessful in this laboratory. Sodium borohydride saponifies the esters and the acids and salts produced are not readily reducible.

Potassium borohydride has also been used to reduce esterified uronic acids [50]. Bishop and Zitko [47] in their paper on the reduction of galacturonan form sunflower pectic acid analysed in detail the effect of solvent on potassium borohydride efficiency. They examined water, 80% aqueous methanol, and 80% aqueous dimethyl sulfoxide. Their results indicated that reductions in 80% aqueous methanol were most efficient. De-esterification was suppressed, a reasonably high proportion of galacturonic acid was reduced and recoveries were good. In water, a high proportion of galacturonic acid was reduced but there was considerable de-esterification and recoveries were low. The low recovery was felt to result from degradation of esterified galacturonan in alkaline media [51]. In 80% aqueous dimethyl sulfoxide [DMSO], de-esterification was greatly suppressed. The greatest reduction was obtained

when a partially reduced product was reacted. These advantages however were offset by low recovery. Indeed it was reported that when this solvent was used for the first reduction of a fully esterified galacturonan, complete degradation occurred. No product precipitable by methanol could be obtained. Reductions were found to be only slightly more efficient with methyl than hydroxyethyl esters.

Bishop and Zitko [47] examined the efficiency of esterification. As galacturonan was reduced heterogeneous esterification with diazomethane became less efficient. At a galacturonic acid content of 11%, no more than 45% of the carboxyl groups could be esterified. The substrate was even freeze dried to provide a large surface area. Esterification with ethylene or propylene oxide gave good results but the products when precipitated by organic solvents, tended to form gels which were difficult to handle. It was possible however to esterify residual carboxyl groups in highly reduced galacturonans with diazomethane in dimethyl sulfoxide. In this procedure the esterified product did not need to be isolated before reduction since the same solvent could be used. However, the main drawback to using the method proposed by Bishop and Zitko was the accumulated decrease in degree of polymerization [from 270 to 21].

Lithium borohydride has also been used to reduce acidic polysaccharides. This reagent was first used by Rees and Samuel [40] for the reduction of alginic acid. A heterogeneous reaction in boiling THF was carried out on both 2-acetoxy-ethyl di-O-acetylalginate and methyl-di-O-acetylalginate. Special precautions were taken to ensure that the polysaccharides were completely accessible. The authors report that reduction yielded a polysaccharide containing 4.7% anhydro uronic acid.

Manning and Green [45] in their examination of the reduction of alginic acid, reduced methyl di-O-propionyl alginate by the method of Rees and Samuel. Methyl di-O-propionyl alginate [89% of its uronic acids esterified] was however soluble in tetrahydrofuran. All n-propionyl and methyl esters were reductively cleaved in the reduced polysaccharide. All reducible terminal sugar units were converted to glycitols. In addition the authors reported a 43% reduction of uronic acid carboxyl group to primary alcohols.

Rees and Samuel suggested that reduction of functional groups on polysaccharides under heterogeneous conditions is incomplete because molecules of the solid phase are inaccesible. Manning and Green [45] however showed that 94% of the hemiacetal end group were reduced under heterogeneous conditions. It was felt that lowered reactivity was not due to lack of accessibility. Their alternate explanation is based on activation energies. Addition of solvation energy for heterogeneous reaction to the activation energy still makes the total energy for reaction of aldehydes lower than that for carboxylic acids. Hence aldehydes react more rapidly than carboxylic acids under heterogeneous conditions. It should be noted that there may be vast differences in accessibility. The reducing end group of a linear molecule such as alginate should, if well dispersed, be accessible to reduction. Uronic acids on the other hand can be strongly associated with hydroxyl functions of other polymer chains. Areas of polysaccharide may thus be rendered inaccessible.

Aspinall and McKenna [52] esterified Araucaria bidwilli gum with ethylene oxide. Acetylation and subsequent reduction by the method of Rees and Samuel gave a product polysaccharide with less than 1% uronic acid.

EVALUATION OF NEW ROUTE TO REDUCED POLYSACCHARIDES

The method of Rees and Samuel [40] for reducing acidic polysaccharides is obviously effective. It does however involve several reactions; polysaccharide esterification, uronic acid esterification, reduction and saponification. Each reaction may have to be repeated and possible fractionation or degradation of the polysaccharide can occur. Removal of any of these steps might increase the chance of obtaining a neutral polysaccharide which retains potential periodate oxidation sites and is closely related to the original polymer.

To prevent unnecessary degradation, esterification should not be carried out in methanol with an acid catalyst because of D.P. reduction. Diazomethane is unsuitable on the unsubstituted polymer due to ether formation. Ethylene oxide has shown none of these detrimental effects. It has certain advantages; water soluble, volatile, if hydrolysed to ethylene glycol, it may be readily removed by dialysis, and it also inhibits any bacterial degradation. Its two disadvantages are long reaction time for esterification as mentioned previously and possible ether formation. The latter point requires further evaluation, however no etherification has thusfar been reported.

Certain reducing agents are unacceptable in the reduction of acidic polysaccharides. Diborane to be effective requires a homogeneous reaction. But, soluble ester derivatives are reduced in part to ethers which block subsequent periodate oxidation. Silyl blocking groups which also make a soluble derivative, hydrolyse as the reaction proceeds producing a heterogeneous reaction. Lithium aluminum hydride complexes free hydroxyls, the result being a heterogeneous reaction. The complex is difficult to break down, introducing

problems in isolating the reduced polysaccharide. Thus the choice of reducing agents is limited to the borohydrides. Their ability to reduce esters and acids is increased with increasing covalent character. Lithium borohydride best fits this requirement [53].

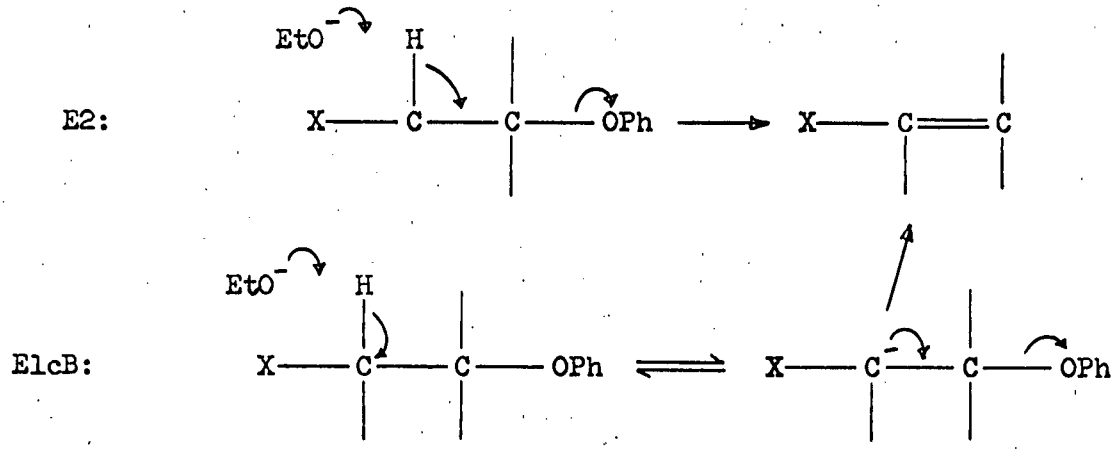
In the work conducted in this laboratory, choice of solvent was a departure from those previously employed. It was shown by Bishop and Zitko [47] that 80% aqueous DMSO greatly suppressed de-esterification and a high proportion of reduction occurred. Since only the hydrolysis of reduction complex involves water in a borohydride reduction it was decided to use pure DMSO. Addition of water after complex formation will complete the reaction. A large number of polysaccharides are soluble in this reagent and tests showed only a slow reaction with lithium borohydride. The only disadvantage in the use of DMSO was reported decomposition of polysaccharide [47]. An explanation as to why depolymerization occurs was not indicated, however there are two possibilities. Alkaline cleavage by β -alkoxycarbonyl elimination could lead to depolymerization. DMSO is effective in preserving methyl ester necessary for this elimination. However, it should not greatly increase the ability to co-ordinate transition states or leaving groups in comparison to water or methanol.

When methyl 4-O-methyl α -D-glucuronic acid methyl ester was reduced with lithium borohydride in DMSO there was gas chromatographic evidence for the presence of small amounts of by-product. The by-product was tentatively assigned the structure of the 4,5-dehydro-D-glucuronate [54]. Confirmation of this structure will require further analysis. The proposed mechanisms for the elimination in uronic acids are outlined in Figure 14.

FIGURE 14

THE β-ELIMINATION MECHANISMS FOR URONIC ACIDS

[It has been recently suggested that the eliminations proceed via an ElcB mechanism [55]]



The second possible explanation is that DMSO altered the precipitation characteristics of pectin. Work in our laboratory on isolation of methylated polysaccharides from DMSO solutions indicated changes in ease of precipitation. Methylated polysaccharides in DMSO occasionally failed to precipitate on addition to water. Methylation mixtures poured directly into dialysis tubing with no addition of water to the DMSO resulted in cases of almost complete loss of partially methylated polysaccharides. Dilution of methylation mixture with water, followed by dialysis to remove DMSO resulted in easily isolated methylated polysaccharide in excellent yield.

Lemon gum was utilized as a model substance to evaluate reduction of esterified polysaccharides dissolved in DMSO. Lithium borohydride was the reducing agent employed. Polysaccharide reduction was monitored by G.L.C.

Inclusion of erythritol during polysaccharide hydrolysis gave quantitative results on retrieval of constituent monomers. The results indicated additional difficulties in obtaining a neutral polysaccharide by reduction. Esterification with ethylene oxide requires reaction for a period of ten days [47]. However, loss of arabinose occurred during the esterification of lemon gum.

TABLE XIII

CARBOHYDRATE ANALYSIS OF LEMON GUM AND ITS REDUCED PRODUCT

	Arabinose %	Rhamnose %	4-O-Methyl Glucose %	Galactose %	Glucose %	Neutral Sugar Recovery %
Lemon Gum	32.7	3.4	--	63.9	--	66.6
Lemon Gum OCH_2CH_2OH	29.9	3.3	--	66.8	--	--
Lemon Gum OCH_2CH_2OH 1 x $LiBH_4$	25.0	1.5	15.9	51.3	5.3	84.0
Lemon Gum 2 x OCH_2CH_2OH 2 x $LiBH_4$	26.25	1.7	18.8	48.6	4.8	89.5

Calculations based on aldobiouronic acid survival during hydrolysis indicated quantitative levels of reduction. These calculations were based on survival of aldobiouronic acids during hydrolysis as well as degradation of individual sugars. The results accounted for all sugars present prior to hydrolysis and correlated well with recovery of neutral sugar in hydrolysate.

The loss of some arabinose during esterification was not unexpected. The polysaccharide was deionized hence all uronic acids were free to cause hydrolysis. Although the large amount of ethylene oxide present provided a

substrate for reaction of ionized acids, there was still water present and cleavage of labile arabinose linkages could occur. As the reaction proceeded the pH decreased to 7 and hydrolytic action was reduced. Hydrolysis could be suppressed by substitution of DMSO for water during esterification. It was necessary however to isolate the polymer before reduction as residual ethylene oxide reacted with lithium borohydride.

Lithium borohydride was added as a DMSO solution to lemon gum 2-hydroxyethyl ester in DMSO. Hydrogen was released during the initial vigorous reaction through a mercury gas trap to exclude moisture. By the 12th hour of reduction a thick gel had formed. This gel could be partially broken down by stirring or gentle shaking. A slow evolution of hydrogen continued for several days. The solution was then treated with small portions of dilute acetic acid to destroy excess lithium borohydride. The solution was diluted with water and dialysed. Saponification occurred during isolation making re-esterification necessary for continued reduction.

The initial ethylene oxide esterification did not fully block all uronic acids. The lack of complete reduction with an excess of lithium borohydride suggested either a problem of accessibility or hydrolysis of esters during reduction. Similar results in subsequent reductions could be attributed to effects of either of these factors. Some losses occurred as a result of work-up and dialysis. However yields were generally good.

The mild nature of the reaction was evident from the yields of L-arabinose in relation to D-galactose on subsequent reduction. Yields of arabinose increased in relation to galactose as the polymer was further reduced. In addition, losses which one might expect in arabinose should have been accompanied by

losses in 4-O-methyl-D-glucuronic acid, since this acid is the major acid lost on mild hydrolysis [1]. Acid hydrolysis of lemon gum resulted in reduction of 4-O-methyl compared to unsubstituted glucuronic acid. On reducing lemon gum ester with lithium borohydride in DMSO, the percentage of 4-O-methyl-D-glucose increased as reduction proceeded. Had any oligosaccharides been formed during reduction, they would have been lost during dialysis. This would have resulted in lower 4-O-methyl-D-glucose levels as was the case on mild acid hydrolysis.

The reduction method as evaluated on lemon gum was successful in reducing 75% of available uronic acids. It seems probable that this method would be more successful on smaller polymers. Central portions of a large molecule like lemon gum may be inaccessible to a reagent such as lithium borohydride. Borohydrides can complex readily with free hydroxyls, blocking access to the interior of this highly branched polymer.

Reduction with lithium borohydride in DMSO appears to be very useful for convenient detection of the presence of different uronic acids such as glucuronic and 4-O-methyl glucuronic. If the structures of the major aldobiouronic acids are known, then accurate carbohydrate analyses are also conveniently obtained. The method involves a minimum number of reaction steps and hence would appear to be a very convenient procedure for initial examination of polymer uronic acids. If higher degrees of reduction occur in lower molecular weight or linear polysaccharides, increased use of this reduction sequence could be envisioned.

SIGNAL ENHANCEMENT USING TRIMETHYLSILYL [TMS] DERIVATIVES AND UTILIZATION OF TMS AS A PROBE FOR DETERMINATION OF NUMBER OF HYDROXYLS IN A COMPOUND

During gas-liquid chromatographic examination of products from total hydrolysis of periodate oxidized carbohydrates, samples of per silylated polyol were examined by proton magnetic resonance [p.m.r.]. Spectra for carbohydrate protons were very complex because of similar electronegativity and environment [see Figure 15]. It was noted however that silyl methyl proton resonances were very sharp and in most cases, unless silyl ethers were in identical positions, resolved from one another.

This discovery indicated a possible technique for characterization of polyhydroxy compounds. Introduction of TMS ethers increases the number of hydrogens on each hydroxyl by a factor of nine. Consequently detection of the number of hydroxyls in a compound is possible even though the remaining proton signals are lost in background noise. In addition, compounds which are of unknown structure may be given some partial assignment of structure. It may be possible to determine number of hydroxyls, their positions and degree of substitution at the parent carbon atom. Any compound containing a silyl ether would be amenable to this analytical procedure.

In examinations of carbohydrate molecules, knowing the number of hydroxyls can be a useful addition to information about that compound. The analysis of compounds on G.L.C. can result in a confused picture because of wide variation in retention times for partially methylated sugars and polyols. Although it is possible to bypass many difficulties with mass spectrometric analysis, it may be difficult to distinguish between components by their mass spectra alone.

For example, two methyl sugars may have similar G.L.C. retention times. The mixed mass spectra might be very difficult to interpret. The silyl p.m.r. spectra however may show two anomers and indicate something about their substitution.

FIGURE 15: 100 MH_z P.M.R. SPECTRUM FOR THE ERYRTHRITOL PROTONS IN TRIMETHYLSILYLATED ERYTHRITOL

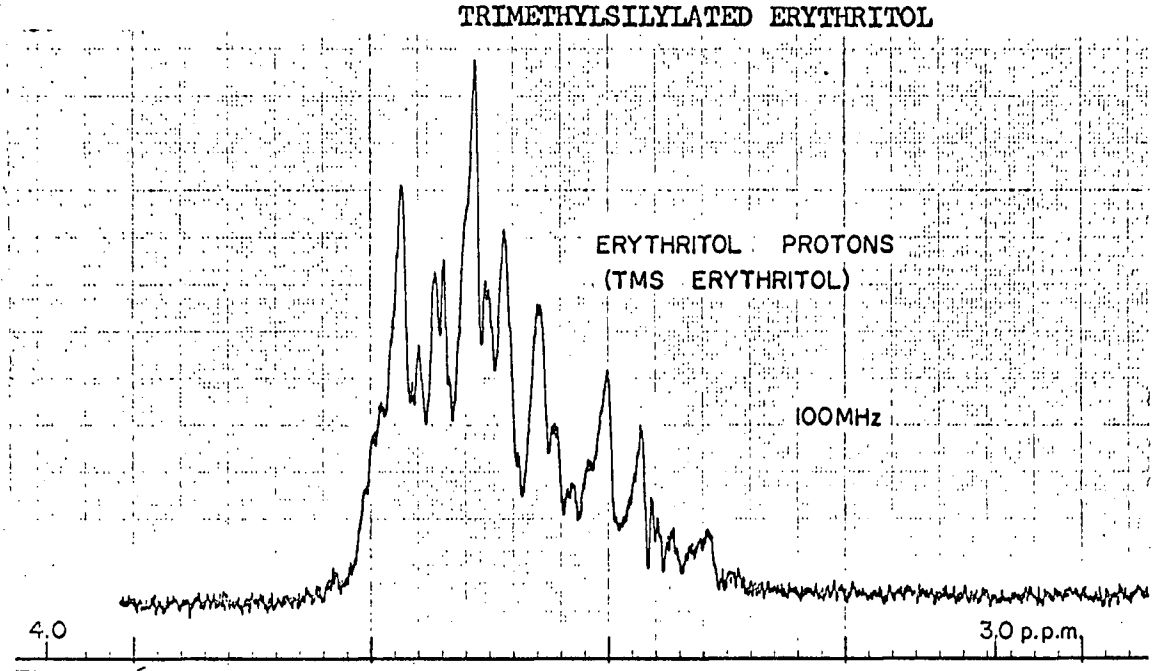


Figure 16 is a list of compounds which have been subjected to silyl ether analysis indicating the number of peaks and their degree of separation.

Primary trimethyl silyl ether protons resonate at higher field than secondary trimethyl silyl ether protons. Barring shielding effects, the resonance signal for a trimethyl silyl group will reflect electronegativity of the hydroxyl to which it is attached. The wide diversity of signal value can be seen in almost complete separation of signal for eight different silyl ethers present in an unresolved collection of two arabinose TMS ether peaks. This p.m.r. technique provides a non-destructive check on component purity as eluted from the gas chromatograph.

FIGURE 16: COMPOUNDS SUBJECTED TO P.M.R. [100 MH_z] ANALYSIS OF TRIMETHYLSILYL PROTONS

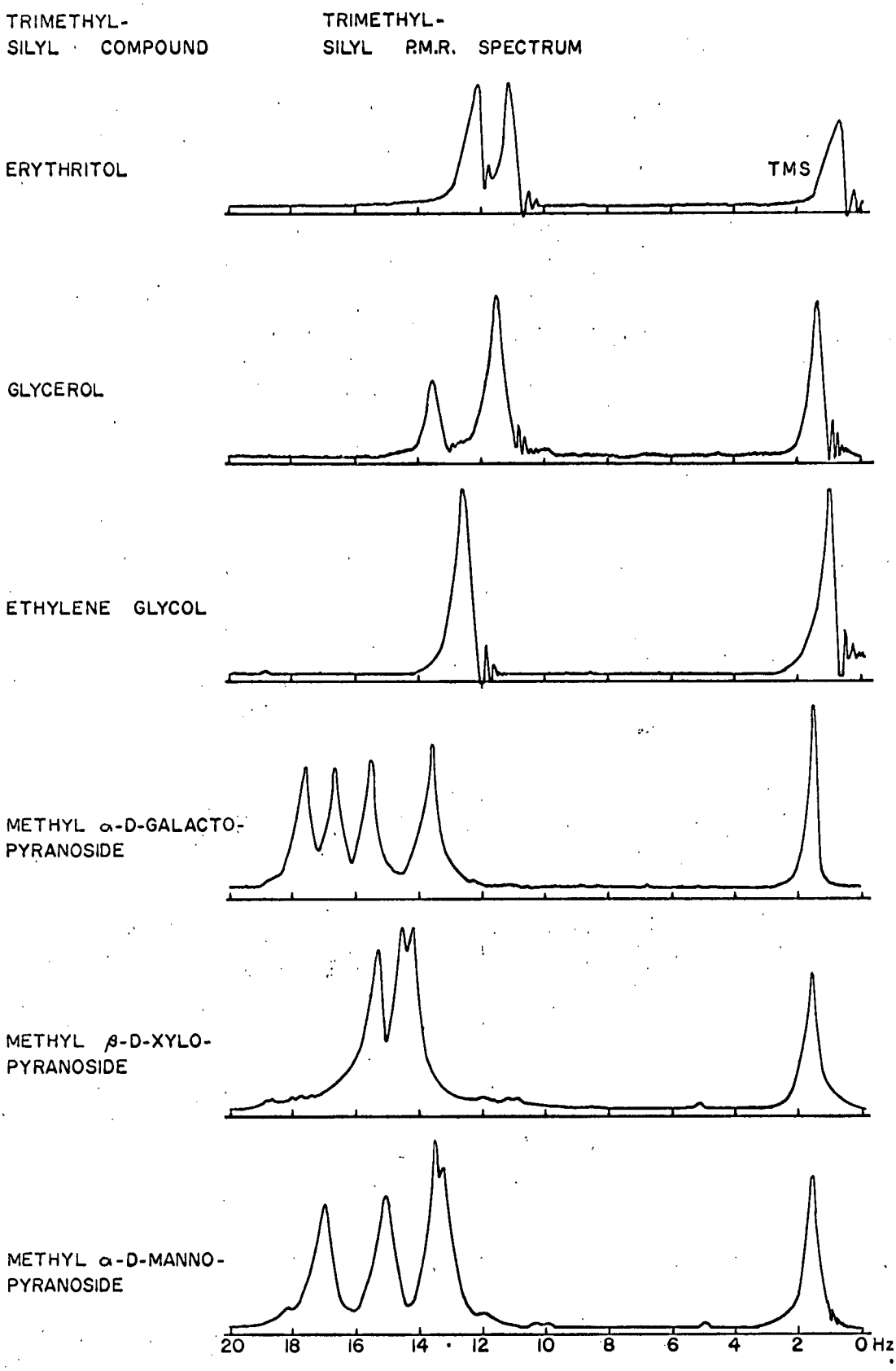
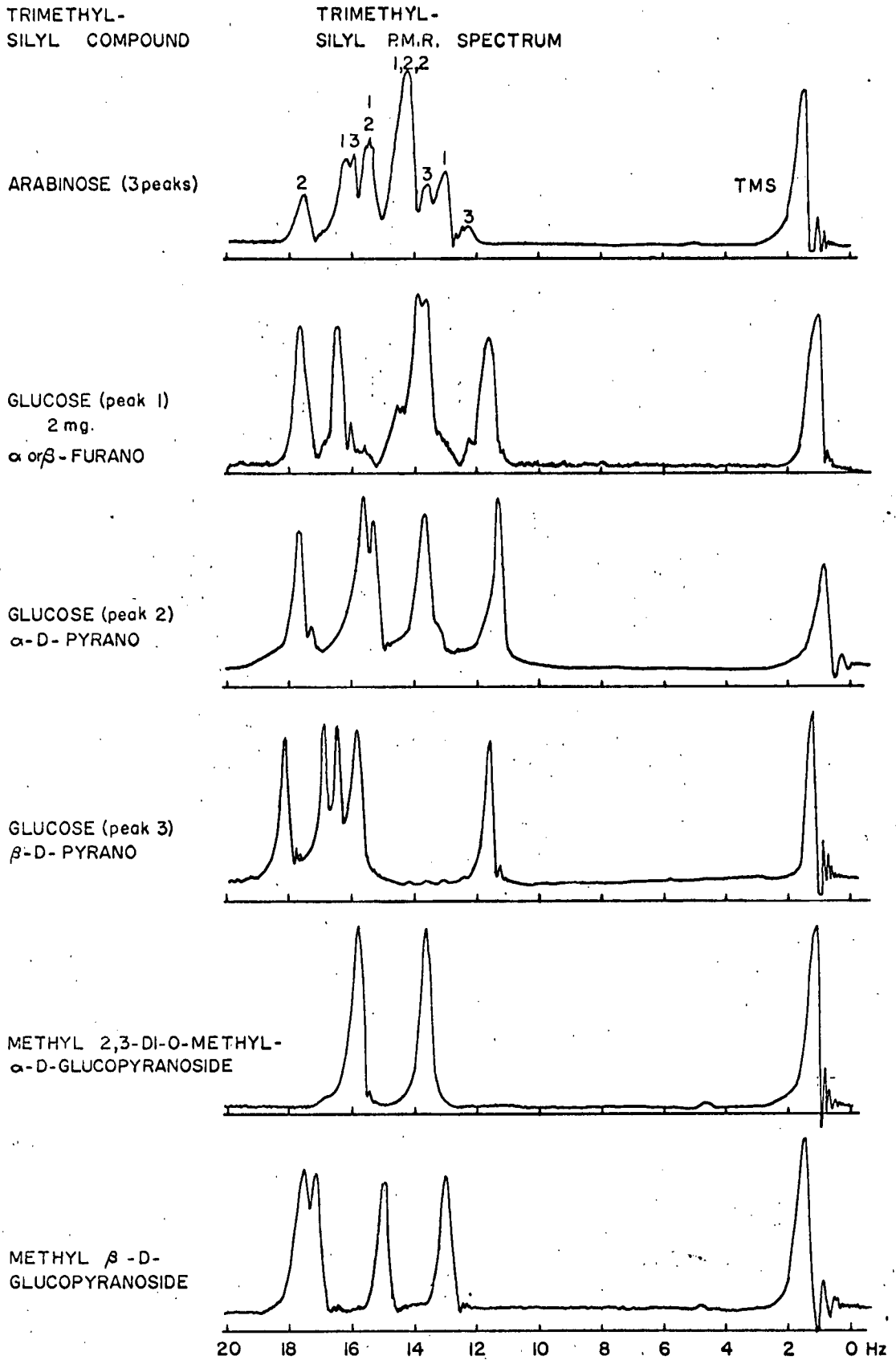


FIGURE 16: COMPOUNDS SUBJECTED TO P.M.R. [100 MH_z] ANALYSIS OF TRIMETHYLSILYL PROTONS [Continued]



Obviously there will be situations where overlap occurs between trimethyl silyl ether proton resonances. Perfect overlap will occur in cases such as erythritol where there are two identical primary and two identical secondary hydroxyl positions. This results in only two peaks of equal intensity. Normally some hint of overlap will be visible. For example, two primary peaks of glycerol overlap but they have, as a result, twice the signal intensity of the secondary TMS ether. In many cases where two compounds produce peaks that overlap, such overlapping will not be absolute making it obvious that there are two contributing components. In cases where some signals are well resolved, integration of component peak area will indicate percentage composition.

This technique for examination of silyl methyl proton resonances provides a useful tool in structural elucidation and detection of component mixtures eluted as a single gas chromatographic peak.

SIGNAL ENHANCEMENT

An additional benefit can arise from examination of p.m.r. spectra of TMS ethers. A nine-fold increase in number of protons occurs on silylation of each hydroxyl position. In effect, TMS ethers act as built-in signal accumulators. The resonance peak for TMS ether protons is very sharp. The signal undergoes only weak proton-proton interactions because of the oxygen bridge between carbon and silicon. Normally, proton resonances are interacting, forming multiplets of lower intensity. TMS resonances are unaffected by hydrogen bonding which can broaden hydroxyl proton spectra of alcohols making

them even more difficult to observe than normal proton resonances. Jackman [56] discusses various factors exerting influence on the appearance of ethanol's hydroxyl proton. It can be broadened, split into a multiplet or increased in signal size by the presence of water.

TMS protons give a sharp signal on the low side of tetra methyl silane. The signal is nearly ten times as large as the hydroxyl proton signal would have been had it been present. This signal enhancing effect allows for examination of very small samples of TMS ethers. The number and structure of hydroxyls may be determined.

A small peak occasionally occurs in glucose TMS ether gas chromatograms. It represents only about one percent of total peak area. Although it was thought to be a furanoside anomer it could not be ruled out that it was due to under silylation or an anhydro form of glucose. Gas chromatography of a large amount of TMS glucose resulted in collection of only two milligrams of this compound. Examination of the p.m.r. spectra of TMS protons indicated a compound containing five hydroxyls. This ruled out under silylation or anhydro sugars as possible causes of this component peak. The G.L.C. retention time of this component agrees with no other common sugar. These points strongly indicate the minor component to be α or β TMS glucofuranoside. This information regarding a minor component was obtained on two milligrams of sample. P.m.r. spectra for the remaining protons of the glucose molecule were completely obscured by spectrometer noise.

It will be possible using TMS proton spectra to obtain number and nature of hydroxyls on very small samples isolated by gas chromatography. Purity of single peaks may also be evaluated.

TRIMETHYLSILYLATION OF GALACTURONIC ACID

During studies of lemon gum a method for estimation of aldobiouronic acids was investigated. Work has been done on estimation of fully methylated aldobiouronic acids and on silylated methyl esters of aldobiouronic acids [57]. It was hoped that a procedure could be developed for separation of fully silylated aldobiouronic acids. A reagent was selected which would react with alcohol and acid substituents to form corresponding TMS ethers and esters. The reagent chosen was bis-trimethylsilyl-acetamide [BTMSA] which was known to react with amino acids to form silyl esters. Galacturonic acid was chosen as a model compound containing the necessary alcoholic and acidic functions.

Silylations were carried out with BTMSA in various solvents; pyridine, dimethyl formamide, dimethyl sulfoxide, hexane and dioxane. Subsequent gas chromatography [SF-96 liquid phase] indicated four components. Two components were well resolved, two overlapped to form a central pair of peaks. Since under silylation is known from our own and other work [58] to produce extra peaks, a check was carried out on the eluted peaks. Possible lactone formation had to be ruled out as well, since lactones are known to have shorter retention times than parent acids.

Other liquid phases were evaluated for gas chromatography of the silylated galacturonic acids. None proved more efficient than standard SF-96 liquid phase used in earlier studies. Preparative gas chromatography was convenient with this liquid phase because of a high maximum operating temperature. Small samples of the four galacturonic acid peaks were isolated and analysed by p.m.r. spectrometry. This preliminary examination indicated that each component contained five trimethyl silyl groups. This fact eliminated lactones or under silylation as causes for any of the four components isolated.

For complete p.m.r. spectral analysis large scale sample separations were carried out. Analysis of the p.m.r. spectrum for ring protons should confirm furano and pyranoside forms for the four peaks. The well separated first and last peak were isolated in a quantity large enough to confirm their structures by p.m.r. spectroscopy. Large scale separation of central peak components was extremely difficult. In addition degradation was observed due to prolonged sample collection.

An experiment was carried out on an easily isolated peak to determine the exact cause of degradation. A sample of peak four of TMS galacturonic acid was treated with a small amount of methanol. The TMS proton region of the p.m.r. spectra was monitored prior to and after addition of methanol. The spectra revealed a rapid loss of TMS ester. It is apparent that although precautions were being taken to keep the samples dry, loss of TMS ester was taking place. Samples of peak one of TMS galacturonic acid were dissolved in dry chloroform. Spectra run at intervals over twenty-four hours underwent changes. The changes in peak height were indicative of changes in substitution of the galacturonic acid isomer isolated. These latter changes were relatively small in comparison to the complete loss of silyl ester observed when methanol was added. Losses, however, were large enough to confuse interpretation of spectra in an unknown compound.

It was possible to completely assign structures to components one and four of TMS galacturonic acid by interpretation of their p.m.r. spectra. Component one was an α-furanoside and component four used the β-pyranoside of galacturonic acid. Component two was isolated in sufficient quantity to indicate that all couplings between protons were very small. This finding is consistent with a β-furanoside structure where all ring protons can have $\sim 90°$

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dihedral angles. Sample purity, however, was insufficient, for assignment of ring protons. Low yield and peak overlap made impossible the large scale isolation of pure component three. The anomeric proton of this third fraction had a coupling constant of 4.3 Hz. The TMS proton spectra indicated a mixture of at least two components. The coupling constant 4.3 Hz was consistent with α-galactopyranosyluronic acid [59].

One furanoside and one pyranoside form of galacturonic acid had been isolated and characterized. Since it is known that anomeric configuration has little bearing on mass spectral fragmentation, confirmation of the structure of the two central peak components was determined by mass spectrometry. A problem known to exist in interpretation of mass spectra of silyl ethers is the tendency of samples on ionization to undergo ring contraction and expansion. As a result, only small intensity differences exist in the m/e 191 and 204 mass peaks. These are sufficient, however, to confirm that the initial two compounds isolated were TMS galactofuranosyluronic acid esters and the latter two peaks pyranoside forms.

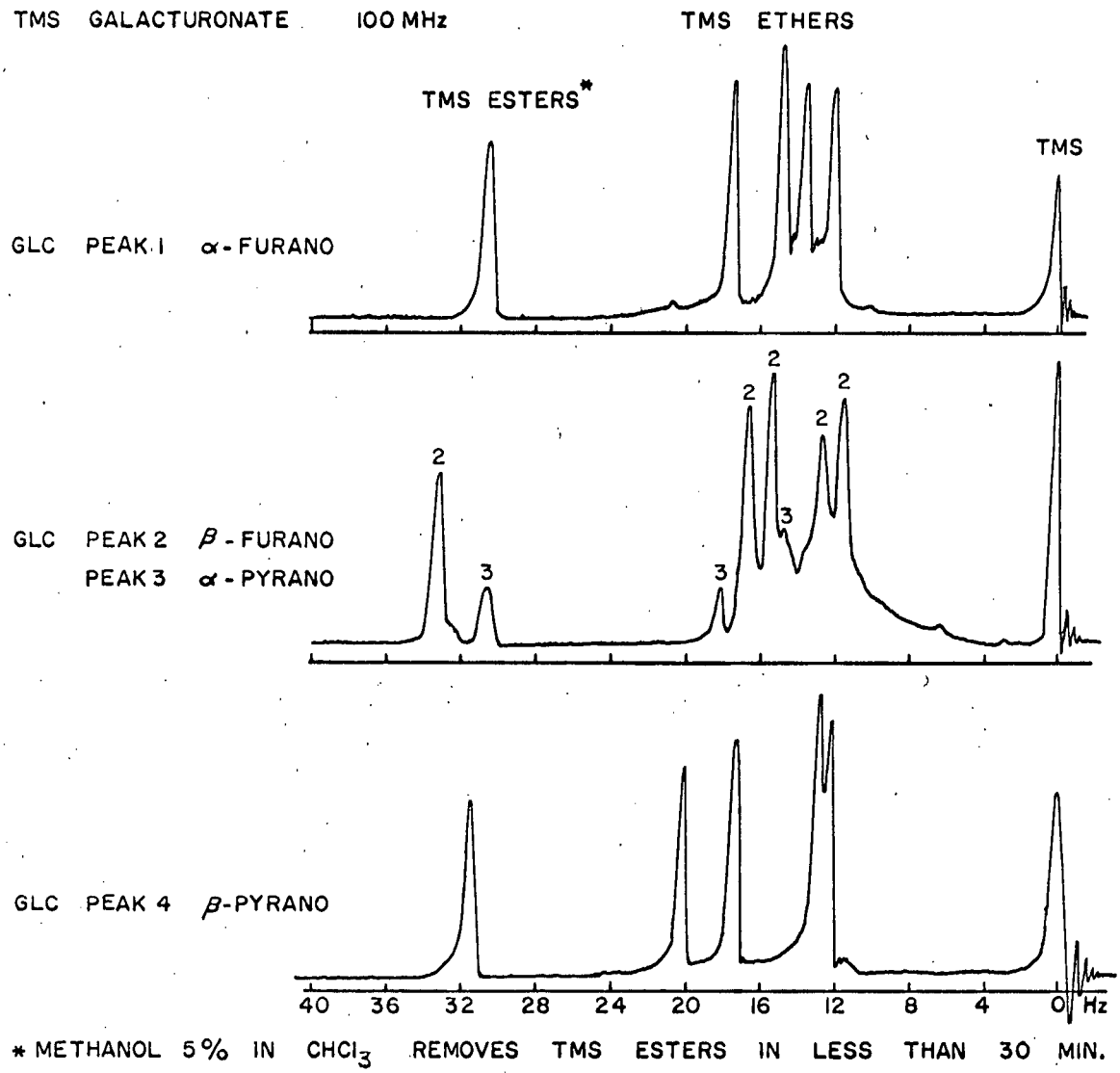
A mixture of aldobiouronic acids obtained from lemon gum was silylated with BTMSA. Although p.m.r. spectral evidence indicated formation of TMS esters in the aldobiouronic acid mixture, no product could be detected on subsequent gas chromatography. It appears that silyl esters are not sufficiently stable at high temperatures to allow separation of disaccharides. It may be possible, however, that part of the problem is associated with the gas chromatograph used in these experiments.

Although BTMSA silylation results in formation of TMS ethers and esters, it does not appear to be a useful reagent for analysis of uronic acids. Comparison of obtained results with other methods of silylation of uronic acids [60],

indicates a marked change in equilibrium concentration for the various isomers. Other methods [60] indicate an isomer distribution more like that of the parent sugar galactose. Galactose has only a small amount of furanose present at equilibrium.

FIGURE 17

PROTON MAGNETIC RESONANCE SPECTRA FOR THE TRIMETHYLSILYL PROTONS OF SILYLATED GALACTURONIC ACID



* METHANOL 5% IN $CHCl_3$ REMOVES TMS ESTERS IN LESS THAN 30 MIN.

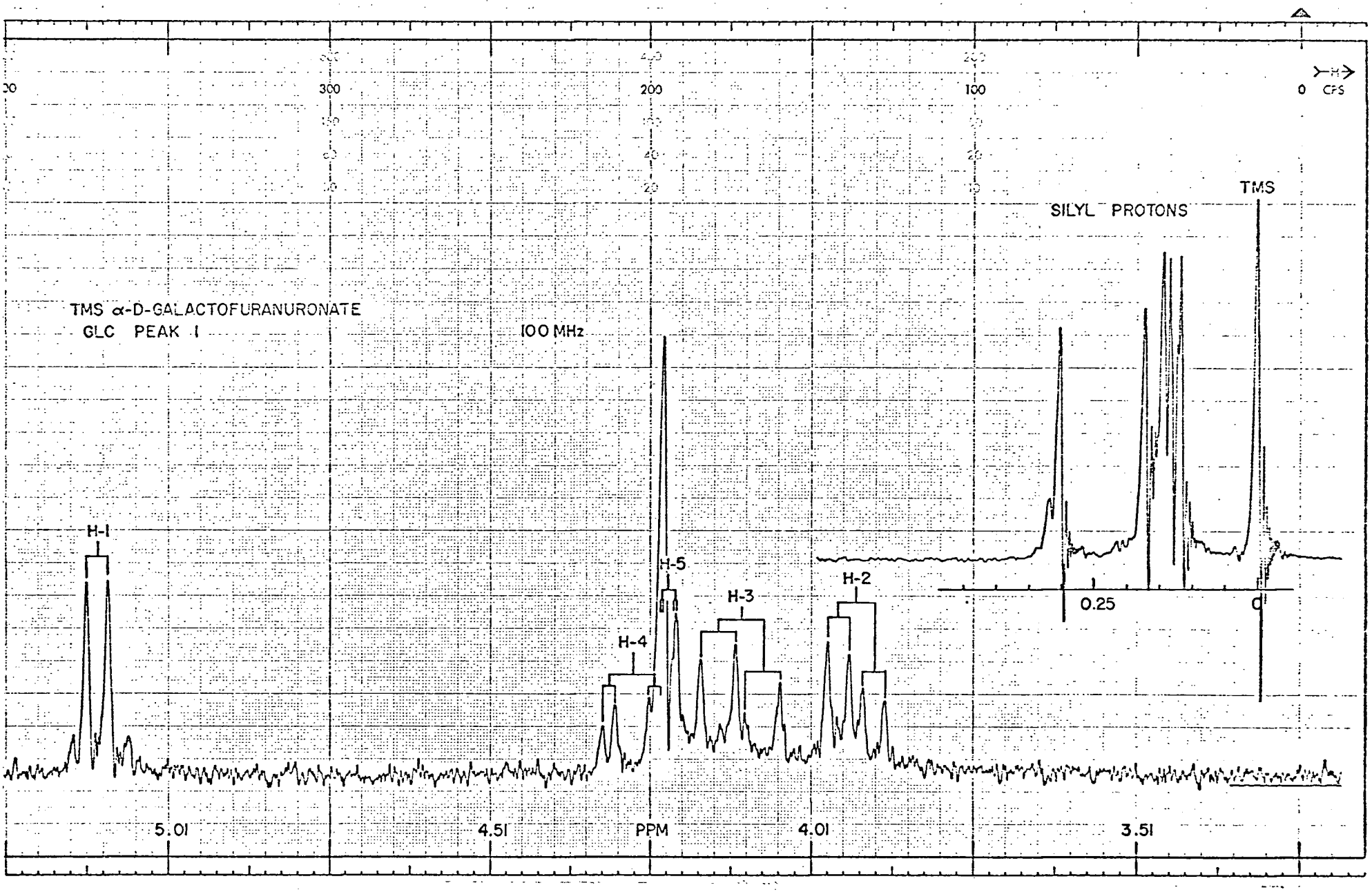


FIGURE 18: P.M.R. SPECTRUM OF TRIMETHYLSILYLATED GALACTURONIC ACID [G.L.C. PEAK 1]

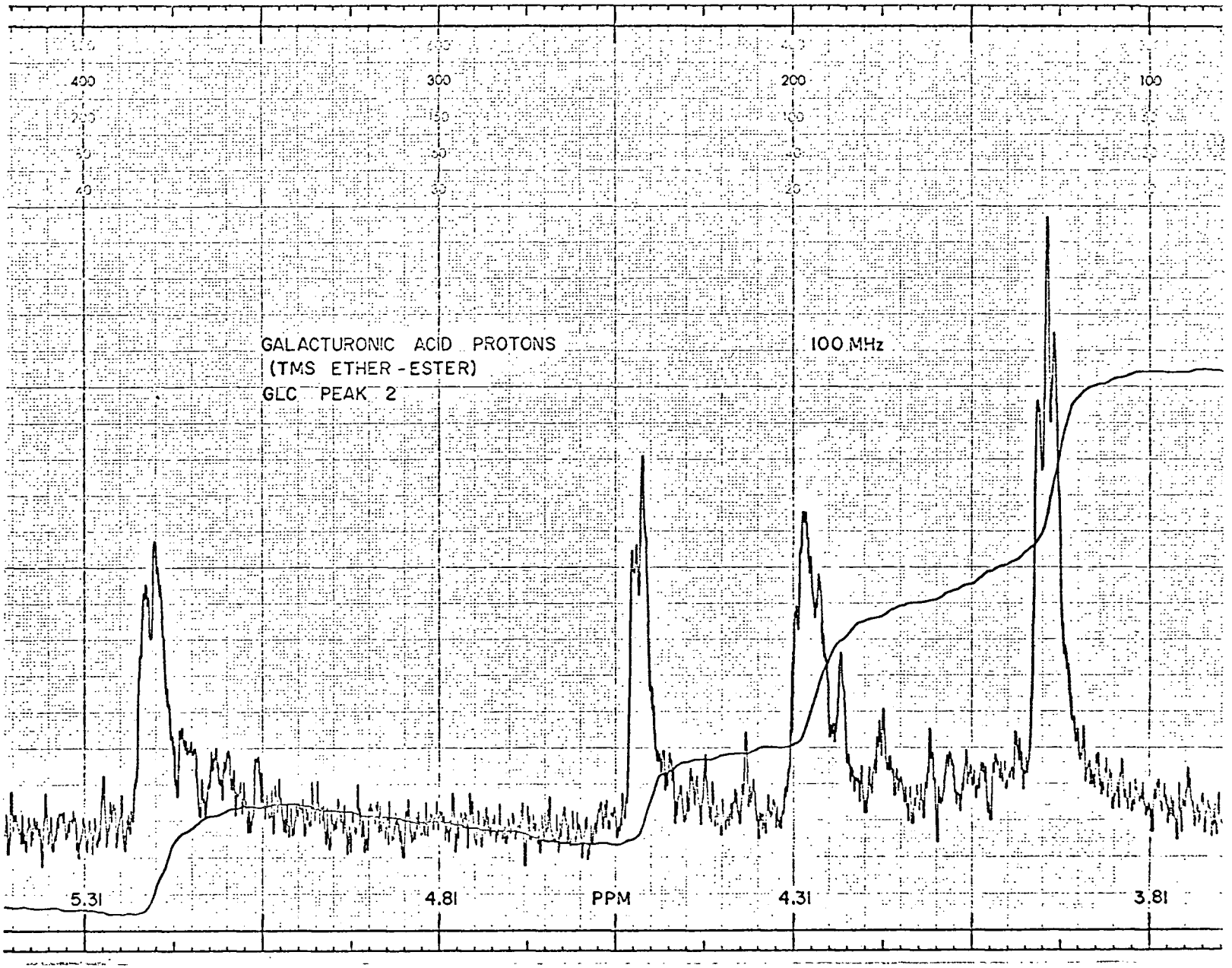


FIGURE 19: P.M.R. SPECTRUM OF TRIMETHYLSILYLATED GALACTURONIC ACID [G.L.C. PEAK 2]

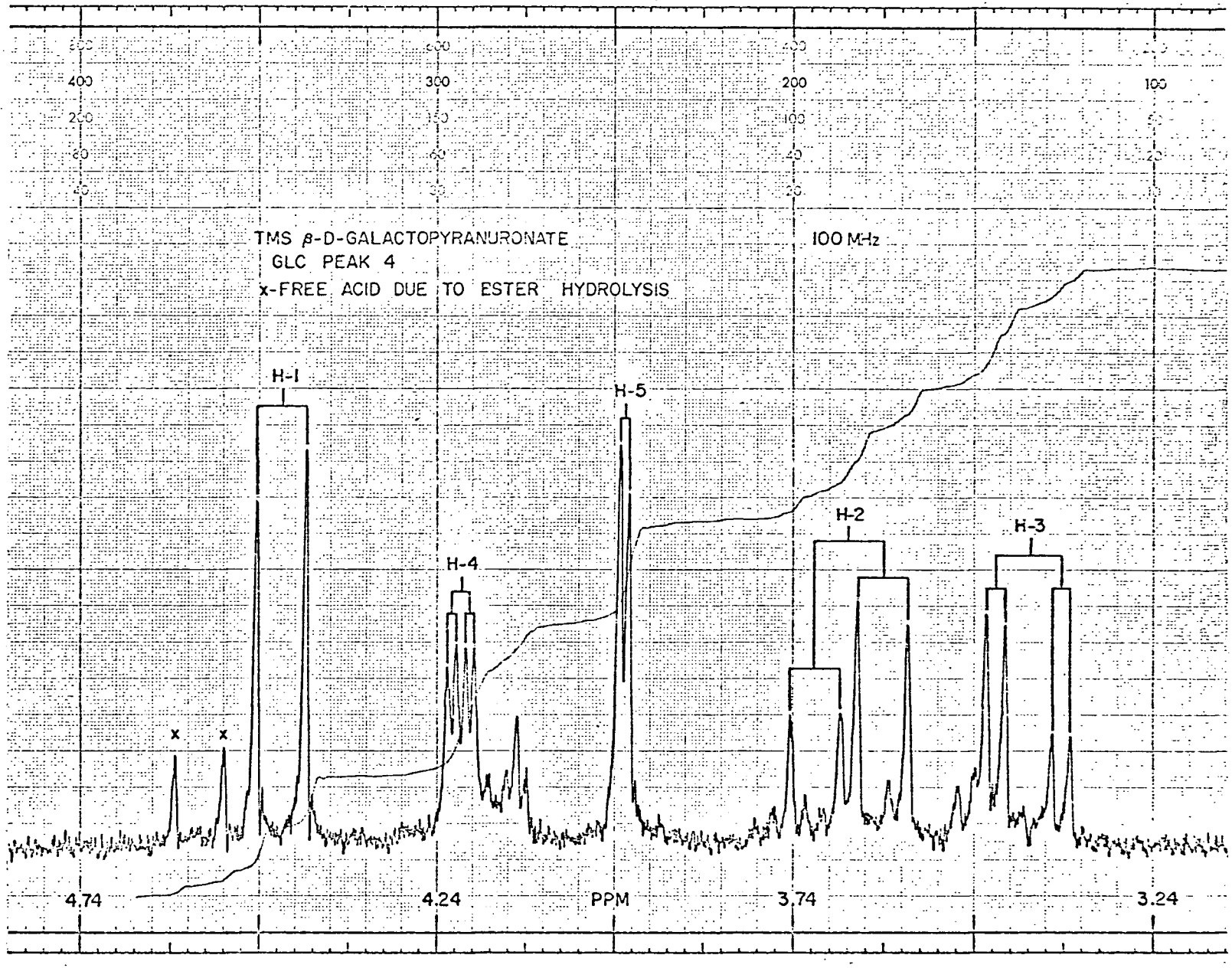


FIGURE 20: P.M.R. SPECTRUM OF TRIMETHYLSILYLATED GALACTURONIC ACID [G.L.C. PEAK 4]

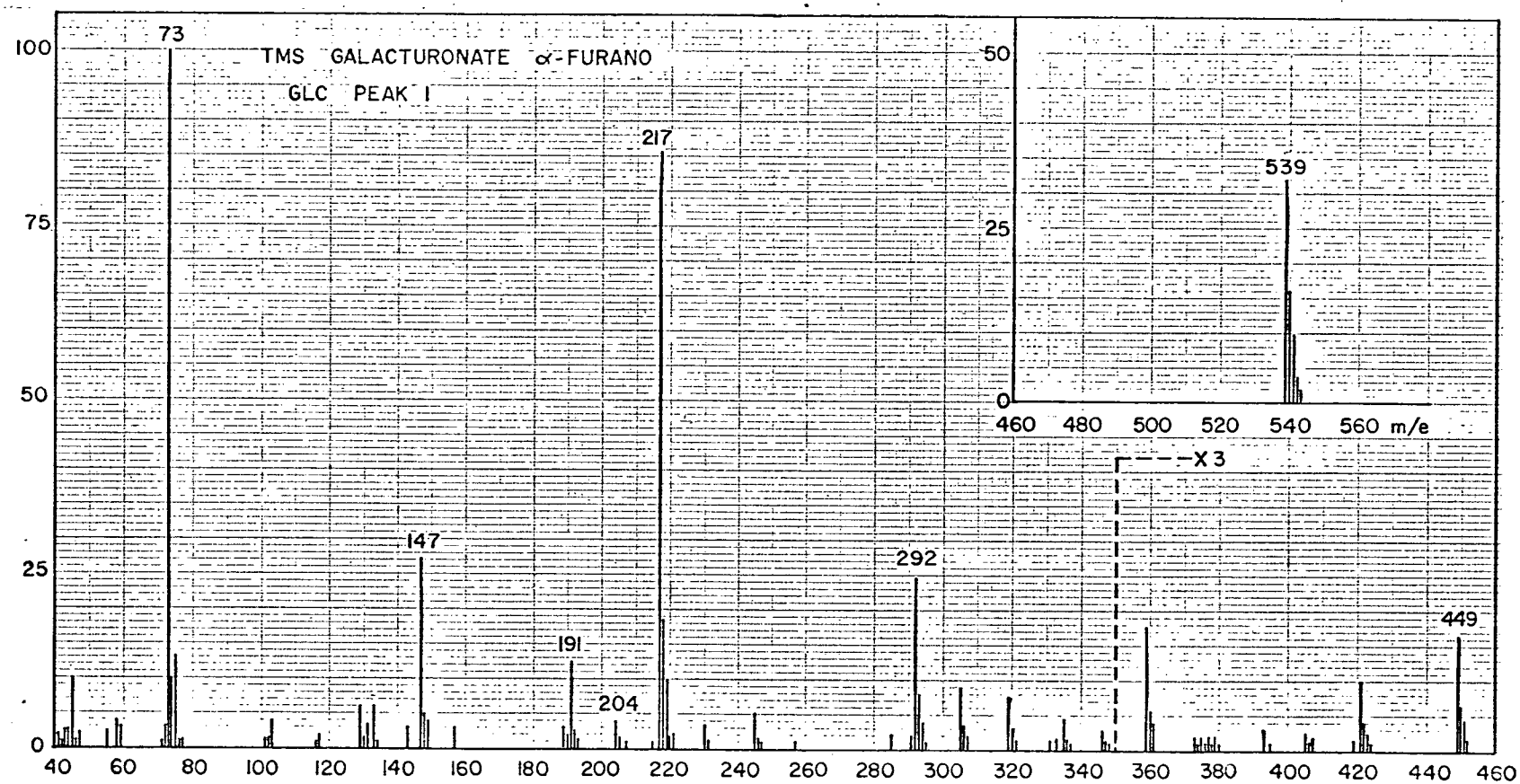


FIGURE 21: MASS SPECTRUM OF THE TRIMETHYLSILYLATION PRODUCT OF GALACTURONIC ACID [G.L.C. PEAK 1]

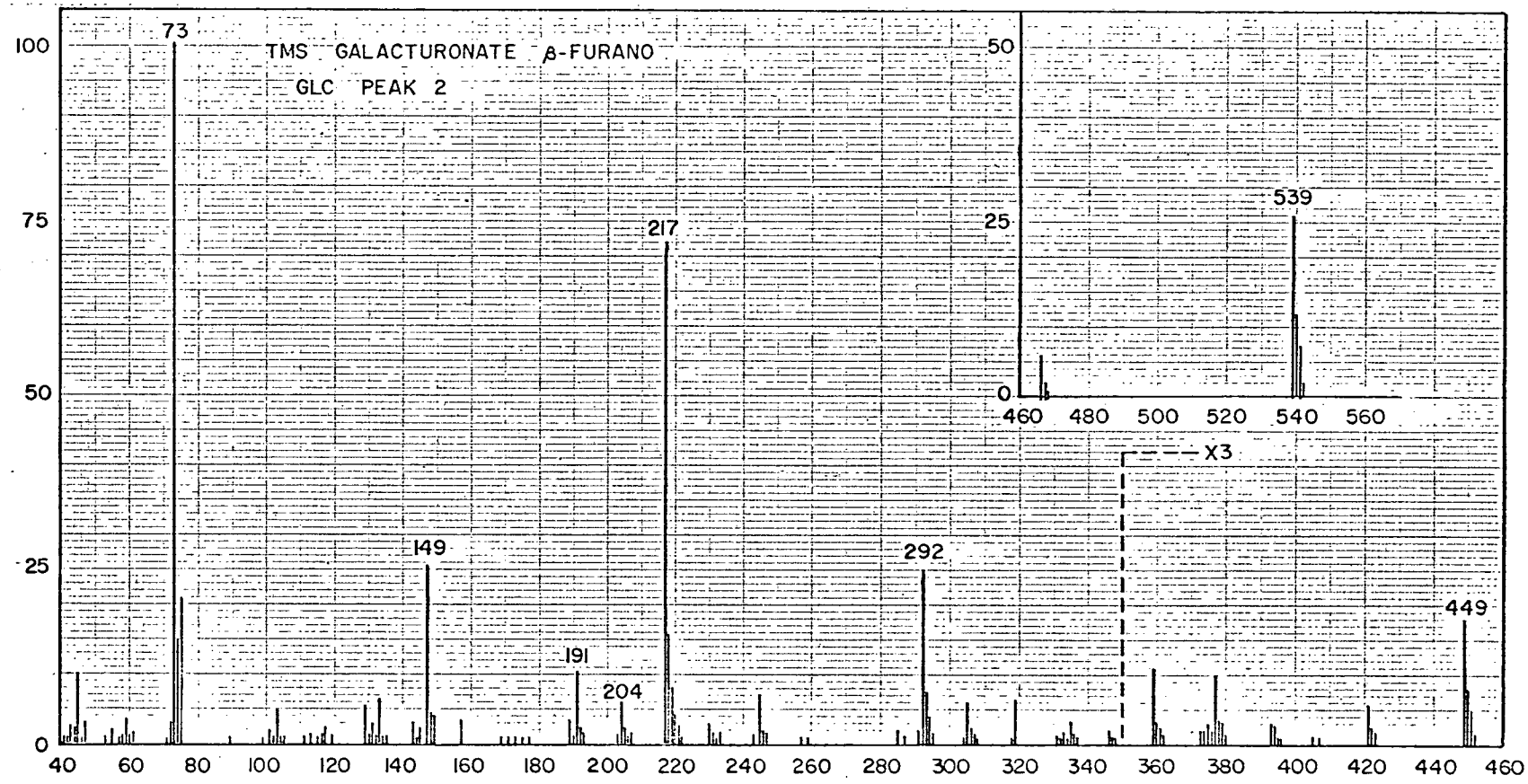


FIGURE 22: MASS SPECTRUM OF THE TRIMETHYLSILYLATION PRODUCT OF GALACTURONIC ACID [G.L.C. PEAK 2]

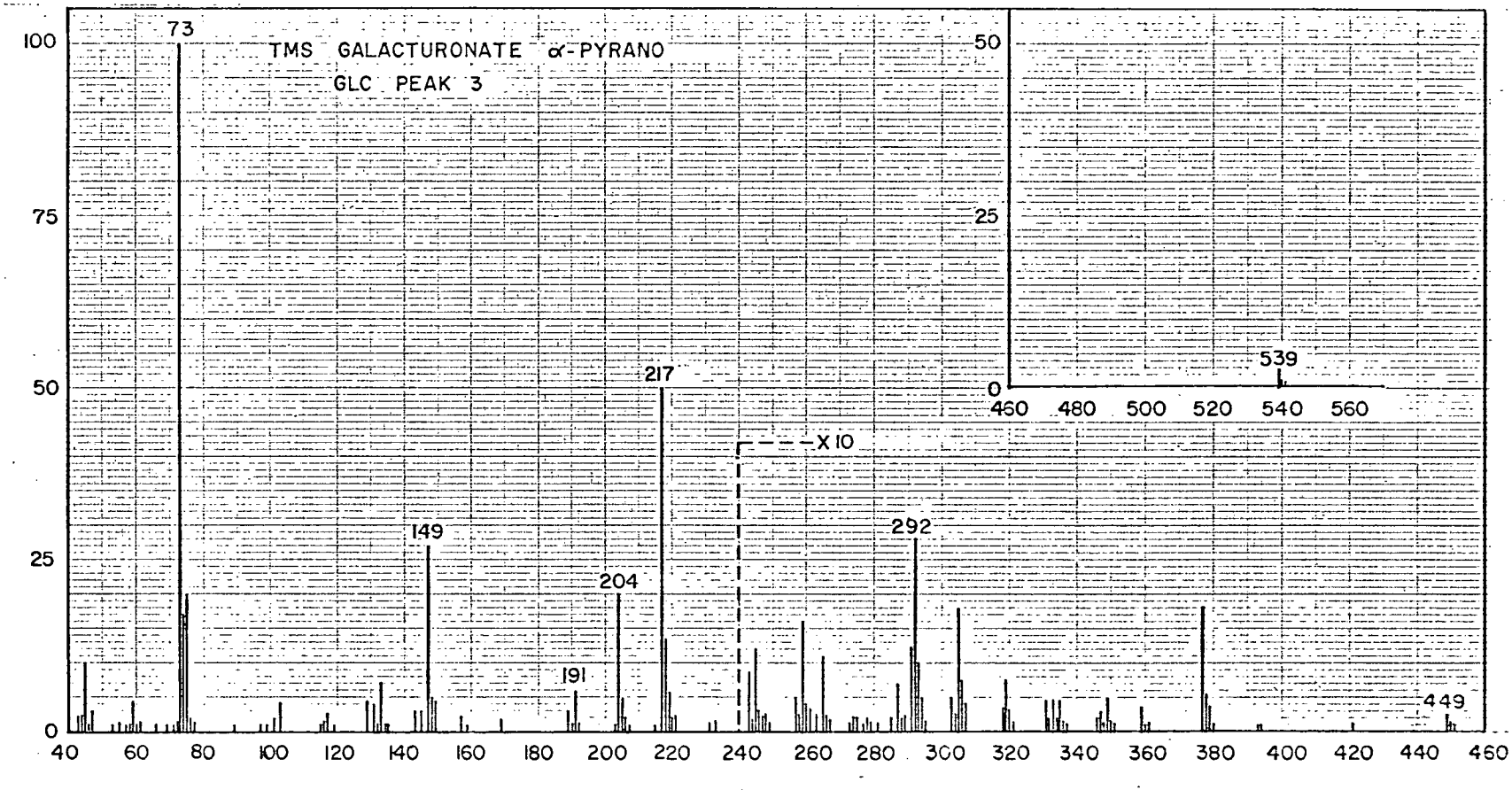


FIGURE 23: MASS SPECTRUM OF THE TRIMETHYLSILYLATION PRODUCT OF GALACTURONIC ACID [G.L.C. PEAK 3]

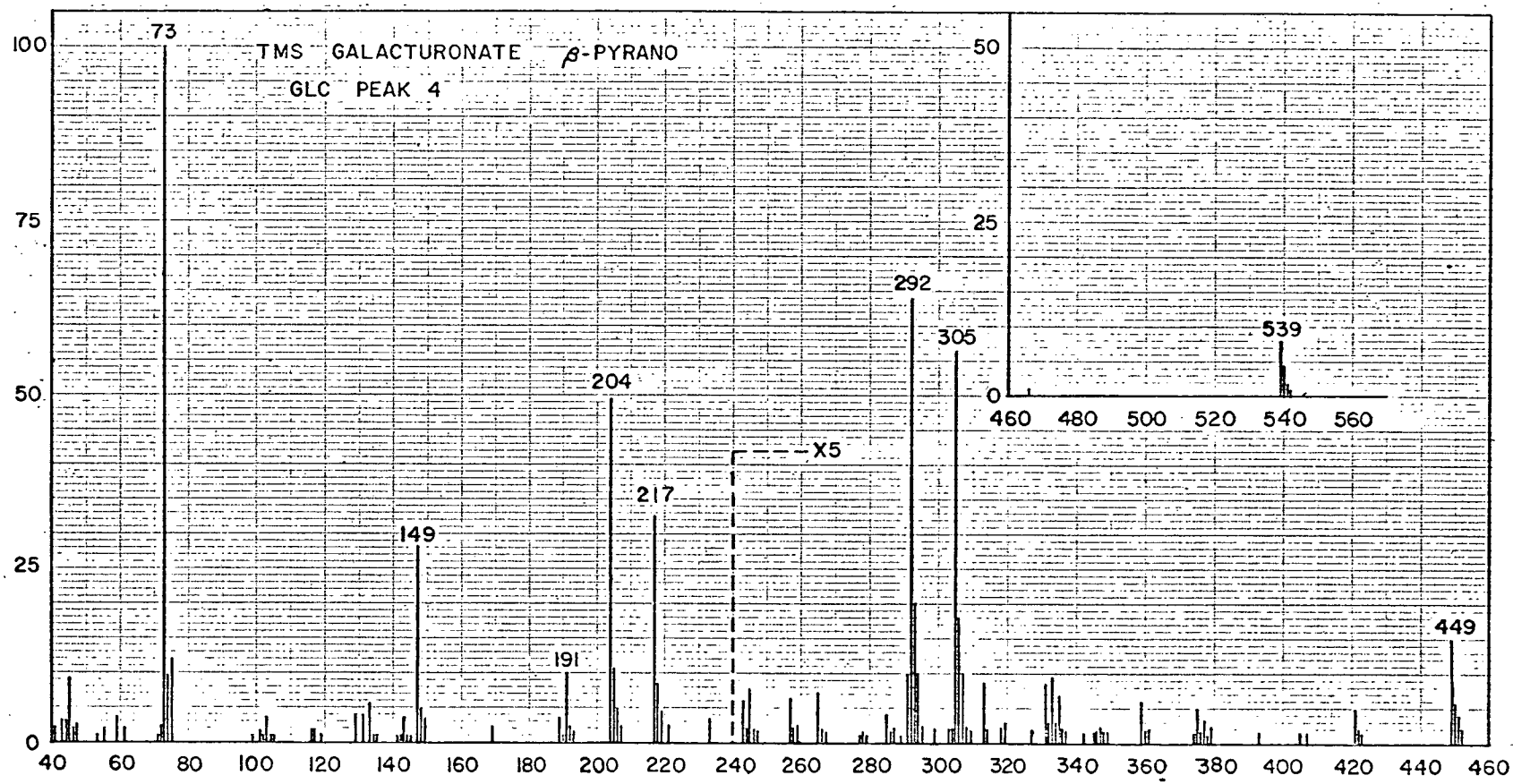


FIGURE 24: MASS SPECTRUM OF THE TRIMETHYLSILYLATION PRODUCT OF GALACTURONIC ACID [G.L.C. PEAK 4]

METHYLATION OF LEMON GUM

Methylation has been extensively employed in structural analysis of polysaccharides. Free hydroxyls in a polymer will be converted to methyl ethers during methylation. Hydrolysis of a methylated polymer will yield carbohydrate fragments characteristic of the original polymer's linkage.

The main problems associated with methylation of polysaccharides are yield and degree of substitution. Methylations using the classical Haworth [61] and Purdie [62] procedures are often long, involving many repeated reactions. Haworth methylations do not normally achieve a high degree of substitution on a single reaction. In addition polysaccharides are in strongly basic solutions [20-30%] for prolonged periods of time. Purdie methylations are normally carried out on partially methylated polysaccharides. Silver oxide used in this methylation adsorbs polymer resulting in poor yields. This adsorption appears to be more pronounced with poorly methylated starting materials. Table XIV gives literature yields and information on polysaccharides methylated by these procedures. Losses incurred on complete methylation cast some doubt on the quality of the methylation analysis. When large losses occur it is impossible to know if some structurally significant portion of the polymer has been missed.

Many other methylation reactions have been proposed in recent years. Table XV lists some of these with references to their use.

Complete methylation of a polymer is necessary to avoid erroneous interpretation of the original structure [63]. Each unblocked hydroxyl detected in the methylated polysaccharide hydrolysate appears as a linkage point in the

TABLE XIV

YIELDS AND DEGREES OF METHYLATION OF VARIOUS POLYSACCHARIDES

Polysaccharide	% Yield Methylated Polymer [A]	Methoxyl Found %	Ref.
Acacia arabica Gum	56	38.2	[64]
Armillaria mellea fruit body heterogalactan	61	41.6	[65]
Amylose polyalcohol	65	44.3	[66]
Arthrobacter viscosus extracellular polysaccharide	60	44.7	[67]
Tamarind kernal polysaccharide	54	39.5	[68]
Aeodes orbitosa polysaccharide [desulfated]	26	35.0	[69]
Aldotriouronic acids from Sapote Gum 1	45	N.R.	[70]
Aldotriouronic acids from Sapote Gum 2	13	N.R.	"
Aldotriouronic acids from Sapote Gum 3	25	N.R.	"
Synthetic araban	78	39.8	[71]

[A] Based on starting material weight over methylated polymer weight a no loss result would be 135-145%.

TABLE XV

RECENTLY DEVELOPED METHYLATION PROCEDURES

I	Methyl iodide with silver oxide	a]	in dimethylformamide [DMF]	[72]
		b]	in dimethylsulfoxide [DMSO]	[73]
II	Methyl iodide with barium oxide or barium hydroxide	a]	in DMF	[74]
		b]	in DMSO	[73]
III	Dimethylsulfate with barium oxide or barium hydroxide	a]	in DMF	[75]
		b]	in DMF and DMSO	[75]
IV	Dimethylsulfate with powdered sodium hydroxide		in DMSO	[76]
V	Methyl Iodide with sodium hydride	a]	ether type solvents	[77]
		b]	DMF	[77]

original polymer. Since molecules such as galactose are known to be difficult to methylate at position four, errors result in proposed structures. An example of undermethylation being interpreted as a more complicated structure is outlined by Sandford and Conrad [78].

The method chosen to provide optimum yield and degree of methylation was the sodium hydride, DMSO and methyl iodide procedure of Hakomori [79]. Some workers have shown degradation of polysaccharides on use of these reagents. It was felt, however, that a polymer more closely resembling original material would be obtained by this method. Degradations which have been reported have occurred in uronic acid portions of carbohydrate polymers. Hakomori methylations were shown to cause O-glucuronides to undergo elimination reactions yielding 4,5-dehydrouronic acids [54]. Conversely, Anderson and Cree [80] examined methylation of some Acacia polysaccharides using this procedure. They looked specifically for condensation of ester groups with methylsulphinyl carbanion to give a sulfoxide or β -elimination to unsaturated acids. No evidence was found for either of these reactions. The slight reaction found with benzyl glucuronide may have been due to unfavourable reaction conditions or simply a higher concentration of uronic acid. Identification of degradation product may be easier in a more concentrated system. Benefits derived from use of this methylation procedure outweighed any possible minor degradation of uronic acid.

One point must be emphasized in the use of Hakomori methylations. In a methylation of an acidic polysaccharide, uronic acids exist as sodium salts which limit any degradation to unsaturated uronate [81]. Methyl esters are known to undergo elimination more readily. Uronic acid sodium salts are converted to

methyl esters during the course of methylation providing an opportunity for elimination. It appears from methylation results that some uronic acid salts remain after methylation and excess sodium hydride has been destroyed. Infrared spectra for the methylated polysaccharides show the presence of carboxylate. Should a polymer undergo depolymerization due to formation of 4,5 unsaturation then reduction employing lithium borohydride in DMSO would eliminate the cause of the problem.

Hakomori methylation produces virtually complete methylation in a single reaction. Several possible methods of analysis are available for the methylated sugars present on hydrolysis. The preferred techniques involve use of gas liquid chromatography. Several methods of sample preparation for G.L.C. are possible. Depolymerization of methylated polysaccharide may be achieved by methanolysis. Dependent on the sugars present, the samples may be analysed directly or further reacted to acetates or silyl ethers. Acid hydrolysis give free sugars which may be reduced by sodium borohydride to alditols. The samples may be made volatile by acetylation or silylation. A common procedure is simple methanolysis, since volatile derivatives are obtained for most partially methylated sugars.

Although G.L.C. retention time offers a good indication of the nature of a product it is by no means a positive identification. Further tests must be carried out to provide positive identification. The classical approach has been to isolate the components and prepare crystalline derivatives. This method of analysis can be extremely difficult. Contaminating products can be completely missed because of purification during derivative preparation.

The need for a more rapid and smaller scale identification has prompted many workers to employ mass spectrometry. Good mass spectra may generally be obtained on samples which pass through a gas chromatograph. Two procedures

have been employed before mass spectral analysis. Methanolysis products have been silylated for greater thermal stability. This method [82] provides very precise identification of components. A drawback, however, is the formation of different ring and anommeric forms. For example, 2,3,6 tri-O-methyl-D-galactose will form at least three different derivatives, fairly large amounts of both pyranosides and smaller amounts of a furanoside. These extra peaks can aid and/or hinder analysis of complex mixtures.

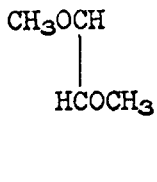
Lindberg [83] has developed an alternate procedure employing partially methylated polyol acetates. Methylated polysaccharides are hydrolysed with 72% sulfuric acid, reduced with sodium borohydride, acetylated and analysed by gas chromatography. Mass spectral fragmentation is dependent on methyl ether substitution of the original polyol and provides for very easy interpretation of structure. Resolution of methylated polyol acetates is less than for corresponding methyl glycosides. This lack of resolution can introduce complications during analysis of complex mixtures.

MASS SPECTROMETRY OF METHYLATED POLYOL ACETATES

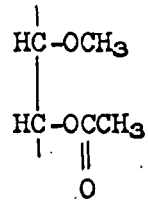
The following is a brief outline of controlling factors in mass spectrometry of partially methylated alditol acetates as outlined by Lindberg [83]. The base peak in almost all determined spectra was m/e 43 [CH_3CO^+]. It was stated that only peaks of intensity, greater than 10% of base peak were included. It should be noted that spectra of isomeric alditols having the same substitution pattern are not sufficiently different to allow for distinction between them. Thus information will sometimes be lost when converting into alditols. For example, mass spectra from 2,3 and 3,4 di-O-methyl pentoses will be identical. Reduction with deuterioborohydride would eliminate this difficulty [83]. The proposal is that intense peaks [> 10% base peak] of the mass spectrum and retention time of the component on G.L.C. in combination with sugar composition of the original polymer will, in most cases, give sufficient evidence for an unambiguous characterization of the methylated sugar.

Chizhov et al. [84] have determined that primary fragments from alditol acetates arise through fission between two carbon atoms of the carbon chain. Either fragment can carry the positive charge. Secondary fragments are formed from primary fragments by elimination of acetic acid [62] and/or ketene [42]. Intensity of primary fragments increases with decreasing mass number. Chizhov et al. [84] have shown that intensities of primary fragments given by alditol acetates are much lower than intensities of corresponding peaks from partially methylated alditol acetates. Fission takes place between carbon atoms in partial structures V and VI in preference to structure VII. Positive ions are stabilized by methoxyl groups. Primary fragments of high intensity

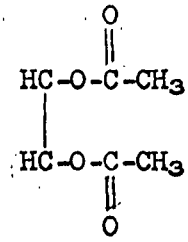
containing two vicinal methoxyl groups are not observed, except when they can be formed by fission between two other vicinal methoxyl groups, indicating that structure V is cleaved in preference to structure VI.



V



VI

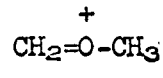


VII

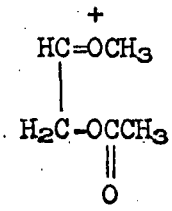
The secondary fragments observed in methylated alditol acetates are derived from primary fragments by single or consecutive elimination of acetic acid [60], ketene [42], methanol [32] or formaldehyde [30].

The lowest molecular weight primary fragment, m/e 45 [A], is produced by substances having a methoxyl group at C-1. No other polyol produces this primary peak. A peak [m/e 45] of low intensity [< 5%] may be seen in mass spectra of other substances and is probably a secondary fragment.

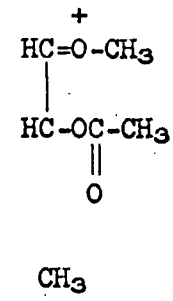
Primary fragment D, m/e 117, is obtained when C-1 is acetylated and C-2 methylated. An analogous primary fragment m/e 131 [E] is obtained from a 6-deoxy hexitol methylated at C-4 but not at C-5.



A

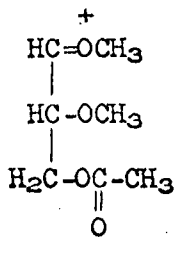


D

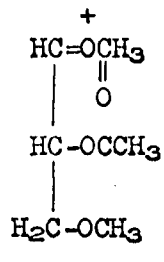


E

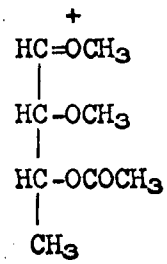
Lindberg [83] reports that two primary fragments have m/e 161, F_1 and F_2. Of these, F_1 is obtained in low intensity from alditols methylated at positions 2 and 3. If 4 is also methylated then it becomes a prominent fragment. Fragment F_2, obtained from alditols methylated at positions 1 and 3, is always of high intensity. The secondary fragments m/e 129 and 101 are obtained from F_1 and F_2 by loss of methanol and acetic acid respectively. Further loss of ketene from m/e 129 gives m/e 87. Loss of formaldehyde from m/e 101 may give m/e 71. Primary fragment m/e 175 [G] is obtained from 6-deoxyhexitols methylated in positions 2, 3 and 4.



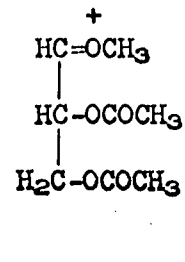
F_1



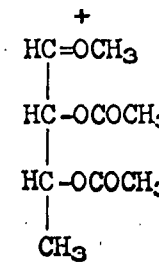
F_2



G



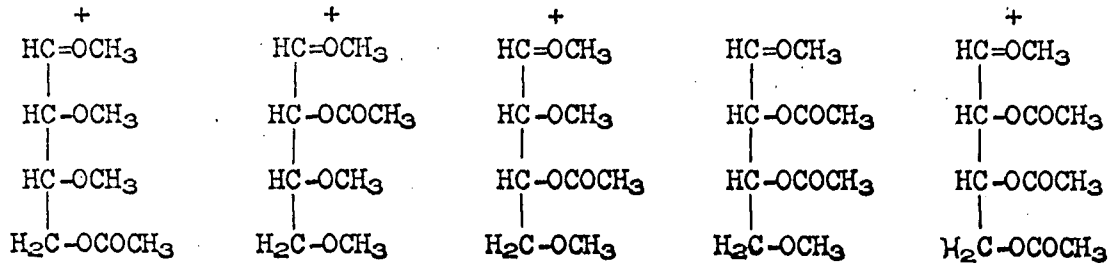
H



I

Primary fragment m/e 189 [H] is given by alditols methylated at position 3 but not at positions 1 or 2. Secondary fragment m/e 129 may be formed by elimination of acetic acid from H. Loss of formaldehyde or ketene from m/e 129 gives respectively m/e 99 or 87. M/e 203 [I] is an analogous primary fragment obtained from a 6-deoxy hexitol methylated at position 3 but not 4 or 5.

Three primary fragments of m/e 205 may be expected [K_1, K_2 and K_3]. The first, derived from alditols methylated at C-2, 3 and 4, is not observed in high intensity. Fragment K_2 is observed from alditols methylated at positions 1, 2, 4 and 5, K_3 from alditols methylated at positions 1, 3, 4 and 5. In either case if position 5 is not methylated, the corresponding peak appears with only low intensity. Several secondary fragments may be obtained from K_1, K_2 and K_3. Loss of acetic acid from K_3 to produce m/e 145 appears to be an especially important secondary fragment.



K_1 K_2 K_3 L M

Three primary fragments of m/e 233 may be expected. One of these [L] is obtained from alditols methylated at C-1 and 4. A prominent secondary peak [m/e 113] is most probably derived from L by loss of two molecules of acetic acid.

The primary peak m/e 261 [M] is the highest mass number of relatively high intensity. Alditols methylated at 3 and acetylated at 4, 5 and 6 produce this peak.

Lindberg et al [83] have used formation of these primary and secondary fragments to analyse partially methylated alditol acetates. Tables of fragments expected under these rules are presented in Lindberg's initial paper on mass spectrometry. Knowledge of the partially methylated polyols has aided their analysis of polysaccharide structure.

The Lindberg procedure for analysis was chosen because it offered an inate simplicity not present in other analytical schemes. Lemon gum has been analysed by methanolysis [1]. It was felt that this sort of complex polysaccharide would give a good evaluation of reduction - acetylation as a method for analysis of methylated polysaccharides.

Four polysaccharides were methylated by the Hakomori method and analysed by Lindberg's mass spectrometry - gas chromatography procedure. The four were:

1. Lemon gum
2. Lemon gum degraded with 1/10N H_2SO_4
3. Lemon gum periodate oxidized and borohydride reduced [before hydrolysis]
4. Lemon gum periodate oxidized and borohydride reduced [after 0.5N H_2SO_4 hydrolysis]

METHYLATION ANALYSIS OF LEMON GUM

Lemon gum was methylated using the Hakomori method. Extreme care was taken to avoid interfering side reactions. After methylation, uronic acids were reduced with lithium aluminum hydride. Reduction avoided problems associated with formation of aldobiouronic acids on hydrolysis. Since lemon gum was fully methylated before reduction, no methylation was carried out to block the six positions in the newly formed neutral sugar. All steps in the reactions outlined produced high yields. After reactions to form acetylated-methylated polyols, analysis was carried out on the gas chromatograph. Two columns were evaluated, one of ECNSS-M, the other butanediol succinate. Resolution of the mixture was virtually identical, with butanediol succinate being slightly superior.

Samples were collected for analysis on the mass spectrometer since access to a coupled GLC-mass spectrometer was not possible. The collected components were analysed by thin layer chromatography [T.L.C.]. T.L.C. revealed that single peaks on the gas chromatograms were, in fact, at least two components. Using thin layer chromatography, samples of the components could be separated. The samples were then reanalysed by GLC and T.L.C. to ensure their purity.

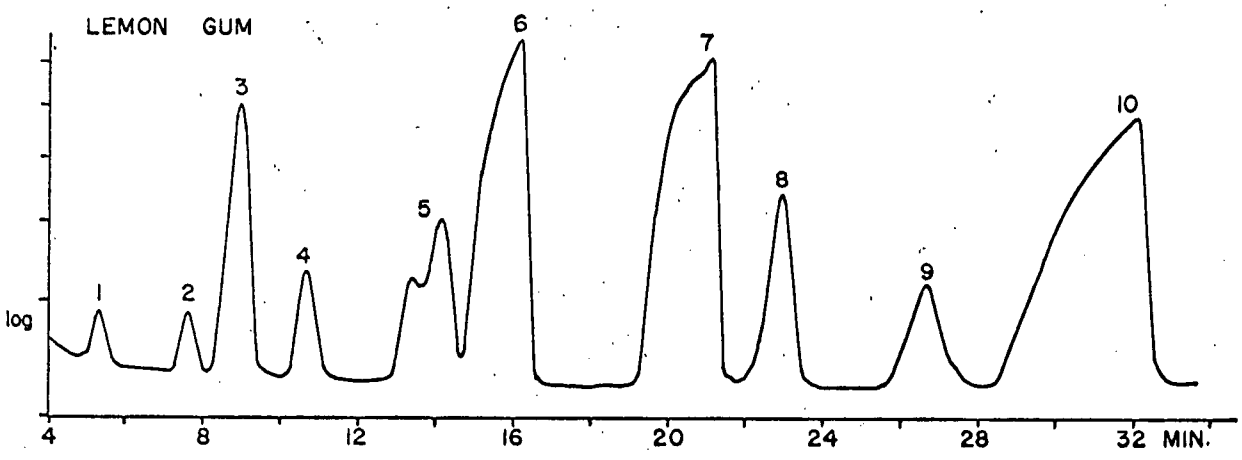


FIGURE 25: GAS CHROMATOGRAM OF LEMON GUM AFTER METHYLATION, LITHIUM ALUMINUM HYDRIDE REDUCTION, HYDROLYSIS, BOROHYDRIDE REDUCTION AND ACETYLATION [140-195°C at 3°/min.]

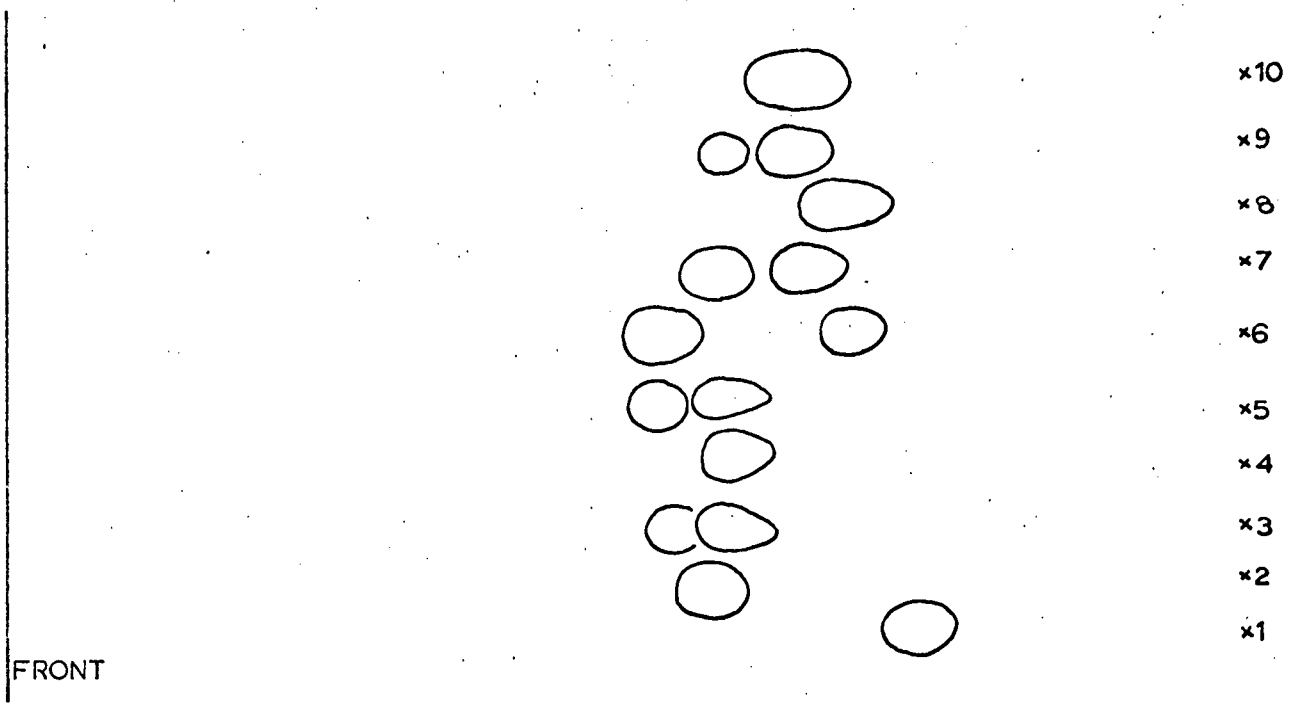


FIGURE 26: THIN LAYER CHROMATOGRAM OF THE TEN FRACTIONS OF LEMON GUM METHYLATED POLYOL ACETATES

During the isolation and purification of these components, a technique was developed for handling microgram quantities of compounds collected from the gas chromatograph. This procedure for micro manipulation is discussed in the appendix to the thesis.

Mass spectra were obtained on all significant components isolated by gas-liquid chromatography. Analysis of the spectra indicated that although simple rules had been set down by Lindberg, intensity of some component peaks was altered. This may be a function of the means of sample introduction. Samples to be analysed were inserted into the spectrometer via the probe. It was not possible to identify components using the simple rules proposed by Lindberg. It was, however, possible to identify the majority by careful consideration of fragmentations.

Care was taken with the spectra to ensure no background contribution which would have lead to misleading peak intensities. Two checks were made on the spectra. A background spectrum was obtained and subtracted from the component spectra. Analysis of the spectra in their order of production confirmed that major peaks were not contributing to subsequent spectra. The spectra are included in the appendix and numbered to indicate both order of elution and order of mass spectra determination.

Although Lindberg and co-workers utilized a coupled gas chromatograph-mass spectrometer, there are advantages in using direct sample collection. The presence of overlapping peaks is a clear example of need for further separations. Peak broadening also occurs in the gas chromatograph-mass spectrometer separator chamber. The method of micro manipulation outlined in the appendix has proven extremely valuable in purification and sample analysis.

Variation in peak intensity for some mass values has created a problem. Peaks which were expected to be of intensity greater than 10% were, in fact, less than this. In addition peaks at lower m/e values had higher intensity than predicted. Availability of complete spectra would have aided considerably interpretation of structure.

Quantitative estimation of the identified components was not possible due to lack of thermal conductivity molar response factors for partially methylated alditol acetates. A relation should exist between subsitution pattern and molar response factor for a given sugar polyol. A relatively large number of molar response factors are available for flame ionization equipment but none are presently available for thermal conductivity detectors.

METHYLATED POLYOLS FROM LEMON GUM

Peak 1: Component one on the gas chromatograph was unidentified. The retention time was less than that reported for the most volatile sugar alditol polyol. The increased volatility of this component was likely due to elimination or anhydride formation. Hydrolytic etherification has been reported for partially methylated polyols and sorbitol [85]. Strong peaks at m/e 117 and 101 indicated the molecule was terminated by an acetyl group adjacent to two methoxyls. A strong m/e 88 peak was not consistent with any reported peak. Strong even numbered fragments do not often occur in mass spectra.

Peak 2: The spectrum for this component does not correspond to any of the known methylated polyol acetates. Its structure does, in fact, appear to be very different from that of a polyol. None of the characteristic primary

fragments except m/e 45 can be seen. Although some masses correspond to secondary fragments, it is difficult to determine their origin. In addition a peak m/e 74 is almost as intense as the base peak m/e 43.

Peak 3: T.L.C. indicated that peak three contained two components. It was confirmed experimentally that 2, 3, 5-tri-O-methyl-L-arabinitol and 2, 3, 4-tri-O-methyl-L-rhamnitol acetates overlap as a single peak. Two components are clearly seen in the mass spectrum. This ability to distinguish two components as a result of knowledge about sugar composition and chromatographic behaviour could be used to determine percentage composition. Only standard spectra of the two components are required.

Peak 4: This component, present in only small amounts, is 2, 3, 4-tri-O-methyl-L-arabinitol acetate. With this component it became clear that the level of 10% of base peak for included peaks was not going to be applicable. In addition to m/e values of 43, 101, 117 and 161 required for identification, m/e values 45, 58 and 87 were all above 10% of base peak.

Peak 5: This peak contains two components, which, being very minor constituents, were impossible to separate. Some information was available from the mass spectrum of this peak. Additional information may be deduced from G.L.C. retention time. Since 2, 3, 4, 6-tetra-O-methyl-D-galactitol acetate comes immediately after these two components their retention times must be less than its value. The only two applicable compounds are 2, 5-and 3, 5-di-O-methyl-L-arabinitol acetates. The mass spectrum also indicated the presence of these two components. Peaks associated with 3, 5-di-O-methyl-L-arabinitol are of greater intensity and correspond in strength to the second

peak of peak 5. Positive identification of the smaller component as 2,5-di-O-methyl-L-arabinitol is clouded by strong signals from 3,5-di-O-methyl-L-arabinitol. Identification is based on m/e 233 and 113 peaks. Reduced intensity of higher mass values increases the difficulty of identification without pure standard spectra.

Peak 6: This peak contained two components as shown by T.L.C. Peak 6 was collected and separated into its components by preparative T.L.C. Reisolation of the constituent parts by gas chromatography indicated no contamination from peaks 5 or 7. Analytical T.L.C. confirmed the clean separation of peak 6 alditols. The two compounds were designated 6T and 6B on the basis of position on ascending T.L.C.

Peak 6T: 2,3-di-O-methyl-L-arabinitol acetate is the major component of peak 6. This molecule is an example of a component that could be derived from two sugars [2,3-or 3,4-di-O-methyl-L-arabinose]. Both sugars reduce to polyols which fragment in an identical manner. Signal strength for higher m/e peaks was reduced in intensity.

Peak 6B: This constituent proved compounds present as minor constituents in a gas chromatographic peak can be isolated and characterized. Peak 6B is 2,3,4,6-tetra-O-methyl-D-galactitol acetate. Except for reduced intensities for expected peaks the mass spectrum is very clear. A standard sample of this compound coincided in retention time with peak 6. This retention time confirmation aided identification of preceeding chromatographic peaks.

Peak 7: Like peak 6 this fraction contained two components. They were separated, numbered and confirmed pure in the same manner as components in peak 6.

Peak 7T: Analysis of this compound's mass spectrum showed it to be a 2,3,4-tri-O-methyl-hexitol. Since all hexitols substituted in a like manner give similar fragmentation patterns it was necessary to consider G.L.C. retention times. Of the two possible 2,3,4-tri-O-methyl-hexitols in reduced lemon gum, glucitol has the shorter retention time. Therefore, peak 7T is 2,3,4-tri-O-methyl-D-glucitol acetate.

Peak 7B: This peak was thought originally to be only one compound. On detailed examination of the mass spectrum it appeared to consist of two components. No one compound could produce the necessary fragments to account for all peaks occurring in the mass spectrum. The strong m/e 233 peak must arise from 2,3,6-tri-O-methyl-D-galactitol acetate. Only two other compounds produce this peak and they require a strong m/e 189 which was not present. M/e 161 was of such intensity that it had to be considered significant. Only 2,4,6-tri-O-methyl-D-galactitol acetate had a fragmentation pattern compatible with the peaks present. All other compounds which give rise to m/e 161 have peaks at m/e 145 or 189, or are chromatographically unacceptable. Component 7B consists of a mixture of 2,3,6-and 2,4,6-tri-O-methyl-D-galactitol.

Peak 8: T.L.C. indicated that this peak was a single component. The mass spectrum was virtually the same as that for 7T, indicating an identical methyl substitution. A longer retention time was consistent with the structure proposed, 2,3,4-tri-O-methyl-D-galactitol acetate.

Peak 9: There were at least two components in this gas chromatograph peak. The small quantity of these constituents made purification by T.L.C. impossible. One component appeared to be 2,3-di-O-methyl-D-glucitol acetate. The gas chromatogram retention time was identical with an authentic sample. The second component in this peak may be 2,3-di-O-methyl-D-galactitol. The

mass spectrum of this peak would not distinguish between these two compounds. Some peak intensities, however, appear to be too intense, for example m/e 85 and 127. These fragments should arise from a 3-O-methyl hexitol, however, the G.L.C. retention times for such compounds are too great. No other polyol accounts for these mass fragments.

Peak 10: This peak typifies the difficulties that may be encountered when trying to interpret mass spectra of methylated polyol acetates. Peaks for mass values which should have been greater than 10% of the base peak did not reach this value. Peaks with intensities less than 10% of base peak can then not be completely rejected. Thus the mass peak m/e 233 in component 10 which would not normally be considered must be evaluated. Complete spectra present a better picture of relative intensities to be expected. Also, a preliminary table such as given by Lindberg et al [83] is a definite aid. If the table listed values of peak intensity relative to the base peak, an easier interpretation of spectra might be possible. Peak 10 is 2,4-di-O-methyl-D-galactitol although values of certain fragments do not exceed the required 10% value.

The component compounds from fully methylated lemon gum are listed in Table XVI, together with peak area for the components.

TABLE XVI

METHYLATION ANALYSIS OF LEMON GUM

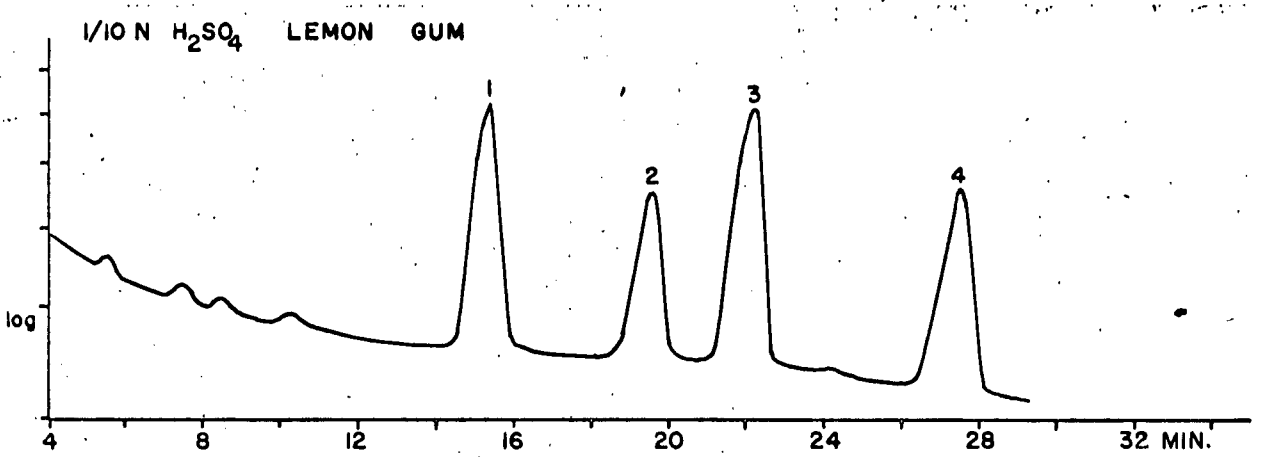
Compound	G.L.C. Peak	Area %
Unknown	1	0.51
Unknown	2	0.70
2,3,5-Me_3-ARAB	3	7.02
2,3,4-Me_3-RHM	3	
2,3,4-Me_3-ARAB	4	1.40
2,5-Me_2-ARAB	5	1.83
3,5-Me_2-ARAB	5	3.62
2,3-Me_2-ARAB	6T	21.7
2,3,4,6-Me_4-GAL	6B	
2,3,4-Me_3-GLUC	7T	28.5
2,3,6-Me_3-GAL	7B	
2,4,6-Me_3-GAL	7B	
2,3,4-Me_3-GAL	8	3.96
2,3-Me_2-GLUC	9	3.16
Unknown	9	
2,4-Me_2-GAL	10	27.6

METHYLATED DEGRADED LEMON GUM

When lemon gum was hydrolysed with 1/10N sulfuric acid a polymer was obtained which contained essentially no L-arabinose. Methylation of this product gave information about the galactan framework of the plant gum. In addition, it provided further proof regarding structures of some partially methylated galactitols derived from lemon gum. The acid degraded polymer was methylated using the Hakomori method [79]. Hydrolysis was carried out by Lindberg's method [5], uronic acids being removed by ion exchange resin. The acids were not examined further, since they represented only a small amount of acidic material. Neutral methylated sugars were reduced and analysed in a manner similar to that described for lemon gum.

There were four principle peaks in the gas chromatogram.

FIGURE 27: GAS CHROMATOGRAM OF DEGRADED LEMON GUM METHYLATED POLYOL ACETATES [140-205°C at 3°/min.]



The peaks were analysed by mass spectrometry with the following results.

Peak 1: The most volatile of the major components exhibited a mass spectrum characteristic of 2,3,4,6-tetra-O-methyl-D-galactitol acetate. Again, intensity of some peaks of lower mass was greater than the 10% cut off suggested

by Lindberg. Two such peaks are m/e 55 and 57. The spectrum, however, was consistent with fragmentation of 2,3,4,6-tetra-O-methyl-D-galactitol acetate in all other respects, clearly indicating the value of the method.

Peak 2: This gas chromatographic peak corresponds to 7B from lemon gum. It contains two inseparable components, 2,3,6-and 2,4,6-tri-O-methyl-D-galactitol. The exact molar balance between these two components will have to await accurate intensity determinations for the two pure compounds.

Peak 3: The component contained in this peak was 2,3,4-tri-O-methyl-D-galactitol, corresponding to peak 8 in the gas chromatogram of methylated lemon gum.

Peak 4: The component of this peak was 2,4-di-O-methyl-D-galactitol acetate and corresponds to peak 10 of lemon gum.

TABLE XVII

METHYLATION ANALYSIS OF DEGRADED LEMON GUM

Compound	G.L.C. Peak	Area %
Initial 4 peaks		1.09
2,3,4,6-Me_4-GAL	1	27.8
2,3,6-Me_3-GAL	2	16.6
2,4,6-Me_3-GAL	2	
2,3,4-Me_3-GAL	3	34.0
2,4-Me_2-GAL	4	20.5

METHYLATION ANALYSIS OF LEMON GUM AFTER PERIODATE OXIDATION AND BOROHYDRIDE REDUCTION

Methylation of periodate degraded lemon gum should generate information concerning free hydroxyl positions of the remaining intact polysaccharide. Total hydrolysis of the methylated polymer would produce sugars from lemon gum which contained no vicinal hydroxyls. Gas chromatographic evidence indicated that this was achieved to a limited extent. However, mass spectra of isolated peaks were very poor.

Peak 1: This component displaying chromatographic characteristics of 2,3,5-tri-O-methyl-L-arabinitol probably resulted from incomplete periodate oxidation and/or basic hydrolysis during methylation. In addition, some mild acid hydrolysis may have occurred during isolation of the borohydride reduced polymer. It was not possible to collect this peak because of sample volatility and the small quantity present.

Peak 2: Like peak 1 this component was present in very low concentration in the mixture of methylated polyol acetates. Retention time was similar to that of 2,3,4-tri-O-methyl-L-arabinitol acetate. The mass spectrum was similar to peak 4 of lemon gum but additional mass peaks indicated the small sample collected was impure.

Peak 3: Like the previous two peaks this was a very minor peak in the gas chromatogram. At most it represented only 3 percent of total sample. A figure of 1.5 mole percent is probably more realistic. The G.L.C. retention time corresponds to that for 2,5-and 3,5-di-O-methyl-L-arabinitol acetate. The mass spectrum can be assigned to these compounds, however, the intensities make absolute assignment difficult.

Peak 4: The first of the major peaks, this component had the gas chromatographic characteristics and mass spectrum of 2,3-di-O-methyl-L-arabinitol acetate. Since this product contains vicinal hydroxyls, blocking groups may have been removed by elimination reactions during the strongly basic methylation. It would appear that a small amount of tetra-O-methyl-D-galactitol may be present in this sample.

Peak 5: This peak consisted of two components on T.L.C. Attempted separation of the small quantities available was unsuccessful. Since this peak corresponded in R_T to peak 7 of lemon gum, one component should have been 2,4,6-tri-O-methyl-D-galactitol acetate. Peaks resulting from this product may be seen in the mass spectrum, however, there are other minor mass peaks consistent with the other consituents of peak 7 of lemon gum, 2,3,6-tri-O-methyl-D-galactitol and 2,3,4-tri-O-methyl-D-gulcitol.

Peak 6: The major component isolated from this oxidized gum was 2,4-di-O-methyl-D-galactitol acetate, accounting for two thirds of the isolated methylated polyols. This information provided further proof that the galactan framework of this polymer is highly branched with many side chains.

Analysis of a periodate oxidized borohydride reduced polysaccharide should be an extremely useful analytical tool. It appears necessary that more study be made of parameters involved in possible hydrolysis during methylation. Lemon gum may not undergo complete oxidation. Alginate has shown this difficulty. This underoxidation may account for the presence of fully methylated sugars in the hydrolysate after methylation.

FIGURE 28

GAS CHROMATOGRAM OF LEMON GUM SMITH POLYALCOHOL METHYLATED POLYOL ACETATES [140-205°C at 3°/min.]

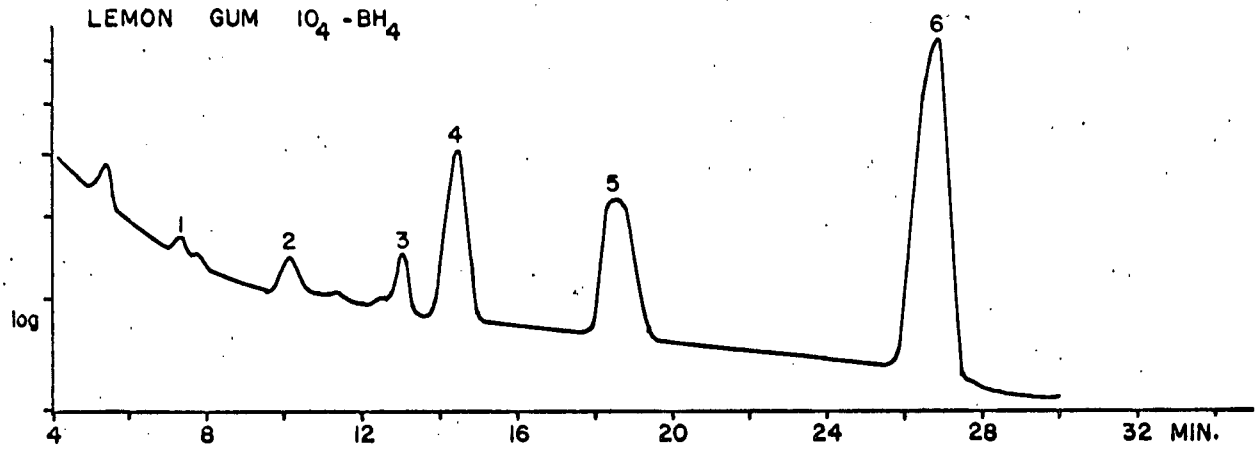


TABLE XVIII

METHYLATION ANALYSIS OF LEMON GUM SMITH POLYALCOHOL

Compound	G.L.C. Peak	Area %
2,3,5-Me_3-ARAB	1	1.03
2,3,4-Me_3-ARAB	2	1.94
2,5-Me_2-ARAB	3	0.37
3,5-Me_2-ARAB	3	3.10
2,3-Me_2-ARAB	4	12.9
2,3,4,6-Me_4-GAL	4	
2,3,6-Me_3-GAL	5	18.4
2,4,6-Me_3-GAL	5	
2,3,4-Me_3-GLUC	5	
2,4-Me_2-GAL	6	62.3

METHYLATION OF LEMON GUM SI

Lemon gum SI was obtained from lemon gum after periodate oxidation, borohydride reduction and mild acid hydrolysis. The reduced and acetylated hydrolysate from methylated lemon gum SI gave a gas chromatogram much like that of lemon gum with some notable exceptions.

Peak 1: The structure of this component was 2,3,5-tri-O-methyl-L-arabinitol. The mass spectrum was similar to that from peak 3 of lemon gum with the minor contribution of 2,3,4-tri-O-methyl-L-rhamnitol removed.

Peak 2: Corresponding to peak 6 of lemon gum in R_T this peak contained only one component. That component was identified as 2,3,4,6-tetra-O-methyl-D-galactitol acetate from its mass spectrum.

Peak 3: Corresponding to peak 7 of methylated lemon gum this peak has no tri-O-methyl glucitol constituent. It contained, therefore, only 2,4,6-and 2,3,6-tri-O-methyl-D-galactitol.

Peak 4: Corresponding to peak 8 of lemon gum this peak was mainly 2,3,4-tri-O-methyl-D-galacitol. The presence of this component indicates the importance of the 1 $\longrightarrow$ 6 linkage in lemon gum. The increased signal strength for m/e 45 may be due to a trace of 2,6-di-O-methyl-D-galacitol acetate. This compound has the correct mobility and might result from undermethylation.

Peak 5: Like peak 9 of lemon gum this was a rather odd peak. Its mass spectrum fragmentation pattern indicated the presence of 3-O-methyl-D-galactitol acetate. The spectrum, however, lacks m/e 189 and it may be that this component both here and in lemon gum is a specific degradation product capable of fragmenting like 3-O-methyl-D-galactitol. This peak also appears to contain 23-di-O-methyl glucitol acetate. This compound may indicate some underoxidation during periodate cleavage.

Peak 6: The final peak is that of 2,4-di-O-methyl-D-galactitol.

FIGURE 29

GAS CHROMATOGRAM OF LEMON GUM SI METHYLATED POLYOL ACETATES
[140-205°C at 3°/min.]

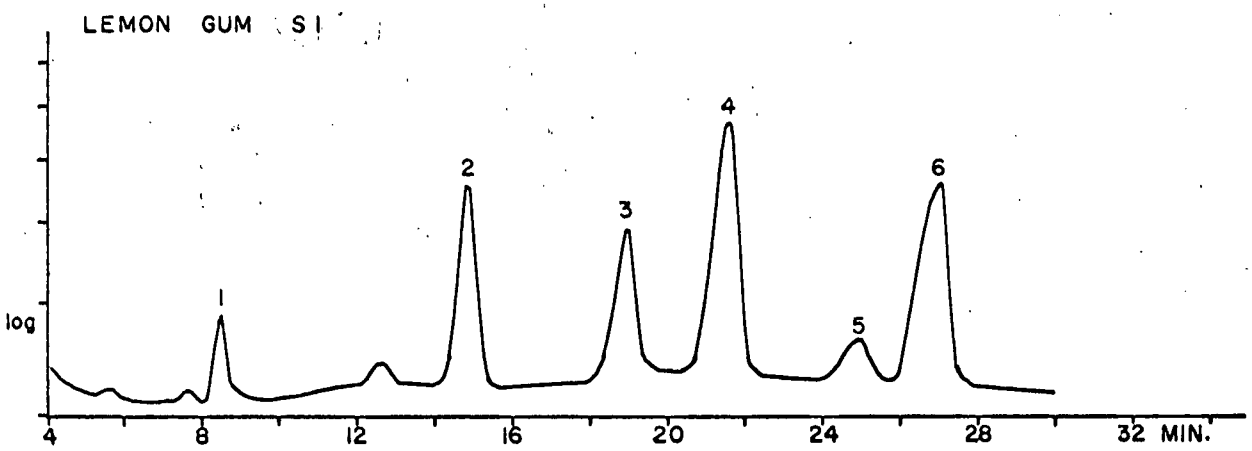


TABLE XIX

METHYLATION ANALYSIS OF LEMON GUM SI

Compound	G.L.C. Peak	Area %
2,3,5-Me_3-ARAB	1	4.69
3,5-Me_2-ARAB	Between 1 and 2	1.86
2,3,4,6-Me_4-GAL	2	15.5
2,3-Me_2-ARAB	2	
2,3,6-Me_3-GAL	3	15.1
2,4,6-Me_3-GAL	3	
2,3,4-Me_3-GAL	4	29.7
2,3-Me_2-GLUC	5	3.4
Unknown	5	
2,4-Me_2-GAL	6	29.0

The present work on lemon gum is the first example of analysis of a plant gum by the Lindberg et al [83] method of mass spectrometry [partially methylated alditol acetates]. The complexity of the lemon gum mixture analysed was such that alternate separations were required on TLC. Micro manipulation, as outlined in the appendex, of less than milligram quantities has facilitated the purification and mass spectrometry of the isolated methylated alditol acetates. Hakomori methylation [79] has given high yields and complete methylation improving the reliability of methylation analysis. The combination of Hakomori methylation and methylated alditol acetate mass spectrometry has reduced the quantity of polysaccharide needed for methylation analysis to the milligram range. The ability to manipulate these sub-milligram quantities of methylated hydrolysis products from polysaccharides will be of immense value to workers faced with complex mixtures or mass spectrometers without a coupled gas chromatograph.

Methylation analysis of lemon gum and degraded lemon gum are in good agreement with results obtained and the structure proposed by Jones and Stoddart. Analysis of Smith degraded lemon gum is also in good agreement with one minor exception. In the previous analysis of lemon gum there was a substantial and increasing amount of 3,5-di-O-methyl-L-arabinose in the Smith degraded gum. The increase has not been confirmed by this work - an unexpected result in view of the generally milder hydrolysis conditions employed in the preparation of the Smith degraded lemon gum.

EXPERIMENTAL

GENERAL METHODS

Paper chromatography was carried out on Whatman No. 1 and 3 paper by the descending method in the following solvent systems:

a] Ethyl acetate-acetic acid-formic acid-water [18:3:1:4].

b] Ethyl acetate-pyridine-water [8:2:2] top layer.

c] Butan-1-ol-pyridine-water [6:4:3].

d] Butanone-water azeotrope.

Thin layer chromatography was carried out on alumina, and silica gel [with and without calcium sulfate binder] by the ascending method in the following solvent systems:

e] Butanone-water azeotrope.

f] Benzene-methanol [96:4].

g] Ethyl ether-toluene [2:1].

Sephadex gel permeation chromatography was performed in a Sephadex column K25/45 fitted with flow adaptors using Sephadex G-15 fine. Prior to packing the column, G-15 was swollen in water for 16 hours. A constant pressure head was maintained by use of a Mariotte flask. Operation of the column was in the ascending mode using water with 0.5% chloroform as eluting solvent. Samples applied to the column through the flow adaptors using a hypodermic syringe barrel with luer lock adaptor were collected on elution by a timed fraction collector.

Reducing sugars on paper chromatograms were detected with either p-anisidine hydrochloride reagent [86] or alkaline silver nitrate dip [87]. Non-reducing carbohydrates and polyhydric alcohols were detected with either the latter dip

or alkaline periodate spray reagent [88]. The rate of movement of substances on paper chromatograms is quoted relative to the solvent front or an internal standard such as galactose.

Visualization of T.L.C. was accomplished by charring [sulfuric acid spray]. When samples were to be isolated, silicone grease was spread in thin ribbons on a clean glass plate. When pressed to the T.L.C. plate, a small amount of adsorbant was transferred, then charred in the normal manner.

Melting and boiling points are uncorrected. Unless otherwise stated optical rotations were measured at 21 $\pm$ 3°C.

Infrared absorption spectra were recorded using a Perkin-Elmer model 257 or 21. Samples were examined as KBr pellets, chloroform or carbontetrachloride solutions [5-10% W/V] in sodium chloride cells, or as thin films on sodium chloride plates.

Nuclear magnetic resonance spectra were determined on a Varian HA-100 or A-60 spectrometer.

Mass spectra were determined on an Atlas AEI MS9 spectrometer.

Gas liquid chromatography was carried out on an F and M model 720 dual column instrument fitted with thermal conductivity detectors. Polyhydric alcohols and sugars were analysed on two columns [8 ft. x 0.25 in. coiled copper] packed with equal weights [to within 20 mg] of 20% SF 96 on 60-80 mesh Diatoport S. The columns were held isothermally at 130° for 6 min. and then programmed at 3° per minute to hold at 220°. During analyses for sugars only, the programme was started at 190° and immediately programmed at 2° per minute to hold at 220°. The injection port was 270°, the detector block 295° and the helium flow 88 ml per minute [6.8 sec. for 10 mls].

For simple systems of the above compounds other columns may be used. Thus silyl derivatives of ethylene glycol, glycerol and methyl β-D-gluco-pyranoside may be separated on a 2 ft. x 0.25 in. column of SE30. This column was run isothermally at 70° until the ethylene glycol emerged, then programmed at 10° per minute to hold at 230°C.

Sugars were dissolved in water containing a small amount of chloroform [0.5% W/V] and allowed to come to equilibrium. Aliquots were removed and concentrated to dryness at 40° on a rotary evaporator. Solutions of the polyhydric alcohols in anhydrous pyridine were added to give the test solutions reported in Tables II - VI. These pyridine solutions [10 parts] were immediately silylated with hexamethyldisilazane [4 parts] and trimethylsilyl chloride [2 parts] and after 5 minutes shaking, portions were injected directly. The values quoted in the Tables represent the mean of at least five determinations.

For the determination of molar response factors separate solutions of the individual compounds with butane-1,4-diol were prepared. In the case of the sugars these runs also served to determine relative concentrations of different forms at equilibrium.

In preliminary experiments it was ascertained that none of the compounds involved were lost on passage through Amberlite IR 120 and Duolite A-4 resins nor on concentration of their aqueous solutions.

Methylated polyol acetates obtained from methylated polysaccharides were examined on columns with ECNSS-M and butanediol succinate liquid phases, the latter giving a slightly improved separation. Methylated polyol acetates were analysed on a stainless steel column [6 ft. x 0.25 in.] with butanediol

succinate [10% W/W] on Diatoport S [60-80 mesh]. Column temperature was programmed immediately from 140-205°C at 3° per minute using the standard flow rate and other settings.

Peak areas were measured initially with a Disc integrator with suitable correction for baseline drift. In later work an Infotronics digital integrator was used.

Silylation of galacturonic acid was carried out in a variety of solvents using bis-trimethylsilyl acetamide as the silylating reagent [89].

Acetylated carbohydrates were prepared by two methods. Some samples were dissolved in anhydrous pyridine and an excess of acetic anhydride added. The samples were allowed to stand 16 hours, then heated to 60°C for 1 hour. The solvents were removed by rotary reduced pressure evaporation. Alternatively, samples were dissolved in pyridine and an excess of acetic anhydride added. The samples were sealed in test tubes and heated at 100°C for 1 hour. Work up was similar to the above case.

Unless otherwise stated hydrolyses were carried out by either of the following methods. A sample [5-20 mg] was dissolved in sulfuric acid [1N] [5-10 ml] and hydrolysed in a sealed tube at 100° for 8-16 hours. Alternatively a sample [5-20 mg] was solubilized with 72% sulfuric acid [1 ml] for 1-2 hours at room temperature, then diluted to 7.2% with distilled water and hydrolysed at 100° in a sealed tube for 4 hours. After cooling the hydrolysates were neutralized partially with barium hydroxide, then completely with a small amount of barium carbonate. Alternatively complete neutralization was achieved using a slurry of barium carbonate. The precipitated barium sulfate together with

the residual barium carbonate was removed by centrifugation. The supernatant was then passed through a small bed of Amberlite IR120 [H + form] ion exchange resin to remove any cations present in solution. The column of Amberlite was often placed in series with a column of Duolite A-4 [OH - form] ion exchange resin to remove sugar acids or other acidic material. The eluant was concentrated to a syrup by evaporation under reduced pressure [ca. 15 mm].

Reduction of free sugars was carried out on the eluant from the ion exchange column [used to remove barium ions still in the hydrolysate]. A large molar excess [3-5 moles] of sodium borohydride was added to the eluant. After 24 hours excess borohydride was destroyed by addition of acetic acid. Sodium ions were removed using a column of Amberlite IR 120 [H + form] ion exchange resin. The solution was evaporated to dryness under reduced pressure, the resulting syrup being repeatedly dissolved in methanol and evaporated to dryness. The repeated evaporation served the dual purpose of removing the boric acid and traces of acetic acid remaining in the reduced sugar sample.

Reductions with lithium aluminum hydride were carried out on methylated polysaccharides and oligosaccharides in the following manner. To a 1% [W/V] suspension of lithium aluminum hydride in T.H.F. was added slowly a 2% [W/V] solution of methylated carbohydrate in anhydrous T.H.F. The reaction mixture was stirred during the addition and for 16 hours afterwards. Excess hydride was destroyed by cautious addition of water to the vigorously stirred suspension. The tetrahydrofuran solution was filtered and the insoluble residue extracted continuously with chloroform. Filtrate and extract were combined and evaporated to dryness.

Moving boundary electrophoresis was carried out by Dr. K. Hunt on a Perkin-Elmer electrophoresis apparatus Model 238 using 2 ml Tiselius cells.

Samples were prepared for analysis by dialysis of a solution of sample [1-3% W/V] in a barbituate buffer [veronal] against the same buffer solution. This latter solution was used in the buffer vessels. During the electrophoresis the power output was adjusted to approximately 2 watts.

Dialysis of samples was carried out in viscose tubing against running water when only the polymer was desired or against a static volume when collecting dialysable materials. Dialysis of DMSO solutions resulted in extensive loses unless water was added to dilute the DMSO before dialysis.

The formation of methyl ethers was carried out by one or a combination of two of the following methods. These were:

I] Hakamori's method [79]

II] Purdie's method [62]

III] Kuhn's method [72]

A generalized account of these methods as applied to the methylation of poly, oligo and monosaccharides is given below.

I] HAKOMORI METHOD [79]

The methylation procedure was essentially that described by Hakomori [79] wherein the methylsulfinyl anion [90] was used to generate the polysaccharide alkoxide prior to the addition of methyl iodide. Routinely, 1 g. samples of polysaccharide were methylated. The procedure described is for this amount.

The methylsulfinyl anion was prepared as follows. Into a dry round bottom flask [250 ml] containing a magnetic stirring bar was weighed 1.5 g. of sodium hydride [55%, coated with mineral oil]. The sodium hydride was washed three times by stirring with 50 ml portions of anhydrous petroleum ether [30-60°] and

decanting the wash. After the third wash the flask was fitted with a serum cap. The residual petroleum ether was removed by successive evacuations with a vacuum pump through an 18-gauge hypodermic needle inserted through the serum cap. After each evacuation, the flask was filled with nitrogen [dried by passage through sulfuric acid and sodium hydroxide]. The flask filled with nitrogen was then placed in a nitrogen filled drybox. The serum cap was removed and 15 ml of anhydrous dimethyl sulfoxide was transferred into the flask. DMSO was dried with and distilled from calcium hydride under reduced pressure then stored over dried molecular sieve [Linde, type 4A]. The flask was stoppered with a mercury gas trap and removed from the drybox. The flask was placed in a force draft oven at 60°C and swirled occasionally until the solution became clear and green and the evolution of hydrogen gas had ceased [ca. 60 minutes].

For generation of the polysaccharide alkoxide, polysaccharide was first freeze dried and then dried overnight at 60° under vacuum. Dried material [1 g.] was added to 50 ml of dried dimethyl sulfoxide in a 250 ml round bottom 2 necked flask containing a magnetic stirrer. The flask was tightly stoppered with a polyethylene stopper and removed from the drybox. The sample was stirred until all the polysaccharide had dissolved. Polysaccharide and methylsulfinyl anion solutions were placed in the drybox. A 50% excess over the number of equivalents of base required for hydroxyl plus carbonyl was added to the polysaccharide. The number of equivalents required was calculated on the basis of the polysaccharide structure. Upon the addition of the anion [$\sim$ 12 ml] to the polysaccharide a gel formed. The flask was stoppered with a serum cap and thermometer, before removal from the drybox. The gel, on stirring, liquified and the reaction mixture appeared homogeneous. The reaction was allowed to run for 4 hours, a time indicated as minimum for complete alkoxide formation [78].

In the methylation reaction the polysaccharide alkoxide solution was cooled to less than 20° in an ice bath. Methyl iodide [5 ml] was added to the stirred solution with a hypodermic syringe at such a rate that the temperature did not rise above 25° [$\sim$ 10 minutes]. The amount of methyl iodide added was not critical as long as it was in molar excess of the base. Shortly after addition of methyl iodide, heat evolution ceased, the solution became clear and the viscosity was markedly reduced. At this stage reaction was complete. The reaction mixture was poured into water, then dialysed overnight against running water. The methylated polysaccharide partially precipitated by water came completely out of solution on dialysis. The methylated polymer was washed from the dialysis tubing and dissolved in chloroform. The chloroform solution was dried with anhydrous sodium sulfate and evaporated to dryness. The methylated polysaccharide was fractionated using chloroform and petroleum ether [30-60°] extractions. The major fractions were dried in vacuo and analysed by IR and microanalysis.

II] PURDIE METHOD [62]

The Purdie method was generally applied only to partially methylated polysaccharides. The partially methylated polysaccharide was dissolved in methyl iodide or a methyl iodide/acetone mixture. The solution was vigorously stirred and boiled under reflux in the presence of Drierite. Silver oxide was added in portions over a period of 6-18 hours. The silver oxide was removed by filtration through a Celite pad and was continuously extracted in a Soxhlet with boiling chloroform. If acetone was a component of the original mixture the silver oxide was washed with acetone or methanol instead of chloroform. The combined filtrate and washings were concentrated to dryness and the material was examined by means of an IR.

III] KUHN METHOD [72]

This method was employed with partially methylated polysaccharides. The partially methylated polysaccharide was dissolved in a mixture of redistilled dimethyl formamide and redistilled methyl iodide. The solution was stirred with Drierite. Silver oxide was added in small portions over two hours and stirring was continued for a further 16 hours. The solid material was removed by centrifugation and was washed with portions of dimethyl formamide and chloroform. The centrifugate and the washings were poured into a solution of sodium cyanide [1% W/V] and this solution was extracted with chloroform. Extracts were concentrated to remove chloroform and residual dimethyl formamide was removed by continual co-distillation with water. In some experiments Drierite was omitted, in others the methylated polymer was isolated by dialysis. Distillation of dimethyl formamide was intentionally carried out without stringent precautions for the exclusion of moisture.

Often traces of silver salts remain in the methylated product after Kuhn and Purdie methylations. These salts were sometimes successfully removed by redissolving the methylated polysaccharide in chloroform and filtering the solution through a Celite pad.

METHYLATION OF OLIGO AND MONOSACCHARIDES

PURDIE AND KUHN METHODS

The methods used were similar to those described for the polysaccharides with the following modifications. During Kuhn methylations a second portion of silver oxide was added after 4 hours of stirring. In addition dialysis was not applicable in these cases.

HAKOMORI METHOD

The method described for polysaccharides was followed for mono and oligosaccharides with the following exceptions and changes. Some of the lower molecular weight samples could not be freeze dried. As well the dialysis step was of necessity omitted. The samples were isolated by extracting with chloroform the water-dimethyl sulfoxide solution used for dialysis in the polysaccharide case. After three extractions or continuous extraction the chloroform was washed with distilled water to remove dimethyl sulfoxide. The products were evaporated to a syrup and residual dimethyl sulfoxide was removed by high vacuum distillation of a part or all of the sample.

PERIODATE OXIDATION

Analytical periodate oxidations were carried out in the following manner. Duplicate samples of the ash-free polysaccharide [$\sim$ 50 mg accurately weighed] were dissolved in water and the pH was adjusted to 7. The solutions were transferred to standard flasks [100 ml] fitted with ground glass stoppers and sodium metaperiodate solution [25 ml] was added. This solution was obtained by dilution of a 0.3M sodium metaperiodate solution [10 ml diluted to 100 ml]. The polysaccharide solution was made up to 100 ml and stored in the dark at 0° or room temperature. Aliquots [5 ml] were removed at intervals for the estimation of periodate consumption. Duplicate solutions which contained sodium metaperiodate and water but no polysaccharide were made up in the same manner to act as reagent blanks.

ESTIMATION OF PERIODATE [91]

An aliquot [5 ml] was added to a solution containing a phosphate buffer at pH 7 [25 ml] and 20% potassium iodide solution [10 ml]. The liberated iodine was titrated against standard sodium thio sulfate [0.01N] using starch as the indicator. The phosphate buffer contained disodium hydrogen phosphate [5.68 g.] and potassium dihydrogen phosphate [3.62 g.] in one liter of distilled water.

SIMULTANEOUS ESTIMATION OF POLYHYDRIC ALCOHOLS AND SUGARS

The experimental work outlined here was part of a group project involving Reid, Jensen and Gibney [92]. The estimation of erythritol with threitol, the computer analysis of these results and examinations of the effects of glycolaldehyde were mainly the work of Jensen.

Columns employing the following liquid phases were evaluated for their potential use in the separation of galactitol, glucitol and mannitol acetates:

Apiezon J, K, L, M, N
Butane 1,4-diol Succinate
Carbowax 20M
FAPP
Neopentyl Glycol Sebacate
Silicone DC QF-1
Silicone GE SE30, 52, SF96, XE-60
Versamide 900

None of these columns would produce the required separation. The literature has since reported two systems which will achieve this separation. However, the columns when used on thermal conductivity have resulted in only limited

success. If large samples are injected the columns overload and the separation is lost [93]. A certain advantage is apparent when galactose is a minor component of the mixture.

Columns were examined for the separation of equilibrium mixtures of neutral sugars using the silylation technique of Sweeley et al [15]. The columns examined were:

Silicone GE SF-96
Silicone GE SE-30
Silicone GE SE-52
Dow Corning Silicon High Vac Grease

The column having the clearest resolution was that reported in this work [SF-96]. It has been shown in the discussion that a scheme can be devised where, knowing the equilibrium values of the components, the total analysis of a sample of the five common neutral sugars may be evaluated. The column length, the programme rate and the flow rate were chosen experimentally to give optimum splitting between the two tetritols, erythritol and threitol.

Samples of the following polyhydric alcohols were purified by vacuum distillation; ethylene glycol, glycerol and butane -1,4-diol. D-Threitol and erythritol were recrystallized to literature melting points. The five neutral sugars were recrystallized and analysed by paper chromatography and gas liquid chromatography for impurities. Pyridine refluxed over sodium hydroxide pellets was distilled. A narrow boiling range fraction was stored over sodium hydroxide pellets. Hexamethyldisilazane and trimethylchlorosiline were used as obtained from Penninsular Chem Research.

Analysis of mixtures of internal standard and each of the five neutral sugars was carried out in the following manner. The sugars were dissolved in water containing 0.5% W/V chloroform and allowed to equilibrate. A period of

24 hours appeared to be ample with no further change being detectable in the equilibrium ratio determined. The sample evaporation to dryness was critical to avoid any change in equilibrium. The samples were evaporated under vacuum on a rotary film evaporator until the sample just became dry, a further minute of evaporation was allowed and then the sample was removed. Immediately a sample of pyridine containing the internal standard was pipetted into the evaporation flask. The samples were swirled and hexamethydisilazane then trimethylchlorosilane were added. Any variation from this procedure will result in alteration of the equilibrium mixture. This was especially pronounced when a component which crystallized easily such as xylose was predominant. The equilibrium ratios found for pyridine are similar to those for water [22] resulting in little change in the equilibrium mixture if these conditions are rigidly followed.

Analysis of single compounds and internal standard butane -1,4-diol yielded data reported in the discussion for molar response factors and equilibrium concentrations for various anomers. Sample size was varied with respect to internal standard over a wide range to confirm that detector response was linear with sample size. All other parameters were held constant to minimize variation in molar response factors.

Sufficient data were evaluated to provide molar response factors and equilibrium anomer ratios [at least three samples were run in duplicate for peaks of the polyols, five samples were run in duplicate for sugars]. Samples were then prepared of the various synthetic mixtures [expected to result from periodate oxidations of plant polysaccharides]. Samples were analysed with three runs in each sample determination. The data have been presented in the discussion.

Data were also obtained using erythritol as the internal standard and employing the shorter programme from 190-220° as a method for the analysis of crude hemicellulose hydrolysates as reported in the discussion.

Accumulated experimental data indicate that for sugar analysis the following limiting percentages may be determined.

Xylose	0.5%
Arabinose	0.5%
Mannose	0.5%
Galactose	1.0%
Glucose	0.5%

The value expressed is normally a practical limit in general hydrolyses. For a clearly detected sugar it may be considerably lower, in the order of 0.01%. Galactose is notably higher because only a single peak containing 30% of the total peak area is visible in hemicellulose hydrolysates.

The final check on this method was the analysis of three samples prepared independently and analysed by the proposed method. The results were in good agreement with expected values.

PURIFICATION OF LEMON GUM

Lemon gum*[crude] was dissolved in water and filtered through several layers of gauze followed by sintered glass. The solution was poured in a thin stream into a five volume excess of alcohol acidified with acetic acid. This procedure, however, left the gum with a high ash so the gum was redissolved and purified by two alternate routes which yielded products which were identical. A solution of gum was acidified with hydrochloric acid

* The sample of lemon gum used in these experiments was received from the late Dr. E. Anderson.

and precipitated into 5 volumes of alcohol. This product showed only a trace of ash. A second portion of gum solution was deionized by passage through Amberlite IR 120 [H+]. The solutions were immediately frozen and freeze dried. Optical rotation of the products gave identical values of $[\alpha]_D$ +19.2°. The polymers were hydrolysed and analysed by G.L.C., for results see discussion. A sample of the freeze dried gum was analysed by Tiselius moving boundary electrophoresis and showed only a single band. The gum was also chromatographed on DEAE cellulose [2.5 x 50 cm] using water [150 mls] followed by 0.05M NaH_2PO_4 [150 mls] increasing by equal steps to 0.25M NaH_2PO_4. The gum was eluted during 0.10M NaH_2PO_4 irrigation. The gum tailed somewhat on the column and as the ionic strength was increased more carbohydrate was eluted from the column. The bulk of the gum was isolated in a volume of 50 ml, tubes 72 to 82. G.L.C. analysis indicated that the product isolated had the same neutral sugar analysis as the starting material and an identical optical rotation of $[\alpha]_D$ +19.3°.

The neutralization equivalent of the ash free gum was determined by titration with 0.100N NaOH using a pH meter. The equivalent weight was calculated to be 793 g.

OPTIMUM HYDROLYSIS OF LEMON GUM - PERIODATE DEGRADED

Analytical periodate oxidation of lemon gum produced results in close agreement with those reported earlier for this gum [1].

Lemon gum [20 g.] dissolved in water [1000 mls], was oxidized with sodium metaperiodate [29 g.] for one week at 5°C. Dialysis was used to removed low molecular weight materials after destruction of excess periodate with ethylene

glycol. The polyaldehyde present in the dialysate was reduced with sodium borohydride [5 g.] for 48 hours. An excess of acetic acid was added to destroy unreacted borohydride and aid in break down of borate complexes. Dialysis was again used to remove low molecular weight materials, yield 12.5 g.

Samples of the polyalcohol [20 mg] were dissolved in water [5 ml] and the acid concentration was adjusted so that the final volume [10 ml] had the following sulfuric acid concentrations; 0.05, 0.1, 0.2, 0.5 and 1.0N. Hydrolysis was continued for 24 hours at room temperature. The acid was neutralized with barium carbonate. The residual polysaccharide was isolated by precipitation and analysed on the gas chromatograph. The 0.5N acid solution, as indicated in the discussion, produced the most satisfactory hydrolysis, as determined by residual glycerol [see page 22].

A sample of polyalcohol [200 mg] was dissolved in water and the normality of the acid and volume were adjusted to 0.5N and 20 mls. Samples [2 ml] were removed from the reaction mixture at the following intervals: 1, 2, 4, 8, 16 and 32 hours. Precipitation of the sample taken was carried out by direct dilution with alcohol [8 ml]. Immediate centrifugation and work up minimized further hydrolysis.

In addition to the samples taken above, a sample of precipitated polysaccharide [32 hours] was dialysed for 24 hours against running water.

Neutralization of a sample of supernatant from the 32 hour hydrolysis precipitation provided a fraction containing material lost during hydrolysis.

Gas chromatographic analysis was carried out on hydrolysates of fractions isolated including a sample of starting material and a sample of unhydrolysed

soluble material at 32 hours. The data from these hydrolyses are presented in Table X [see page 26].

Lemon gum polyalcohol [9.17 g.] was hydrolysed for the required 16 hours with 0.5N H_2SO_4 [400 ml]. The solution was neutralized with barium carbonate. Yield of precipitated polysaccharide designated lemon gum SI on addition of 4 volumes of alcohol, 3.51 g. The supernatant fraction was evaporated to a syrup under reduced pressure. To this thick syrup was added 30 mls of methanol and 30 mls of acetone [2 times] to extract the very low molecular weight fraction. The fractions were centrifuged to remove the insoluble higher molecular weight material. On evaporation of the 120 mls of solution 3.89 g. of product was obtained. The insoluble material was dissolved in water and freeze dried, yield 1.06 g.

Lemon gum SI [100 mg] was oxidized with an excess of periodate until the oxidation levelled off [88 hours]. Periodate consumption was 0.40 moles per anhydro sugar based on galactose and arabinose analysis. A larger scale oxidation of 2.30 g. of polysaccharide yielded 1.22 g. of poly alcohol.

Smith degradations carried out as before indicated that an optimum yield of polysaccharide was obtained using the same hydrolysis conditions as outlined for the first periodate oxidized polysaccharide, namely 0.5N H_2SO_4 for 16 hours. Hydrolysis under these conditions of $2xIO_4/BH_4$ lemon gum yielded a polysaccharide containing only a limited amount of arabinose in comparison to galactose as outlined in the discussion.

SMITH FRAGMENTS

Direct gas chromatography of the soluble portions from the original periodate oxidized gum programmed to elevated temperature on SF-96 and SE-52 [10% on 80-100 Diatoport S, 8' x 1/4" and SE-30 [20% on 60-80 Chromasorb W, 2' x 1/4"] showed only one component in sufficient quantity to isolate and identify. The column temperature [275°C] was such that maltotriose was eluted [SE-52 - 80 min., SE-30 - 30 min.] [94]. Isolation of a small sample and hydrolysis yielded galactose and glycerol. A second sample was isolated, the silyl groups were removed by methanol/water and the sample was methylated by the Hakomori procedure. Hydrolysis, reduction and acetylation produced a partially methylated alditol acetate with the same mobility as in authentic sample of 2,3,4,6-tetra-O- methyl-D-galactitol acetate. Analysis of the mass spectrum confirmed this assignment.

THE REDUCTION OF ACIDIC POLYSACCHARIDES

ESTERIFICATION

Lemon gum [1.00 g.] which had been deionized by passage through Amberlite IR-120 [H+-form] and freeze dried was dissolved in water [50 mls]. Ethylene oxide [12.5 g.] was bubbled into the polysaccharide solution cooled in an ice bath. The vessel was sealed and stored at room temperature. During the next fourteen days the sample was cooled daily in an ice bath and the pH of the solution was measured. The solution pH rose from an initial value of 3.2 to slightly over 7 at the end of the fourteenth day. The gum solution was dialysed against running water for twenty-four hours and freeze dried, yield 0.93 g.

Analysis of the degree of esterification was carried out using acid hydrolysis with 1N H_2SO_4 at 100°C for 20 hours. The quantity of ethylene glycol and what is presumed to be diethylene glycol were in excess of the molar requirements for complete esterification.

REDUCTION OF ETHYLENE GLYCOL ESTER OF LEMON GUM

Lemon gum ester [1.00 g.] was dissolved in dry dimethyl sulfoxide [dried as for Hakomori methylations] [50 ml.]. To this solution was added lithium borohydride [0.5 g.]. Such a large molar quantity was required for interaction with free hydroxyl functions in the polysaccharide. The solution formed a gel during the first hours of reduction. The gel was relatively stable and persisted throughout the reduction. The gel could be broken down partly by swirling the mixture. The reaction was maintained anhydrous and hydrogen gas was vented through a mercury trap. The reduction was monitored by daily analysis of aliquots. All samples were worked up in the same manner. Acetic acid was added slowly to the DMSO solution to destroy the excess borohydride and to break down borate complexes. The samples were dialysed then freeze dried, yield 0.83 g.

Material [1.00 g.] from a large scale esterification and reduction was subjected to a second esterification to re-esterify any acids which were saponified. Salts of uronic acids were converted to free acids by ion exchange chromatography as in the previous reduction. Quantities of ethylene oxide and duration of reaction were as before, yield 0.96 g.

This freeze dried ester was reduced by the method indicated previously, yield 0.90 g.

The samples were analysed by gas chromatography and the results are reported in the discussion.

SIGNAL ENHANCEMENT

Compounds which were examined by silylation of free hydroxyls [Figure 16] were synthesised by known pathways or purified by preparative gas chromatography as reported in the discussion. Pure starting materials were silylated and purified by both gas chromatography and simple removal of silylating reagents by evaporation under reduced pressure.

TRIMETHYL SILYLATION OF GALACTURONIC ACID

A commercial sample of D-galacturonic acid was silylated using each of the following solvents: pyridine, dimethyl formamide, dimethyl sulfoxide, hexane and dioxane, by warming and triturating the sample in the solvent with added bis-trimethylsilyl acetamide [BTMSA]. There were significant variations in the percentages of sample silylated. Those solvents which dissolved the galacturonic acid showed better yields of silylated product. In addition, silylation was carried out in pure BTMSA. The percentage of anomers present when these solutions were analysed on the gas chromatograph varied widely. The best general yield and the most reproducible result was obtained with pure BTMSA.

TABLE XX

ANOMERIC RATIOS FOR BTMSA SILYLATION
OF GALACTURONIC ACID IN DIFFERENT SOLVENTS

Solvent	Peak 1 %	Peak 2 and 3 %	Peak 4 %
BTMSA	49.5	24.3	26.1
DMF	53.1	18.1	28.8
Dioxane	52.5	43.3	4.2
Pyridine	18.8	33.4	47.8
DMSO	36.2	33.6	30.2
Hexane	45.0	30.9	24.1

METHYLATION ANALYSIS

Lemon gum [1 g.] was methylated by the Hakomori procedure described earlier, yield 0.90 g. A second methylation of this methylated material [0.70 g.] was carried out and the DMSO solution was poured directly into a dialysis sack without dilution by water. The weight of recovered lemon gum was only 0.020 g.

A second methylation [5.0 g.] was carried out, yield 6.198 g. This sample was fractionated using chloroform in 30-45° petroleum. The extracted fraction insoluble in 20% $CHCl_3$ but soluble in 30% $CHCl_3$ contained 6.011 g. of the total sample. Analysis: OMe 39.46%. A small sample [1.0 g.] of methylated lemon gum was acidified with anhydrous hydrogen chloride in chloroform. A small amount of solid material [NaCl] was centrifuged off and the polysaccharide was precipitated with 30-45° petroleum. The polysaccharide was dried under high vacuum over sodium hydroxide. This methylated polysaccharide was given a Purdie methylation in order to complete the methylation of the uronic acids. Analysis: OMe 40.2%

This polymer, although still showing traces of uronic acid as the salt form, was reduced with lithium aluminum hydride to give a product showing only a trace of carboxyl, yield 90%.

The following polymers [100 mg] were also methylated by the Hakomori procedure:

a] Lemon gum degraded with 1/10N H_2SO_4, yield 105 mg.

b] Lemon gum periodate oxidized and borohydride reduced before hydrolysis, yield 82 mg.

c] Lemon gum periodate oxidized and borohydride reduced after 0.5N H_2SO_4 hydrolysis, yield, 95 mg.

None of these polymers was reduced with lithium aluminum hydride.

All four of these methylated polymers were analysed by the method outlined in the introduction to the experimental section.

The following monosaccharides were prepared by known routes to facilitate the peak identification on the gas chromatograph:

2,3,4,6-tetra-O-methyl-D-galactose
2,3,4-tri-O-methyl-D-rhamnose
2,3,5-tri-O-methyl-L-arabinose
2,3-di-O-methyl-D-glucose
2,3,4-tri-O-methyl-L-arabinose

APPENDIX I

ESTIMATION OF DEGREE OF POLYMERIZATION

During the course of evaluating G.L.C. as a method for the estimation of polyhydric alcohols and sugars, it became apparent that these procedures could be applied to an estimation of D.P. of oligosaccharides and small polyaschar-ides. Reduction of the reducing end group with sodium borohydride produces on hydrolysis the terminal component which may be estimated with respect to the remainder of the non reduced carbohydrate. Work by Reid, Rowe, Rowe and Dutton [95] has shown that a D.P. of 150 may be estimated in this manner.

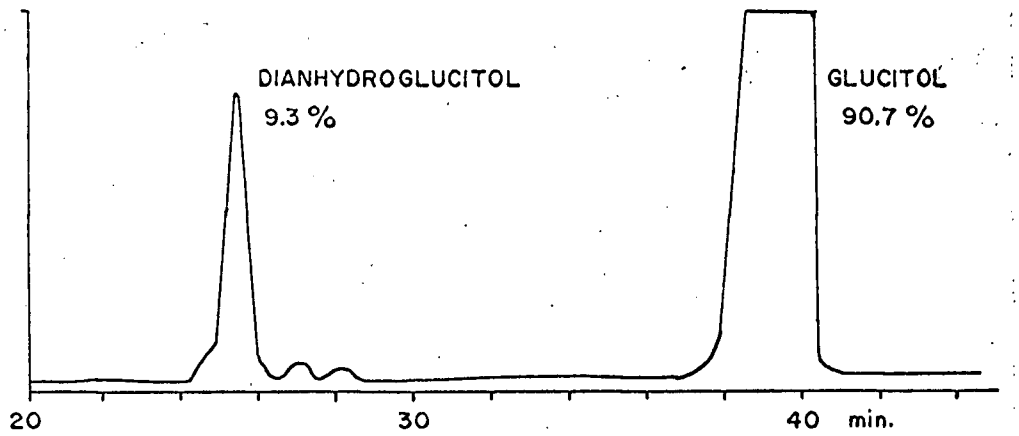
Previous experience with hydrolysis of solutions containing polyols in-dicated a need to examine closely the hydrolysis of reduced oligosaccharides. Certain factors must be taken into consideration before D.P. may be estimated by the method of Reid et al. The compound[s] being reduced must be known. Also within limits complete reduction must occur. The complexity of the sugar mix-ture on hydrolysis must be such that the polyol produced on reduction is clearly resolved on G.L.C. from the remaining anomeric carbohydrate forms. Only with very low D.P. oligosaccharides can analysis be successfully carried out when overlap occurs. It is known that sugars decompose during the hydrolysis re-action. Previous work in this laboratory has indicated that dehydration of polyols to anhydropolyols can also occur [85].

A series of maltosaccharides were obtained by enzymatic hydrolysis of starch with α-amylase. Isolation of the higher D.P. maltosaccharides [D.P. > 4] in a pure state was not possible although paper and Sephadex chromatography were employed in the attempted purifications. Prior to examination of the re-duced maltosaccharides the effects of hydrolysis on glucitol were investigated.

Prolonged acid hydrolysis confirmed that dianhydro-D-glucitol was formed as the major dehydration product. This was in agreement with previous hydrolysis findings [85]. The more volatile component in the gas chromatogram was confirmed to be 1,4,3,6 dianhydro-D-glucitol by mass spectrometry and comparison with an authentic sample.

FIGURE 30

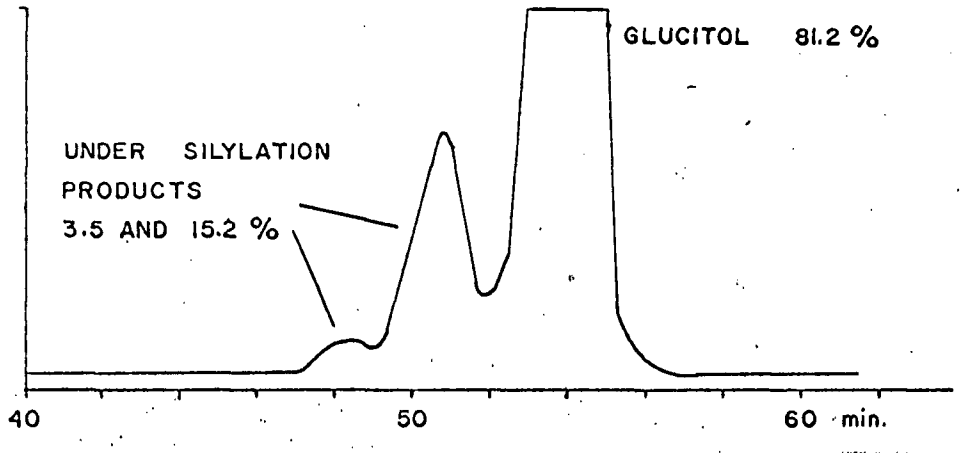
GAS CHROMATOGRAM OF GLUCITOL HYDROLYSED WITH 1.0N H_2SO_4 FOR 24 HOURS
Standard analysis conditions but programmed at 10°/min.



During these hydrolysis studies it was discovered and has now been reported independently by another laboratory [58], that undersilylation can occur. The undersilylated product is eluted before the fully silylated derivative. The silylation of hexitols is much slower than for lower molecular weight polyols or the parent sugars. Glucitol in the presence of excess reagent requires two hours of reaction before the under silylated component becomes insignificant. Further work is being carried out to confirm the structure of these under silylated hexitols.

FIGURE 31

GAS CHROMATOGRAM OF GLYCITOL SHOWING UNDERSILYLATION AFTER 10 MIN. OF REACTION



In order to obtain the maximum D.P. accuracy using reduction, hydrolysis and G.L.C., careful consideration must be taken of carbohydrate degradation and loss of polyol by dehydration.

EXPERIMENTAL

A series of glucose oligosaccharides was isolated by enzymatic hydrolysis of starch [95]. Initial purification was achieved using a stubby charcoal column [10 cm in diameter x 2.5 cm deep]. The glucose and maltose were displaced from the column by gradually increasing the alcohol to water ratio in the elution solvent. The alcohol percentage was increased to 50% and the higher D.P. oligosaccharides were eluted as a group. These higher oligosaccharides were spotted on Whatman No. 3 filter paper and chromatographed in solvent C.

Components were detected by development of guide strips. The bands of oligosaccharides were eluted with water and the sample was isolated in an easily handled form by freeze drying. Samples of the major components were rechromatographed on paper and Sephadex. Only in the case of the tri- and tetrasaccharide were relatively pure compounds isolated. Gas chromatographic analysis of the reduced oligosaccharides did not produce accurate [± 5%] D.P. values because of trace quantities of higher oligosaccharides present as contaminants.

Samples of glucitol were hydrolysed and analysed by gas chromatography. The presence of 1,4; 3,6-dianhydro-D-glucitol was confirmed by comparison of mass spectra obtained from an authentic sample and one isolated by gas chromatography.

APPENDIX II

ENZYMATIC HYDROLYSIS OF LEMON GUM

Enzymatic hydrolysis has been employed successfully in the glycolytic cleavage of certain classes of polymers. Notable is the extensive work which has been carried out on various starches [97]. A wide variety of different enzymes has been discovered and utilized in preparing the maltosaccharides for the earlier work on D.P. and amylo 1,6-glucosidase, the enzyme which debranches 1,6 linkages. The enzymatic hydrolysis of cellulose and some of its derivatives is well known. The fact that ruminants convert cellulose containing plants into useful food sources, microorganisms destroy cellulosic fabrics, and the host of wood rots decompose wood, has also focussed attention on nature's abundant cellulase activity. The food industry employs pectinase preparations for clarifying wines, fruit juices and liquefaction of pectin containing gels. Xylans from wood and plant sources have been hydrolysed with pectinase and xylanase preparations to yield a product containing xylose and a series of oligosaccharides [98]. As a result of the success encountered with these enzymatic degradations an examination was carried out on the effects of various general enzyme preparations on lemon gum. Table XXI lists the enzyme preparations employed in this survey.

TABLE XXI

ENZYME PREPARATIONS EXAMINED FOR ACTIVITY ON LEMON GUM

α -Amylase [salivary]
α -Amylase [BDH[A] -plant source]
Pectinol 41-P concentrate [Rohm and Haas]
Pectinase [BDH]
Hemicellulase [BDH]
HP-150 [Rohm and Haas]
Cellulase [BDH]
Cellulase 36 [Rohm and Haas]
β -Glucosidate [BDH]
Xylanase [BDH]

[A] BDH - British Drug House

Although some of the preparations listed in Table XXI were not expected to show activity they were examined because of possible activity of minor impurities. Two of the preparations [Hemicellulase and HP-150] were expected to show the highest activity. This was especially true of the latter material since it is designed to reduce viscosity in plant gum solutions.

Assay of the α-amylases, pectinases, β-glucosidase and xylanase with lemon gum showed no activity. The enzymes present in these preparations were active as was evidenced by formation of maltosaccharides and a series of xylose oligossacharides from birch xylan [98]. The remaining enzymes could not be assayed by dialysis [99] due to high cellulase activity. The dialysis tubing was attacked, resulting in release of glucose oligosaccharides and rupture of the tubing. Activity of these preparations on lemon gum was examined after the substrates had been in contact for two days. The enzyme was inactivated by a short heat treatment. There were several minor low molecular weight components in the solutions. They were, however, attributable to contamination of the enzyme preparation or autohydrolysis of the lemon gum substrate.

Lack of activity with these latter preparations was unexpected. A sample of HP-150 was fractionated with ammonium sulfate into a series of precipitates. Assayed against lemon gum these fractions were still not active. Fractionation was also carried out on Pentinol 41P. No activity was found in the isolated fractions. The only oligosaccharides present on dialysis of fractionated HP-150 or Pectinol 41P treated lemon gum were attributable to autohydrolysis. This fact was verified by running blanks containing only lemon gum and distilled water. During these experiments a parallel set was being conducted on mesquite gum by Rowe [100]. Equally negative results were obtained. In addition, it was

discovered that HP-150 had transferase activity capable of forming arabinose oligosaccharides. Since, on hydrolysis of either lemon or mesquite gums large amounts of arabinose are expected, any oligosaccharide isolated which contained arabinose would be suspect. The lack of activity against lemon gum and the presence of transferase activity would appear to rule out convenient hydrolysis of lemon gum.

When the role of the transferase activity is more fully known [100] it may be possible to utilize HP-150 for the hydrolysis of oligosaccharides obtained by hydrolysis of lemon gum. Careful examination will be necessary to ensure no other transferase activity [for example galactose transferase] exists.

The closing discussion is speculation regarding low activity against a polymer such as lemon gum. Lemon gum is a highly branched and extremely complicated molecule containing many different subunits. The gum is exuded on damage to the tree bark. To be effective as a barrier the exudate must not be readily attacked by organisms. In an evolutionary sense those plants which exuded a barrier which was difficult to degrade would have had the best chance of survival. The inability of any of these enzyme preparations to degrade lemon gum may be a reflection of this evolutionary protection. An interesting experiment would be the examination of micro-organism growth on media containing lemon gum. When samples of gum have become contaminated in our laboratory, after a short growth period further growth seems to stop. This growth stoppage may reflect a lack of nutrients such as nitrogen or complete utilization of the carbohydrate available as a result of autohydrolysis. It is possible also, that a pH change or formation of a metabolite may be factors which are limiting growth. Experiments designed to test these factors may indicate whether there is limiting growth rate or ability to utilize lemon gum as a carbon source.

EXPERIMENTAL

ENZYMATIC HYDROLYSIS OF LEMON GUM

The enzyme preparations listed in Table XXI were screened for activity on lemon gum by the following procedure. A small sample of ash free gum [$\sim$ 1.0 g.] was dissolved in water [50 ml] and a sample [5 ml] was pipetted into solutions of the various enzyme preparations. The samples were allowed to interact for 48 hours, then excess enzyme and polysaccharide were precipitated with alcohol. The reactions were carried out in centrifuge tubes [50 ml] to facilitate work up. A second solution of lemon gum was adjusted to pH 7 with sodium bicarbonate and a similar series of analyses repeated.

Paper chromatograms were spotted with samples from the concentrated supernatants of the two experiments, with a blank from a pure gum solution and a blank of each enzyme. The chromatograms showed no spots which could not be attributed to either autohydrolysis of the lemon gum or contaminating carbohydrate from the enzyme preparations.

Samples of the enzymes and lemon gum were also examined by dialysis [99], since it was possible that a certain concentration of oligosaccharide could inhibit further enzymatic hydrolysis. The results as before were negative except with lemon gum acid which showed autohydrolysis products. Four enzyme preparations were not amenable to dialysis as a means of oligosaccharide isolation. Hemicellulase, HP-150 and the two cellulase preparations show such high cellulase activity that the viscose tubing, even though forming a heterogeneous reaction site, was weakened to the point that it ruptured. In addition new compounds formed by enzymatic hydrolysis of viscose tubing were released into the solution.

These four preparations were examined by a second dilute reaction solution and the enzyme was inactivated by heating. Alcohol precipitation and concentration of the whole solution were both used to prepare samples for paper chromatographic examination. The results for the dialysis and the latter experiments were as before negative showing no increase in oligosaccharides which could be attributed to enzymatic hydrolysis.

FRACTIONATION OF PECTINASE, CELLULASE AND HP-150

Pectinol 41-P concentrate, Cellulase and HP-150 were all fractionated using ammonium sulfate by the following general scheme. Enzyme preparation [10 g.] was dissolved in water [200 ml] containing sodium bicarbonate [4 g.]. Additions of ammonium sulfate were made to the solution in approximately 10 g. lots. When a precipitate formed it was centrifuged off and further additions of ammonium sulfate were made. No attempt was made to determine quantitative data on various fractions. Rather a general qualitative result was determined.

PECTINOL 41-P CONCENTRATE

A significant portion of enzyme preparation was insoluble in water because of filler added to the preparation. The prepared enzyme fractions were reacted with dilute solutions [1-2%] of lemon gum. All samples gave results similar to an autohydrolysed gum sample.

PECTINOL 41-P FRACTIONATION

Ammonium Sulfate [g.]	Molish on Enzyme	Samples Retaining Molish Color	Amount of Ppt
0	+	--	large
40	++	++++	small
50	++	--	small
60	++	--	fair
70	++	++	fair
80	++	--	fair
90	++++	++++	fair
100	++++	+++	fair
110	+++++	+++++	fair
Final Solution	+++	++	--

CELLULASE 36

A slightly cruder fractionation was obtained on this enzyme.

Ammonium Sulfate [g.]	Amount of Ppt	Molish Test
0	large [filler]	+
60	small	+++
120	fair	+10
120 [over night]	small	++++
Supernatant		++

Examination of activity against lemon gum was similar to that for Pectinol 41-P and showed no specific increase in components isolated over autohydrolysis.

HP-150

A similar crude fractionation was obtained for this enzyme preparation and no activity increase was noted in any of the five fractions examined. Extensive purifications of this preparation were carried out by Rowe [100] but because of the transferase activity noted, work was not carried further.

APPENDIX III

CARBON-CARBON BOND CLEAVAGE, DIALYSIS AND DESTRUCTIVE PURIFICATION AN UNDERGRADUATE ORGANIC EXPERIMENT

Carbohydrates are among the most abundant natural products. They are widely distributed in both the plant and animal kingdoms. Their variety is vast, ranging from cellulose and starch to glycogen, deoxyribonucleic acids, streptomycin and blood group glycoproteins. Carbohydrates have unique and varied biological activity, ranging from stores of potential energy in animals to supporting tissues in plants. Bacterial polysaccharides frequently carry the dominant immunological character of the bacterial cell and, in the case of pathogenic species, may form essential ingredients of vaccines.

Experiments in undergraduate laboratories with carbohydrate compounds have been very limited. Recently a procedure for conversion of polymeric glucose into erythritol was developed. Since this carbohydrate experiment involved a variety of techniques and reactions not often utilized in undergraduate laboritories it was felt to be of possible general interest.

This experiment, as described below, can be used to illustrate heterogeneous and hemogeneous reactions, oxidative carbon-carbon bond cleavage, dialysis, reduction, destructive purification, decolorization, crystallization, melting point and chromatography.

This experiment allows for a development of varied pathways to the final product. Through the use of prepared samples the duration of the experiment may be shortened from one involving several lab periods to only two lab sessions. The quantity used in the oxidations may be large or sufficient for only chromatographic identification of the final product. Table XXII below lists the various reagents and purification steps involved in the experiment. Not all pathways are possible, therefore, if desired a student may be allowed to determine a feasible pathway.

TABLE XXII

VARIABLE STEPS IN PREPARATION OF ERYTHRITOL

Polymer	Oxidant	Purification	Reduction	Purification	Isolation
Cellulose	$NaIO_4$	Dialysis	$NaBH_4$	Dialysis	Precipitation
Soluble starch	HIO_4	Filtration		Filtration	Freeze drying
Insoluble starch					Not necessary
Glycogen					a] filtered off
					b] next reaction on solution

Hydrolysis	Neutralization	Decolorization	Characterization
Time and acid strength may be varied	Ion Exchange	Short charcoal column	Melting point - mixed melting point
	$Ba[OH] - BaCO_3$	Repeated Crystallizations	Chromatography - gas liquid - paper - thin layer

As a result of the wide variety of pathways different points may be illustrated with the experiment. The carbon-carbon bond cleavage may be carried out as a homogeneous or heterogeneous reaction depending on the solubility of the glucose polymer. Depending on the homogeneity of the reaction, products from the oxidation and reduction may be purified by dialysis or filtration. As an alternate, soluble polymers may be purified by precipitation if periodic acid is the oxidant. Barium hydroxide will precipitate iodate ions remaining after the oxidation.

The following experimental procedure outlines the necessary details to convert one of the polymers into erythritol. The quantities and time may be altered by the use of prepared samples and method of characterization. Where prepared samples are used students should carry out the reactions, but pool their sample at each stage in order to supply subsequent laboratory periods with prepared samples. Alternate reaction pathways differ from the experiment below as noted.

Starch [16.2 g.] was dissolved by heating in distilled water [400 ml]. Sodium metaperiodate [23.4 g.] was added to the cooled solution. The reaction was allowed to stand in the dark for 24 hours [Note 1] at room temperature. At the end of this time the reaction was stopped by the addition of ethylene glycol. The solution was poured into a piece of cellulose tubing double knotted at one end. The air was expelled from the tubing and the top end was double knotted. The tubing was completely immersed in running water for 24 hours [Note 2]. Sodium borohydride [4.0 g.] [Note 3] was added to the dialysate and after 24 hours the excess reducing agent was destroyed by the addition of a small amount of acetic acid [5.0 ml]. Dialysis was employed to remove the sodium borate [Note 4] and the solution was concentrated to a small volume [approximately 100 ml]. The solution was made 2N with sulfuric acid and refluxed for 3 hours. The acid was neutralized with slightly less than an equivalent amount of barium hydroxide. The slight excess of acid was neutralized with a small amount of barium carbonate. The precipitate was removed by suction filtration through a pad of diatomaceous earth. The filtrate was concentrated on a rotary evaporator to dryness. The resulting syrup was dissolved in boiling methanol and the solution filtered to remove traces of inorganic salts. The solution was

diluted with an equal volume of water and passed through a stubby charcoal column [101] [Note 5]. A wash solution of the same solvents removed small amounts of erythritol from the column. The column eluate was concentrated to dryness and the clear syrup was dissolved in a small amount of methanol. On cooling erythritol crystallized from the solution. Yield 5.8 g. m.p.t. 121.5.

NOTE 1: Reaction rates with soluble or insoluble polymers may be reduced to 3 hours [102].

NOTE 2: Insoluble polymers when oxidized may be purified by filtration rather than dialysis. Washes with 50 ml. of water and intermediate filtration are necessary until essentially free of iodate, usually involving about 6 washes.

NOTE 3: It has been recommended that a 16 fold excess of borohydride be used when reducing oxycelluloses [103]. For this reaction however a small amount of unreduced product will not effect the results. It is recommended as well that insoluble polymers be shaken or stirred during reduction to prevent material being lifted out of the reduction solution.

NOTE 4: Insoluble polyalcohols are filtered out, washed with water, steeped for 30 minutes in 10% acetic acid, and washed several more times with water.

NOTE 5: Purification may be achieved by repeated crystallizations but the colored matter produced during hydrolysis is difficult to remove.

CHARACTERIZATION

The crystalline erythritol may be characterized by melting point and mixed melting point. The effectiveness of the oxidation may be investigated by chromatographic means. Examination of the hydrolysis solution will show up to three components; glucose from unoxidized polymer, erythritol, and from terminal glucose residues, glycerol. The amount of glycerol present is an indication of the degree of branching in the polymer [95]. The presence of these three components may be shown on paper [104] or thin layer chromatograms [105]. Gas liquid chromatography can be employed to obtain quantitative results regarding the effectiveness of the oxidation and the degree of branching of the starting polymer [95].

APPENDIX IV

LEMON GUM: DEGRADATION WITH 0.1N SULFURIC ACID

Further proof of the galactan branch on branch structure of lemon gum may be seen on analysis of the residual polysaccharide isolated after hydrolysis of lemon gum with 0.1N sulfuric acid. The residual polysaccharide contains essentially no L-arabinose units. This polymer contained 98.2 mole percent galactose as neutral sugar. This material was further analysed by methylation as reported earlier.

EXPERIMENTAL

Lemon gum [20 g.] was dissolved in water. The gum solution was adjusted to the desired 0.1N sulfuric acid strength and a final volume of 250 mls. The solution was refluxed for 12 hours. The residual galactan was obtained on precipitation of the neutralized solution with 4 volumes [1000 ml] of alcohol, yield 8.20 g. Analysis of the galactan showed only a trace of L-arabinose [D-galactose 98.2 mole %].

AUTOHYDROLYSIS OF LEMON GUM

Lemon gum contains a large amount of uronic acid [approximately one residue in five]. Free acids were obtained when the gum was acidified or deionized with ionexchange resin prior to precipitation. The pH of the gum acid is nearly three and when a solution is heated extensive hydrolysis occurs. Oligosaccharides were isolated from autohydrolysis and from 0.1N sulfuric acid hydrolysis. The components from both hydrolysis procedures were identical in their chromatographic behaviour and composition. Close comparison of the oligosaccharides isolated

by these two procedures indicated they were identical chromatographically in several solvents. Table XXIII is a list of the oligosaccharides isolated. Included in this table are two neutral D-galactose containing oligosaccharides obtained when the 0.1N sulfuric acid residual polysaccharide was subjected to stronger acid hydrolysis. All oligosaccharides had been isolated previously [1 and ref. therein]. Identification was achieved by comparison with known mobility on paper chromatograms. The pure oligosaccharides were isolated from paper chromatograms and hydrolysed to indicate the component sugars. Oligosaccharides containing uronic acids were reduced in DMSO with lithium borohydride prior to hydrolysis.

TABLE XXIII

OLIGOSACCHARIDES ISOLATED ON HYDROLYSIS OF LEMON GUM

1] 3-O-β-D-galactopyranosyl-D-galactose

2] 6-O-β-D-galactopyranosyl-D-galactose

3] 4-O-[4-O-methyl-α-D-glucopyranosyluronic acid]-L-arabinose

4] O-[4-O-methyl-α-D-glucopyranosyluronic acid]-[1$\rightarrow$4]-O-α?-L-arabinopyranosyl-[1$\rightarrow$5]-L-arabinose

5] 4-O-[4-O-methyl-α-D-glucopyranosyluronic acid]-D-galactose

6] 6-O-[β-D-glucopyranosyluronic acid]-D-galatose

EXPERIMENTAL

The pH of a lemon gum solution [10 g. in 80 ml of water] was measured as 3.17 for a sample of Amberlite IR-120 deionized and freeze dried gum. Deionized lemon gum [16 g.] was dissolved in water [200 ml]. The solution was placed in a dialysis tube previously inserted in a column [99] fitted with a side arm to allow intake of liquid and a bottom outlet to remove the dialysate silution as it passed by the bag. The unit was wound with 8 feet of heating tape and the temperature was maintained at 70-80°C [measured on a thermometer inserted between the heating tape and the column]. After one week of dialysis the solution in the tube had expanded sufficiently to prevent liquid from passing around the dialysis sack. A small amount of liquid was removed from the dialysis sack and dialysis was continued for a further ten days. The polysaccharide removed earlier and this final solution were precipitated into alcohol and a total of 11.90 g. of polysaccharide was recovered. The collection of solutions from the autohydrolysis had been made in four separate fractions. Paper chromatography indicated that all four fractions had a similar composition. Fractions 2, 3 and 4 were combined and fraction 1 was further divided into an acid and a neutral fraction by absorption on Duolite A-4 [OH] and subsequent elution with 10% acetic acid.

OLIGOSACCHARIDES

Oligosaccharides isolated from the 0.1N sulfuric acid and the autohydrolysis were chromatographed on paper and on inspection proved to be virtually identical. Only minor variation could be detected in the concentrations of some of the components isolated.

Samples of the oligosaccharides present were isolated by elution from paper chromatograms. Since all compounds isolated were known, data are present in Table XXIV.

TABLE XXIV

NEUTRAL AND ACIDIC COMPONENTS OBTAINED ON HYDROLYSIS OF LEMON GUM OLIGOSACCHARIDES

Neutral Components	R_{GAL} Solvent [A]	Components on Hydrolysis
Rhamnose	2.15	Rhamnose
Arabinose	1.30	Arabinose
Galactose	1.00	Galactose
1	0.53	Galactose
2	0.37	Galactose

Acid Components	R_{GAL} Solvent [B]	Components On Hydrolysis	Reduced Components on Hydrolysis
3	1.13	arabinose	arabinitol 4-O-Me-Glucose
4	0.69	arabinose	arabinose, arabinitol 4-O-Me-Glucose
5	0.73	galactose	galactitol, 4-O-Me-Glucose
6	0.23	galactose	galactitol, glucose

APPENDIX V

A TECHNIQUE FOR HANDLING MICRO SAMPLES COLLECTED FROM THE GAS CHROMATOGRAPH

A problem exists when samples are collected in capillary tubes on their elution from the column of a gas chromatograph. If these samples are to be used for TLC or Mass Spectra they may be dissolved in a solvent which can then be evaporated away on the TLC plate or on the probe for the Mass Spectrometer. However, for micro analysis, IR and often with the Mass Spectra it is imperative to avoid contamination of the sample with any solvent. To avoid this problem and still allow 25 μg samples to be handled requires a technique which avoids losses due to adhesion to loops, wires or other transferring systems.

A system has been developed which can handle small quantities with little or no loss. The technique involves manipulation of the sample in a glass collecting tube in the same way it was collected, heat and flowing gas. If a sample can be handled on the gas chromatograph without degradation it may be handled by this technique. If the sample is reasonably stable it may be manipulated using only heat since, as the sample is heated its viscosity is reduced and the sample will flow as the tube is drawn through the furnace. A gentle flow of dry nitrogen or gravity may be used to increase the movement of the sample for less stable compounds, minimizing any degradation. In reality a portion of the gas chromatograph can be taken to the analyst or mass spectroscopist. In addition, purity of collected samples can be determined by analysis on the gas chromatograph, TLC or IR spectrometer. In order to handle 25 μg samples it is necessary to reduce the bore of the melting point capillary to the size of a vacuum leak capillary. This can be done on all samples since the capillary action helps draw the

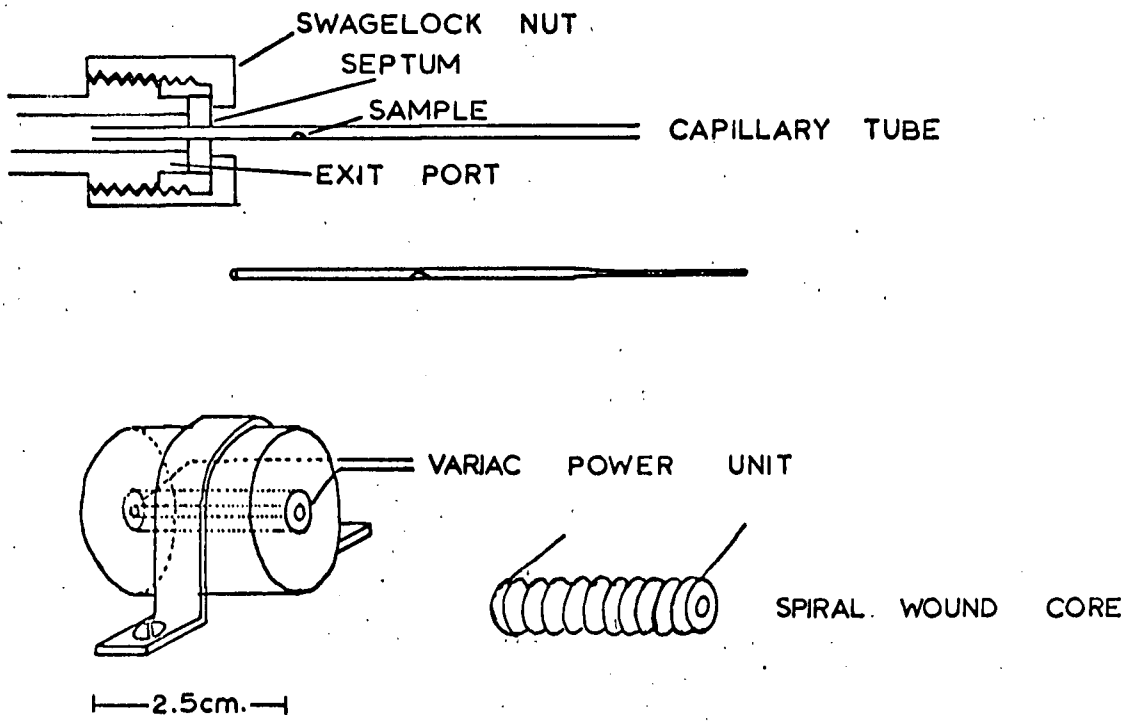
compound to the tip of the melting point tube. Positioning in the fine capillary facilitates removal of the sample to the tip of the mass spectrometer probe, for spotting on a TLC or IR plate. In Mass Spectroscopy, since only a μg sample is required ample sample for examination by other techniques is still available.

This technique of moving the condensed sample in a melting point capillary by the use of a thermal push overcomes the problems faced by workers using one of the simplest and yet still efficient collection techniques. The capillary tube acts as an air condenser and sample container. Samples may be stored by simply sealing both ends in an oxygen -natural gas flame. Should the sample spread in the tube on long standing, slow insertion of the tube in the heater will allow the sample to flow and collect in a small band. Even if the sample should crystallize, its melting in the oven will cause it to flow in the tube.

The melting point capillary should be fire polished at both ends for clean insertion through the holed septum in the exit port of the gas chromatograph. By inserting the tube in this manner all the carrier gas and compound will pass through the tube and maximum collection will be achieved. The fine capillary must be drawn after the collection because the extremely fine bore desired on the end of the tube would restrict gas flow and increase the pressure drop across the column. The minimum sample that can be handled this way was weighed at 25 μg and represented an approximate one inch high peak on an F and M 720 gas chromatograph at maximum sensitivity. The upper limit of size on the collection of a peak, even when the tube has been blown slightly would appear to be about 7-10 mg. A sample larger than this is simply blown through the tube.

FIGURE 32

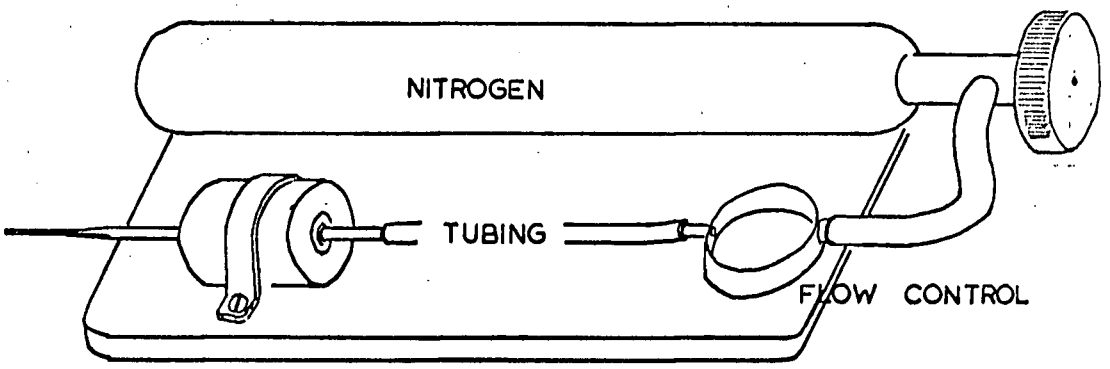
COLLECTION OF GAS CHROMATOGRAPHY SAMPLES IN GLASS CAPILLARIES AND OVEN DESIGN FOR THEIR MICROMANIPULATION



If gas is required to help move the sample, a lecture bottle of pure nitrogen may be mounted on the board with the small oven. Extreme care must be used when using gas as it is very easy to blow the sample out of the micro capillary tip. A large opening in the gas line, such that when open there is no gas flow out the exit tube and into the capillary is an excellent safety valve and avoids loss of sample due to blow through. This opening was closed with an oversized rubber stopper, since it was easily removed when the sample neared the tip of the drawn tube. A small section of rubber tubing can be used as a bulb to force out sample. The tubing can be placed on the capillary without forcing sample out. A finger over the tubing end will then move as much of the sample out of the tube as desired. Utilizing this collection

FIGURE 33

OVEN AND GAS SUPPLY FOR MICROMANIPULATION



technique and method of removal, it has been possible to analyse a single run on the gas chromatograph of a complex mixture of partially methylated alditol acetates. Individual components varied in size as much as 100:1.

This method of micromanipulation will have wide application to the collection of gas chromatography samples for the entire range of organic compounds. This technique will permit analysis of gas charomatograph runs much in the way one would with a GLC - Mass Spectrometer combination.

APPENDIX VI

COMPUTER SEARCHING THE CURRENT CARBOHYDRATE LITERATURE

The information explosion has affected all chemists. As journals multiply and papers increase it has become increasingly difficult to cover all the journals which contain important references. Services such as "Chemical Titles," published by the American Chemical Society, have eased the need to search for journals among different library branches, finding on occasion that they are missing, borrowed or have not arrived. It is, however, a prodigious task to review all the probable journals and scan the key words to ensure that one has not missed interesting or pertinent references. It is now possible, however, to utilize one of the new computerized Chemical Title search systems to retrieve a comprehensive list of titles of papers of interest to carbohydrate chemists. Not only the laborious work of searching but also of writing is immeasurably reduced since the computer print out provides the subscriber with a printed reference sheet for each title retrieved. The programme developed here is designed to cover carbohydrate chemistry from synthetic to polysaccharides. Two restrictions were eventually imposed on the developed programme because of interests and economy of terms. The nucleotides were removed from the search profile as well as certain specific names. The names were included initially but later removed when it was clear that there were few retrievals that could not have been picked up by checking the key words of Chemical Titles. The extra possible profile words were more valuable in limiting retrievals in more difficult areas. The profile development will be outlined in order that individuals with specific or broader interests who may wish to develop their own search profiles will be able to improve and shorten their profile development period.

The search profile was designed to retrieve references to carbohydrate chemistry using the CAN/SDI[1] Project for computerized searching of Chemical Titles. Since nomenclature for the field of carbohydrates is systematic, it is possible to use these systematic endings and structural words to retrieve all references to carbohydrates.

Profile words used in the search are given a letter code. Initial lists of terms were prepared. These were shortened by truncation and utilization of common letter groups. Truncation allows retrieval of words using letter groups preceeded or followed by other letter groups.

	Truncation	Retrieval
1.	OX*	ox, oxen, oxide
2.	*OX	ox, box, fox, Xerox
3.	OX	ox
4.	*OX*	all the above, plus hydroxy, foxes, boxes, dioxide, etc.

The list of words of desired retrievals should be scanned for common letter groupings, for example:

Cerebr o side s
Gangli o side s
Furan o side s
Pyran o side s
Glyc o side

Incorrect truncation will result in excess retrieval.

SIDE besides the terms above picks up side, consider, residence, prus-side, sidewise, inside and sidechain, however, *OSIDE* picks up none of these.

---

[1] National Science Library, National Research Council of Canada

TABLE XXV

COMPUTER PRINT OUT OF PROFILE ONE

```
JUN 4 '69   P 526                    PROFILE  DUTTON, G G
            T     A *ALD*                      T     AF POLYOL*
            T     B *AMYL*                     T     AG *SACCHAR*
            T     C *CELL*                     T     AH *STARCH*
            T     D *ERYTHR*                   T     AI *SUGAR*
            T     E *FUC*                      T     AJ *ARIC*
            T     F *HEX*                      T     AK *ASE*
            T     G *KET*                      T     AL *FURANO*
            T     H *PENT*                     T     AM *ITOL*
            T     I *RIB*                      T     AN *LACTONE*
            T     J *SORB*                     T     AO *ONIC*
            T     K *TAL*                      T     AP *OSAMINE*
            T     L *TETR*                     T     AQ *OSE*
            T     M *THRE*                     T     AR *PYRANO*
            T     N *XYL*                      T     AS *SIDE*
            T     O *ALTR*                     T     AT *URON*
            T     P *ARAB*                     T     AU AGAR*
            T     Q *FRUCT*                    T     AV ALGIN*
            T     R *GALACT*                   T     AW CARRAGEENAN*
            T     S *GUL*                      T     AX CHITIN*
            T     T *LYX*                      T     AY CHONDROITIN*
            T     U *MANN*                     T     AZ DEXTR*
            T     V *RHAMN*                    T     BA INULIN*
            T     W *GLUC*                     T     BB KERATAN*
            T     X *GLYC*                     T     BC LEVAN*
            T     Y CARBOHYDR*                 T     BD NEURAMIN*
            T     Z EXUDATE*                   T     BE PECT*
            T    AA GUM*                       T     BF SIALIC*
            T    AB MUC*                       T     BG TEICHOIC*
            T    AC *MYCIN*
            T    AD *NUCLEOTID*
            T    AE POLY HYDROXY

            T    BH ACETOLYSIS
            T    BI EPOXID*
            T    BJ METHYLATION*
            T    BK MUTAROT*
            T    BL KLEBSIELLA
            T    BM PER IODATE
            E01 T 99     (A|B-N)&(AJ|AK-AT)
            E02 T 99     (O|P-V)
            E03 T 99     W|W
            E04 T 99     X|X
            E05 T 99     (Y|Z-AI)
            E06 T 99     (AJ|AL|AM|AO|AP|AR|AT)
            E07 T 99     AQ|AQ
            E08 T 99     AS|AS
            E09 T 99     (AU|AV-BM)
```

Another example:

Muc ilage
Muc ic
Muc o polysaccharide

MUC* however, picks up as well mucosa, mucosal, mucous, mucoid, muconic, mucor; however, MUCI* and MUCO are completely selective.

The first search profile was over-truncated and the pairing of most terms was not sufficient to eliminate many unwanted references. Thus:-

Profile Word	Unwanted Retrieval
ALD	aldrin, diels-alder
AMYL	carbamylation
CELL	cells, sub-cellular
HEX	hexane, hexanol
PENT	pentoxide
RIB	distribution, ribosomyl, ribosomes
SORB	adsorbed
TAL	metal, skeletal, congential, digitalis, orbital, crystalline, etc.

Removal of the prefix truncation on a large number of the root words was attempted with some degree of concern since if the root were in a compound name, such as trimethyl-D-ribose, the computer might view this as an unbroken word and no retrieval would take place. Fortunately this was not the case and all roots preceeded by a hyphen were printed out.

The following additional changes were made in the preparation of the second profile.

AO *ONIC* from E 06 because of terms ending in "onic"
electronic, anionic, embryonic, paraconic, carbonic, etc.

AC *MYCIN* because of too many references to drug use.

TABLE XXVI

COMPUTER PRINT OUT OF PROFILE TWO

```
U 526                    PROFILE  DUTTON, G G S
T      A  ALD*
T      B  AMYL*                    T     AF  POLYOL*
T      C  CELLULOSE                T     AG  *SACCHARI*
T      D  ERYTHR*                  T     AH  *STARCH*
T      E  FUC*                     T     AI  *SUGAR*
T      F  HEX*                     T     AJ  *ARIC*
T      G  KET*                     T     AK  *ASE*
T      H  PENT*                    T     AL  *FURANO*
T      I  RIB*                     T     AM  *ITOL*
T      J  SORB*                    T     AN  *LACTONE*
T      K  TAL*                     T     AO  *ONIC*
T      L  TETR*                    T     AP  *OS AMIN*
T      M  THRE*                    T     AQ  *OSE*
T      N  XYL*                     T     AR  *PYRANO*
T      O  *ALTR*                   T     AS  *SIDE*
T      P  ARAB*                    T     AT  *URONIC
T      Q  *FRUCT*                  T     AU  AGAR*
T      R  *GALACT*                 T     AV  ALGIN*
T      S  GUL*                     T     AW  CARRAGEENAN*
T      T  *LYX*                    T     AX  CHITIN*
T      U  MANN*                    T     AY  CHONDROITIN*
T      V  *RHAMN*                  T     AZ  DEXTR*
T      W  *GLUC*                   T     BA  INULIN*
T      X  *GLYCO*                  T     BB  KERATAN*
T      Y  CARBOHYDR*               T     BC  LEVAN*
T      Z  EXUDATE*                 T     BD  NEURAMIN*
T     AA  GUM*                     T     BE  PECT*
T     AB  MUC*                     T     BF  SIALIC*
T     AC  GLYCAN*                  T     BG  TEICHOIC*
T     AD  *NUCLEOTID*
T     AE  POLY HYDROXY

T     BH  ACETOLYSIS
T     BI  EPOXID*
T     BJ  METHYLATION*
T     BK  MUTAROT*
T     BL  KLEBSIELLA
T     BM  PER IODATE
E01 T 99      (A|B|D-N)&(AJ|AK-AT)
E02 T 99      (O|P-V)
E03 T 99      W|W
E04 T 99      X|X
E05 T 99      (Y|Z-AC)
E06 T 99      (AD|AE-AI)
E07 T 99      (AJ|AL|AM|AP|AR|AT)
E08 T 99      AQ|AQ
E09 T 99      AS|AS
E10 T 99      (AU|AV-BM)
E11 T 99      C|C
```

X *GLYC* and replacement with *GLYCO* and GLYCAN* because of references to glycine, hypoglycemic, etc.

AT *URON* to *URONIC because of neuron, pleuroncipes.

AG *SACCHAR* to AG *SACCHARI* avoids saccharomyces.

AP *OSAMINE* to AP *OS AMIN* CT word splitting.

C *CELL* to cellulose to many words with CELL

EQUATIONS

E01 Remove C *CELL* to new equation E11

E05 Made into two equations E05 and E06 to many retrievals

E06 → E07 Remove term A0 because of terms inding in onic; electronic, anionic, embryonic, paraconic, carbonic, etc.

PROFILE NUMBER 2

EVALUATION

E01 Still too many titles which contain, by chance, two of the required terms, i.e. THRE, ASE

Triplet State effects in dye lasers at threshold.

HEX, OSE

Binding energy and compressibility of body centered cubic and close packed hexagonal sodium.

Therefore at the risk of losing a few references [2] E01 was split into two search equations. The equations were selected so that the [3] E01 contained all those root words which were found to commonly occur in other

TABLE XXVII

COMPUTER PRINT OUT OF PROFILE THREE

NE 20 69

U 526 PROFILE DUTTON, G G S

T	A	ALD*			
T	B	ERYTHR*	T	AF	*SACCHARI*
T	C	HEX*	T	AG	*STARCH*
T	D	KET*	T	AH	*SUGAR*
T	E	PENT*	T	AI	*ARIC*
T	F	RIB*	T	AJ	*ASE*
T	G	TETR*	T	AK	*FURANO*
T	H	THRE*	T	AL	*ITOL*
T	I	AMYL*	T	AM	*LACTONE*
T	J	FUC*	T	AN	*ONIC*
T	K	SORB*	T	AO	*OS AMIN*
T	L	TAL*	T	AP	*OSE
T	M	XYL*	T	AQ	*OSES
T	N	*ALTR*	T	AR	*PYRANO*
T	O	ARAB*	T	AS	*SIDE*
T	P	*FRUCT*	T	AT	*URONIC
T	Q	*GALACT*	T	AU	ADIPOSE*
T	R	GUL*	T	AV	CLOSE*
T	S	LYX*	T	AW	DOSE*
T	T	MANN*	T	AX	MANNICH
T	U	RHAMN*	T	AY	SIDE*
T	V	*GLUC*	T	AZ	METABOLISM
T	W	*GLYCO*	T	BA	*ENE*
T	X	CARBOHYDR*	T	BB	GLUCONEOGENESIS
T	Y	EXUDATE*	T	BC	GLYCOLYSIS
T	Z	GLYCAN*	T	BD	MUCOSA
T	AA	GUM*	T	BE	CELLULOSE*
T	AB	MUC*	C	BF	CRBRA
T	AC	*NUCLEOTID*	T	BG	PHOSPH*
T	AD	POLY HYDROXY			
T	AE	POLYOL			

E01	T	99	(A|B-H)&(AI|AK|AL|AO|AP|AQ|AR|AT)¬(AU|AV|AW|AZ)
E02	T	99	(I|J-M)&(AI|AJ-AT)¬(AU|AV|AW|AY|AZ)
E03	T	99	(N|O-U)¬(AX|AZ|BG)
E04	T	99	BE|BE
E05	T	99	V¬(AZ|BB|BG)
E06	T	99	W¬(AZ|BA|BC)
E07	T	99	(X|Y-AB)¬(AZ|BD)
E08	T	99	(AC|AD-AH)¬(AZ)
E09	T	99	AK|AL|AO|AR|AT
E10	T	99	(AP|AQ)¬(AU|AV|AW)
E11	T	99	AS¬AY
E12	T	99	BF|BF

fields of chemistry. The following very common endings were removed:

ase , lactone , onic , side

In addition certain not terms were added to remove some commonly occurring words.

adipose, close, dose, metabolism

The remainder of [2] E01 was the same as before with the addition of the not words above and the word AY SIDE* .

Equation [2] E02 formed [3] E03 with the not terms MANNICH, METABOLISM and PHOSPH* added.

A number of not terms were added to the rest of the equations in order to eliminate commonly occurring non-carbohydrate retrievals, i.e. *ENE* was added as a not term for the equation [3] E06 to remove terms such as polyethylene glycol.

In order to reduce the profile to 60 terms and to incorporate the required not terms it was necessary to delete the terms AU to BG of profile No. 2. As a check on this completeness of the retrieval of references the coden for the Journal Carbohydrate Research has been given as the last equation in the profile in order to pick up any titles not previously printed in order to see why they have not been included.

PROFILE NUMBER 3

The following changes were made in profile No. 3 to produce the final profile.

Profile No. 3 Term		Profile No. 4 Term		Reason
AB	MUC*	AB	MUCO	Too many non-carbohydrate words
AC	*NUCLEOTID*			Removal of nucleotides ref. from profile
		AC	MUCI	MUCI and MUCO replace MUC
AS	*SIDE*	AS	*OSIDE*	Improved truncation
AY	SIDE*			Replaced because of improved AS term
		AY	GLUCAGON	New not term
AZ	METABOLISM	AZ	METABOLI*	Better truncation now will pick out metabolic as well
BB	GLUCONEOGENESIS			Term AB *ENE* covers this term
		BB	INSULIN	New not term
BC	GLYCOLYSIS	BC	GLYCOLY*	Better truncation [glycolytic]
BD	MUCOSA			No longer needed [AB]
		BD	CORTICO*	New not term
		BH	TRANSPORT	New not term

The equations for the search expressions were modified to include the new not terms. Amylase references have been eliminated as well as most references to carbohydrate phosphates. The equations are, however, open to general references such as sugar phosphates.

TABLE XXVIII

COMPUTER PRINT OUT OF PROFILE FOUR

OCT 3 '69

U 526 090669 PROFILE DUTTON, G G S

T	A	ALD*			
T	B	ERYTHR*	T	AF	*SACCHARI*
T	C	HEX*	T	AG	*STARCH*
T	D	KET*	T	AH	*SUGAR*
T	E	PENT*	T	AI	*ARIC*
T	F	RIB*	T	AJ	*ASE*
T	G	TETR*	T	AK	*FURANO*
T	H	THRE*	T	AL	*ITOL*
T	I	AMYL*	T	AM	*LACTONE*
T	J	FUC*	T	AN	*ONIC*
T	K	SORB*	T	AO	*OS AMIN*
T	L	TAL*	T	AP	*OSE
T	M	XYL*	T	AQ	*OSES
T	N	*ALTR*	T	AS	*OSIDE*
T	O	ARAB*	T	AR	*PYRANO*
T	P	*FRUCT*	T	AT	*URONIC
T	Q	*GALACT*	T	AU	ADIPOSE
T	R	GUL*	T	AV	CLOSE*
T	S	LYX*	T	AW	DOSE*
T	T	MANN*	T	AX	MANNICH
T	U	RHAMN*	T	AY	GLUCAGON
T	V	*GLUC*	T	AZ	METABOLI*
T	W	*GLYCO*	T	BA	*ENE*
T	X	CARBOHYDR*	T	BB	INSULIN
T	Y	EXUDATE*	T	BC	GLYCOLY*
T	Z	GLYCAN*	T	BD	CORTICO*
T	AA	GUM*	T	BE	CELLULOS*
T	AB	MUCO	C	BF	CRBRA
T	AC	MUCI*	T	BG	PHOSPH*
T	AD	POLY HYDROXY			
T	AE	POLYOL			

T	BH	TRANSPORT*	
E01	T	99	(A|B-I)&(AK|AL|AO|AP|AQ|AR|AS|AT)¬(AU|AV|AW|AZ|BG)
E02	T	99	(J|K-M)&(AI|AJ-AT)¬(AU|AV|AW|AZ|BG)
E03	T	99	(N|O-U)¬(AX|AZ|BG)
E04	T	99	BE|BE
E05	T	99	V¬(AZ|BB|BG|AY|BA|BD|BH)
E06	T	99	W¬(AZ|BA|BC|BB|BG)
E07	T	99	(X|Y-AC)¬(AZ|BB|BH)
E08	T	99	(AD|AE-AH)¬(AZ|BB|BH)
E09	T	99	(AK|AL|AO|AR|AT)¬(AZ|BG|BH)
E10	T	99	(AP|AQ)¬(AU|AV|AW|AZ|BB|BG)
E11	T	99	AS¬(AZ|BB|BG|BH)
E12	T	99	BF|BF

TABLE XXIX. EVALUATION SEARCH PROFILES

PROFILE NO. 1

Search Expression	C.T. No. 11, 1969			C.T. No. 12, 1969		
	Yes	No	Total	Yes	No	Total
1	12	87	99*	18	81	99*
2	8	46	54	10	52	62
3	15	34	49	6	24	30
4	16	65	81	10	35	45
5	30	69	99*	29	53	82
6	5	94	99*	6	78	84
7	12	62	74	13	39	52
8	4	13	17	3	10	13
9	7	11	18	5	15	20
Total	109	481	590	100	387	
% of Total	18.5	81.5		20.5	79.5	

* Maximum number of retrievals 99.

PROFILE NO. 2

Search Expression	C.T. No. 13, 1969			C.T. No. 14, 1969		
	Yes	No	Total	Yes	No	Total
1	13	61	74	23	72	95
2	9	10	19	18	11	29
3	8	22	30	19	33	52
4	15	19	34	11	25	36
5	12	7	19	12	16	28
6	22	20	42	18	26	44
7	2	7	9	5	7	12
8	11	42	53	15	35	50
9	7	15	22	6	15	21
10	6	17	23	7	19	26
Total	105	220	325	134	259	393
% of Total	32.3	67.7		34.1	65.9	

TABLE XXIX [Cont'd]

PROFILE NO. 2

Search Expression	C.T. No. 15, 1969			C.T. No. 16, 1969		
	Yes	No	Total	Yes	No	Total
1	20	79	99	16	65	81
2	13	10	23	7	7	14
3	6	26	32	15	29	44
4	12	29	41	10	15	25
5	9	16	25	6	7	13
6	23	44	67	16	21	37
7	6	21	27	1	8	9
8	11	60	71	4	34	38
9	5	18	23	2	17	19
10	4	9	13	7	14	21
Total	109	312	421	84	217	301
% of Total	25.9	74.1		27.9	72.1	

PROFILE NO. 3

Search Expression	C.T. No. 17, 1969			C.T. No. 18, 1969			C.T. No. 19, 1969		
	Yes	No	Total	Yes	No	Total	Yes	No	Total
1	16	4	20	13	7	20	8	7	15
2	6	4	10	1	2	3	1	9	10
3	7	5	12	5	9	14	4	5	9
4	12	11	23	6	11	17	15	11	26
5	8	20	28	7	21	28	3	18	21
6	11	16	27	12	14	26	7	8	15
7	10	9	19	10	2	12	5	5	10
8	13	30	43	9	18	27	17	34	51
9	2	4	6	1	6	7	2	1	3
10	4	16	20	2	11	13	3	13	16
11	5	16	21	2	13	15	1	9	10
Total	94	135	229	68	114	182	66	120	186
% of Total	41.0	59.0		37.4	62.6		35.5	64.5	

TABLE XXIX [Cont'd]

PROFILE NO. 4

Search Expression	C.T. No. 20, 1969				C.T. No. 21, 1969			
	Yes	No	No But Valid	Total	Yes	No	No But Valid	Total
1	14	1	4	19	9	0	6	15
2	4	2	0	6	1	3	2	6
3	13	1	0	14	9	1	11	21
4	10	0	7	17	7	0	8	15
5	8	0	10	18	6	0	17	23
6	8	2	9	19	8	3	15	26
7	10	0	2	12	7	0	7	14
8	10	3	9	22	28	0	23	51
9	2	3	1	6	1	0	1	2
10	4	3	2	9	5	7	2	14
11	2	0	2	4	2	0	10	12
12	5	0	1	6	0	0	0	0
Total	90	14	48	152	83	14	102	199
% of Total	59.2	9.2	31.6		41.7	7.0	51.3	

Search Expression	C.T. No. 22, 1969				C.T. No. 23, 1969			
	Yes	No	No But Valid	Total	Yes	No	No But Valid	Total
1	11	0	6	17	8	0	4	12
2	3	0	1	4	2	2	0	4
3	10	2	7	19	13	0	2	15
4	10	0	12	22	9	0	9	18
5	9	0	17	26	14	0	14	28
6	10	2	8	20	6	3	9	18
7	3	2	8	13	3	0	0	3
8	13	2	12	27	15	3	12	30
9	2	1	2	5	1	2	2	5
10	2	6	9	17	6	3	8	17
11	1	0	4	5	7	0	9	16
12	3	0	0	3	0	0	0	0
Total	77	15	86	178	84	13	62	166
% of Total	43.3	8.4	48.3		50.6	7.8	41.6	

The final search profile shows extremely good reference retrieval. The references which were not useful retrievals have been broken down into two sub-groups, references retrieved because of carbohydrate words and references retrieved because of other factors.

Many of the non-useful references are legitimate retrievals but outside our fields of interest. The retrieval has been based on the presence of a carbohydrate word in the title of the article. One example for each search expression is listed below.

E01	HEX, OSE	... A Genetic Reappraisal of Hexose Transport by Kidney and Intestine
E02	SORB, ITOL	... Effects of Ethanol, Sorbitol and Thyroid Hormones
E03	ARAB	L-Arabinose Binding Protein ...
E04	CELLULOS	Chromatography ... on Columns of Benzoylated di ethyl-amino ethyl cellulose
E05	GLUC	Effect of Serotonin on Glucose ... in Rabbit Brain
E06	GLYCO	Catabolism of Plasma Glyco Proteins ...
E07	CARBOHYDR	... Chickens Fed Carbohydrate Free Diets
E08	SUGAR	... Effects on Sucrose Yield of Sugar Beets ...
E09	ITOL	Effect of Myo Inositol on the Prevention ...
E10	OSE	Nature of Lactose Fermenting Salmonella ...
E11	OSIDE	Radio Denaturation of DNA Deoxy Nucleosides.

The other terms which are non-useful are normally less than 10% of the retrievals and fall into one of the following groups of retrievals.

1] Accidental combination of two words

XYL, ASE	Xylene, bases
ALD, FURANO	Diels-Alder, furanophane

2] Truncation retrievals

GALACT	Galactic
SACCHARI	Saccharin
OSE	Rose, purpose, those, etc.

3] Specific words found in other fields of chemistry

OS, AMIN	Di ethyl nitros amine
GLYCO	Glycolate
POLY, HYDROXY	Poly hydroxy phenylenes

It is clear that the final profile will retrieve a highly significant portion of the carbohydrate literature present in any issue of Chemical Titles. The profile may be modified as well to be more specific and to retrieve references missed by altering the profile words and search equations.

If at first glance the number of non-useful retrievals seems large it should be borne in mind that it is a matter of moments to peruse the print out and discard those not wanted. In practice a high percentage of marginal material can be tolerated before the mechanical retrieval system ceases to be competitive with a manual search of the literature. The computer print out has in effect carried out a preliminary screening and "short listed" possible references for closer attention. Furthermore, each entry of the print out is on a separate [perforated] sheet of paper which may serve as the basis of a personal filing system. The time saved in avoiding the necessity of transcribing references of interest should also be considered when assessing the merits of the method.

The use of the journal coden for Carbohydrate Research allows a check on the percentage of papers which would not be retrieved by the profile. Of over two hundred references in Carbohydrate Research only 8.6% would not have been

retrieved by the profile. A portion of these were intentional exclusions by not terms and a few references would have been obtained by examination of Chemical Titles keyword lists for the twenty or so profile words eliminated to include the not terms and shorten the profile [compare profiles No. 1 and No. 4]. If these references were included only 5.4% would not have been retrieved. Examination of several title pages of Carbohydrate Research will indicate the type of references which will not be retrieved by the search profile. In general, it will be seen that the titles often give no clue to their carbohydrate nature. In terms of this evaluation the profile proposed is 95% effective in retrieving desired references from journal title pages.

The same profile has also been used to search Chemical Abstract Condensates with good results although the percentage of undesirable retrievals is higher. This situation could now easily be improved by the addition of a profile word PATENT as a not term. This possibility was unavailable at the time profile 4 was developed.

APPENDIX VII

LEMON GUM

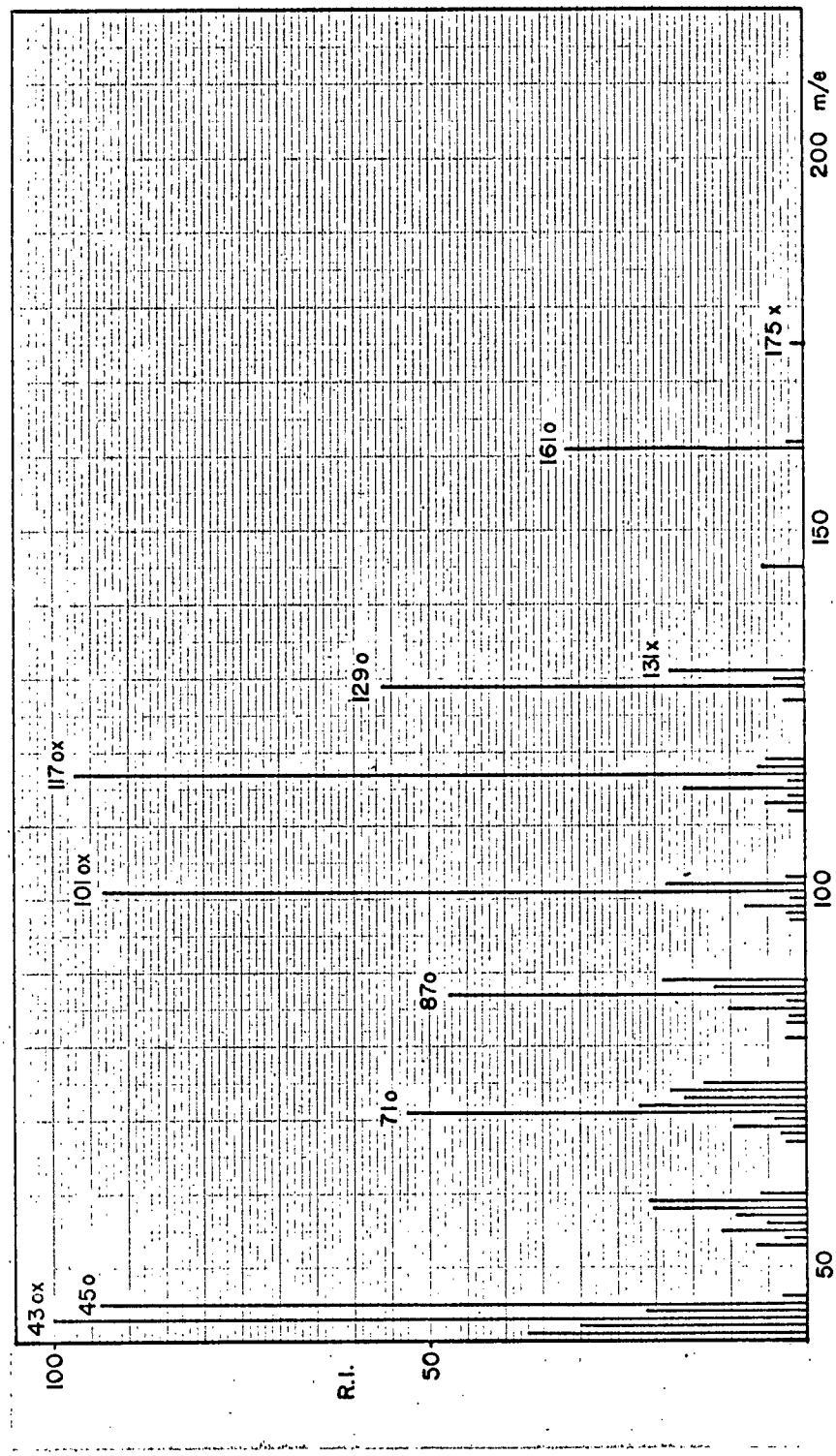


FIGURE 34: LEMON GUM

PEAK 3

o 2,3,5-Me_3-ARAB

x 2,3,4-Me_3-RHM

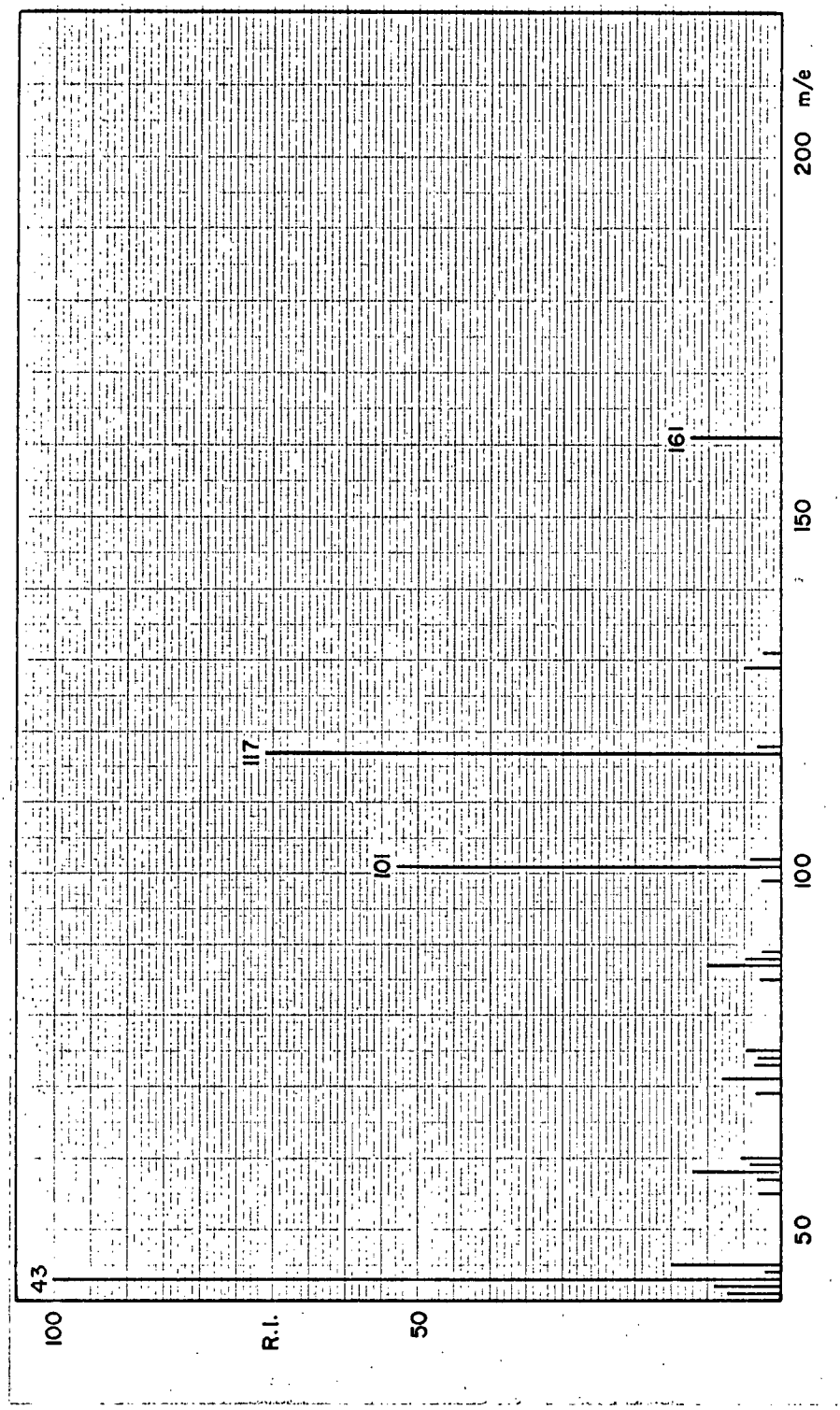


FIGURE 35: LEMON GUM
PEAK 4
2,3,4-Me_3-ARAB

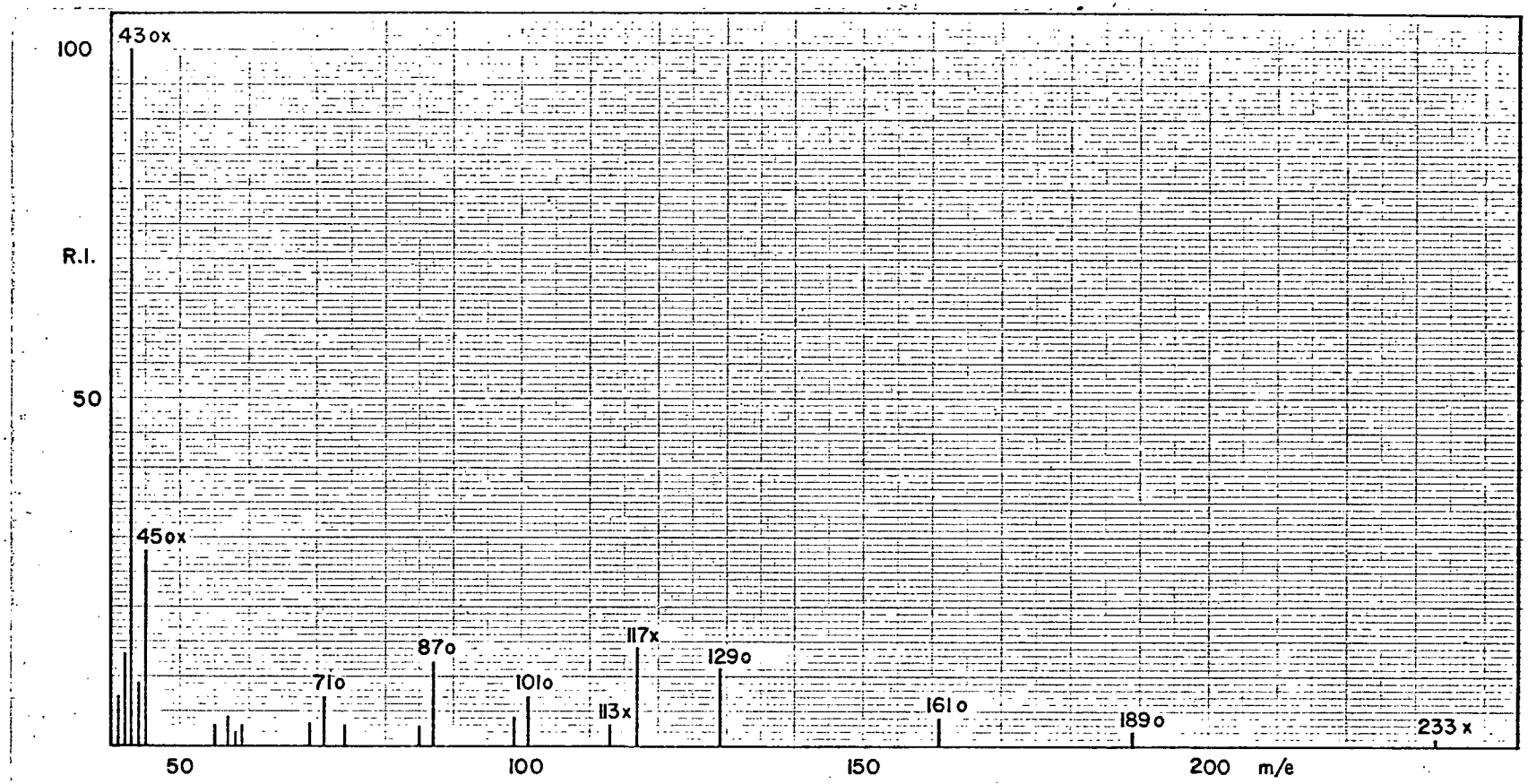


FIGURE 36: LEMON GUM

PEAK 5

x 2,5-Me_2-ARAB

greater amount o 3,5-Me_2-ARAB

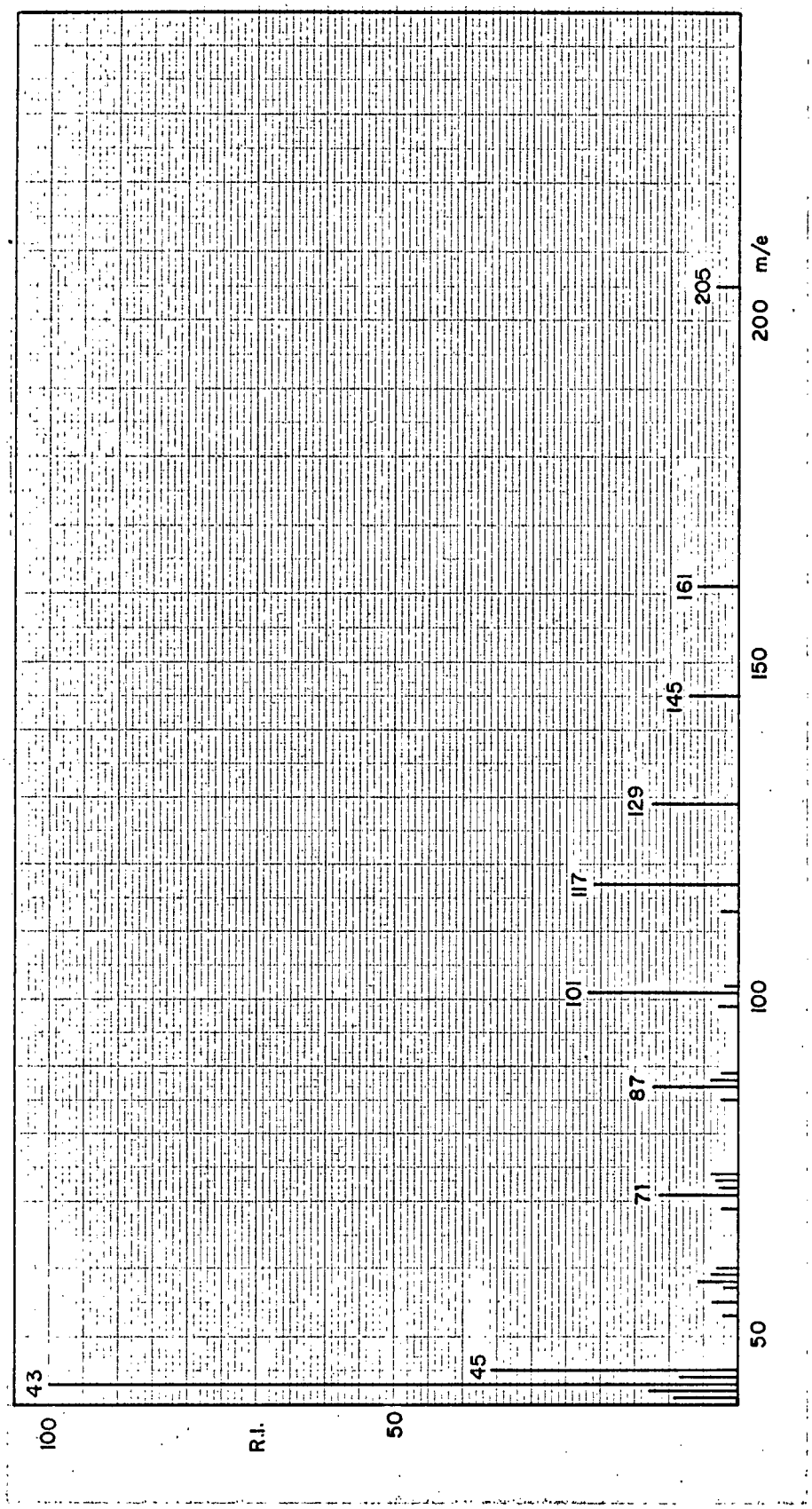


FIGURE 37: LEMON GUM
PEAK 6B
2,3,4,6-Me_4-GAL

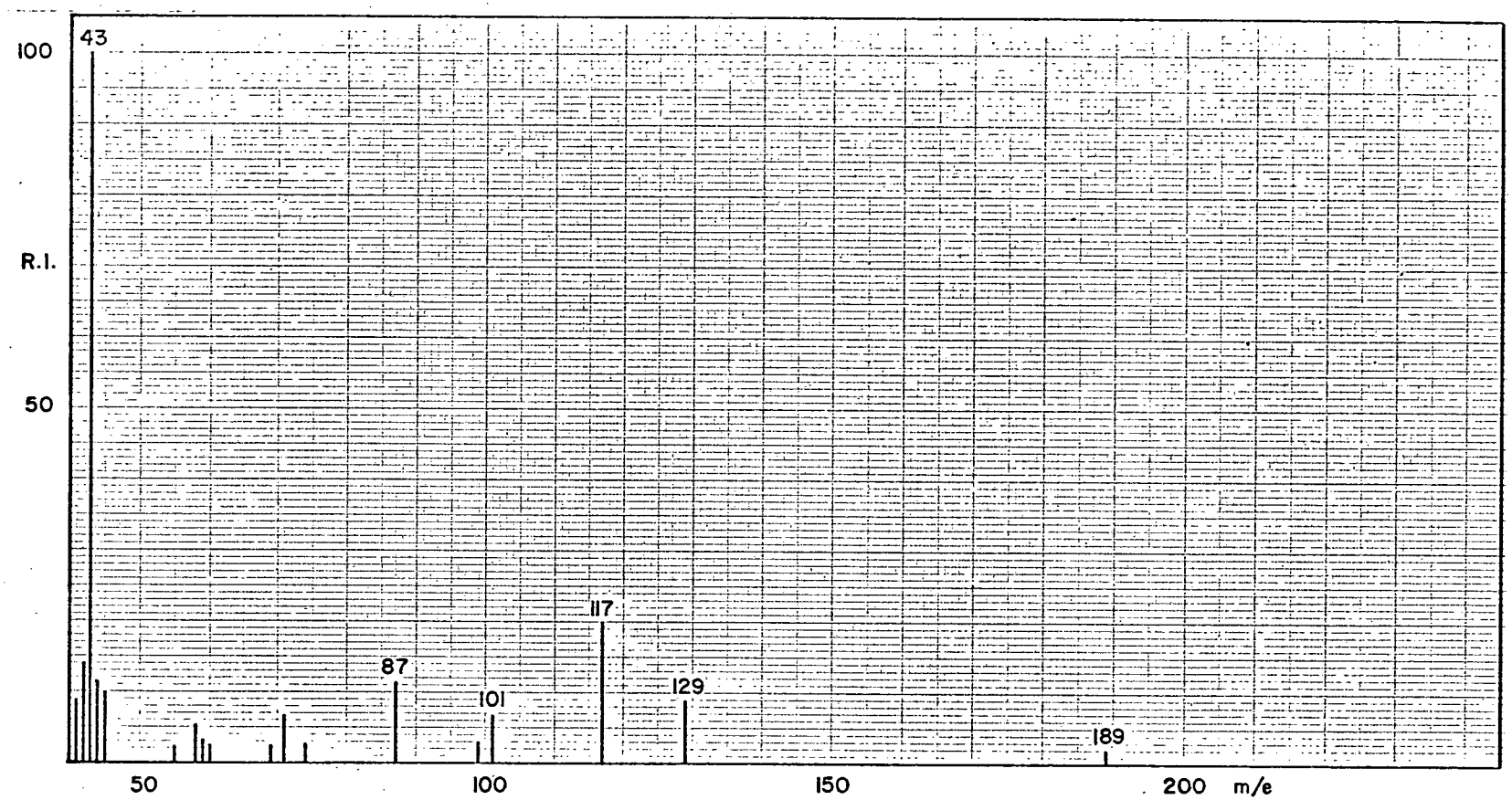


FIGURE 38: LEMON GUM
PEAK 6T
2,3-Me_2-ARAB

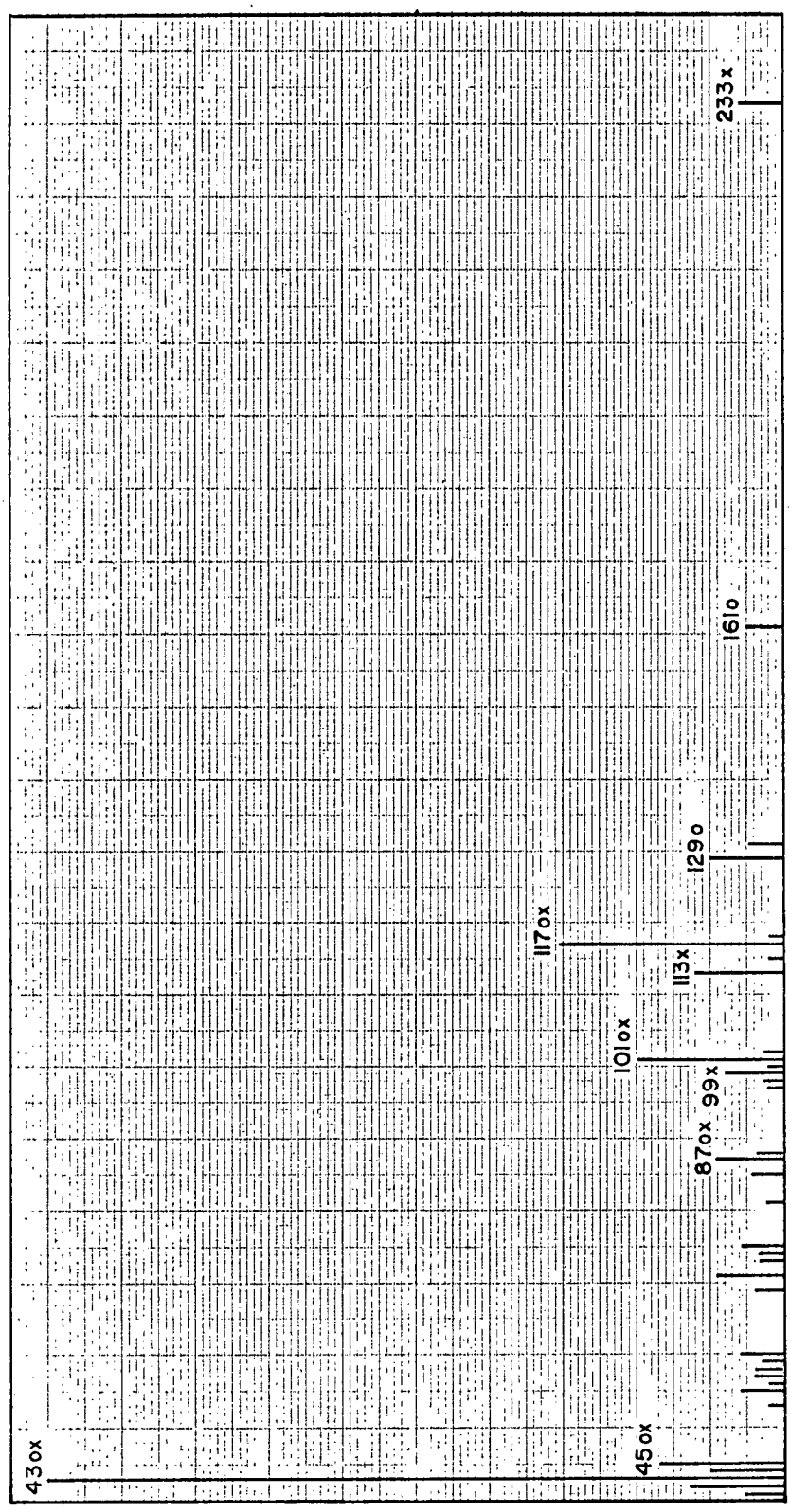


FIGURE 39: LEMON GUM

PEAK 7B

o 2,4,6-Me3-GAL

x 2,3,6-Me3-GAL

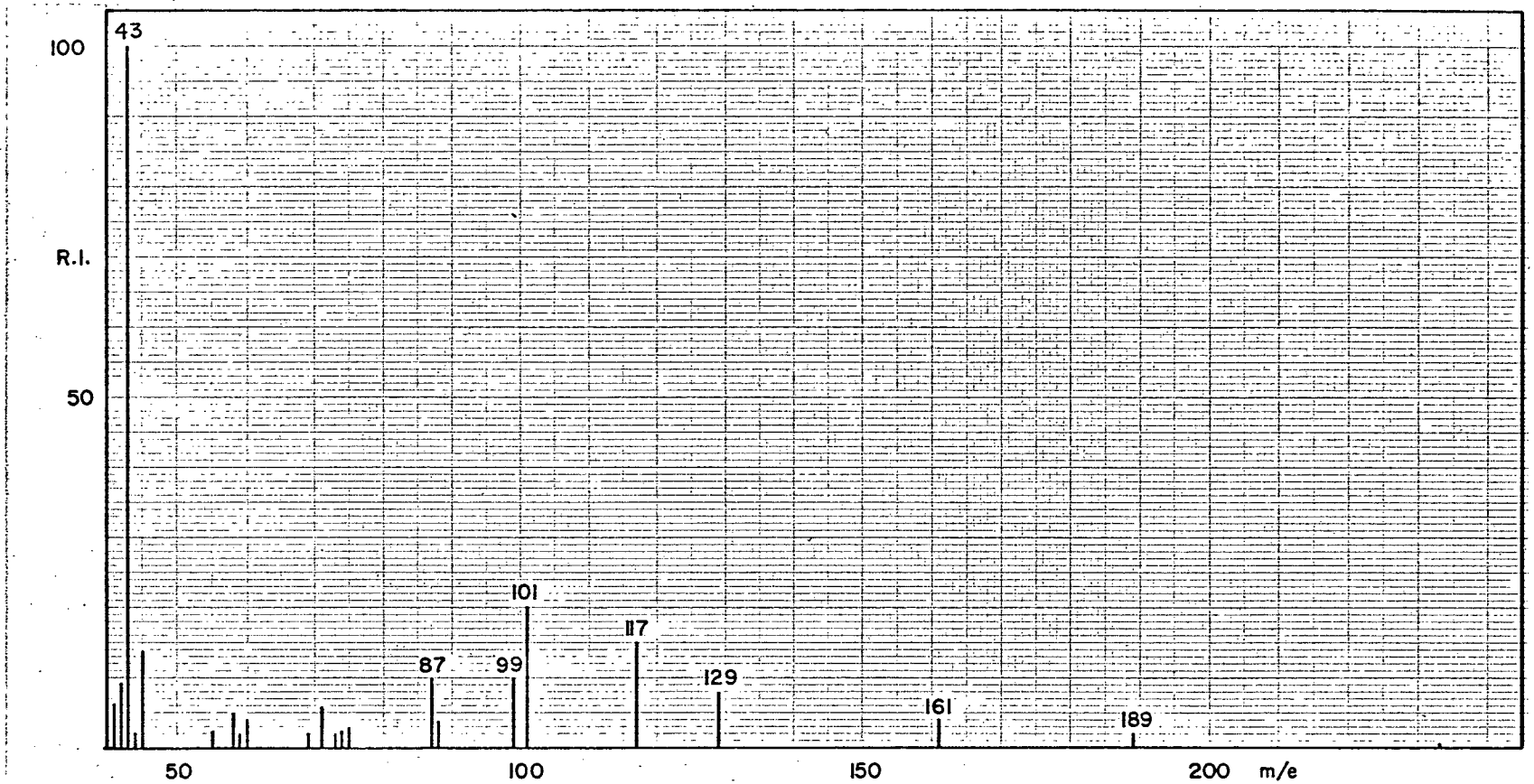


FIGURE 40: LEMON GUM
PEAK 7T
2,3,4-Me_3-GLUC

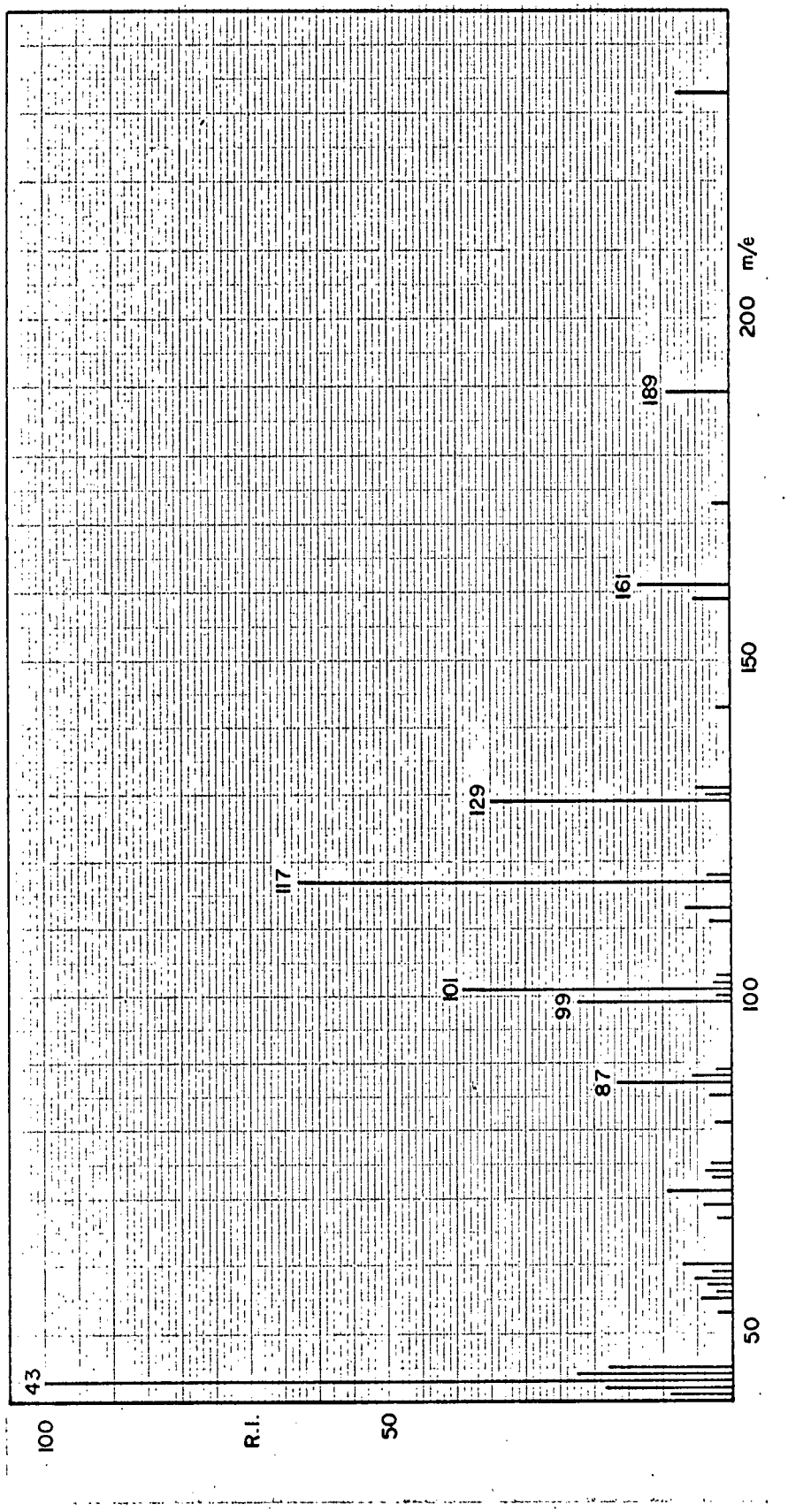


FIGURE 41: LEMON GUM
PEAK 8
2,3,4-Me_3-GAL

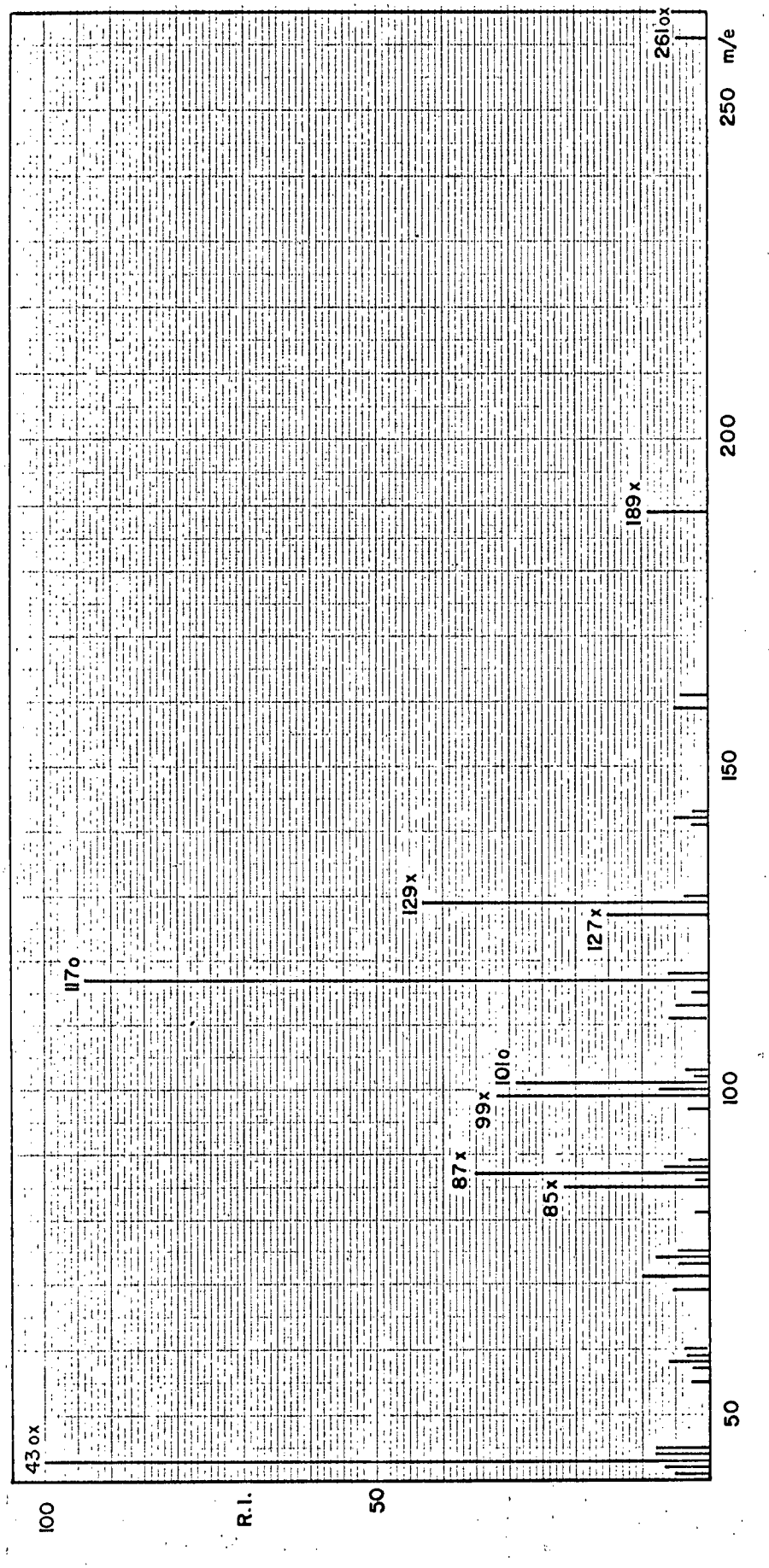


FIGURE 42: LEMON GUM

PEAK 9

o 2,3-Me_2-GLUC

x 3-Me-GAL

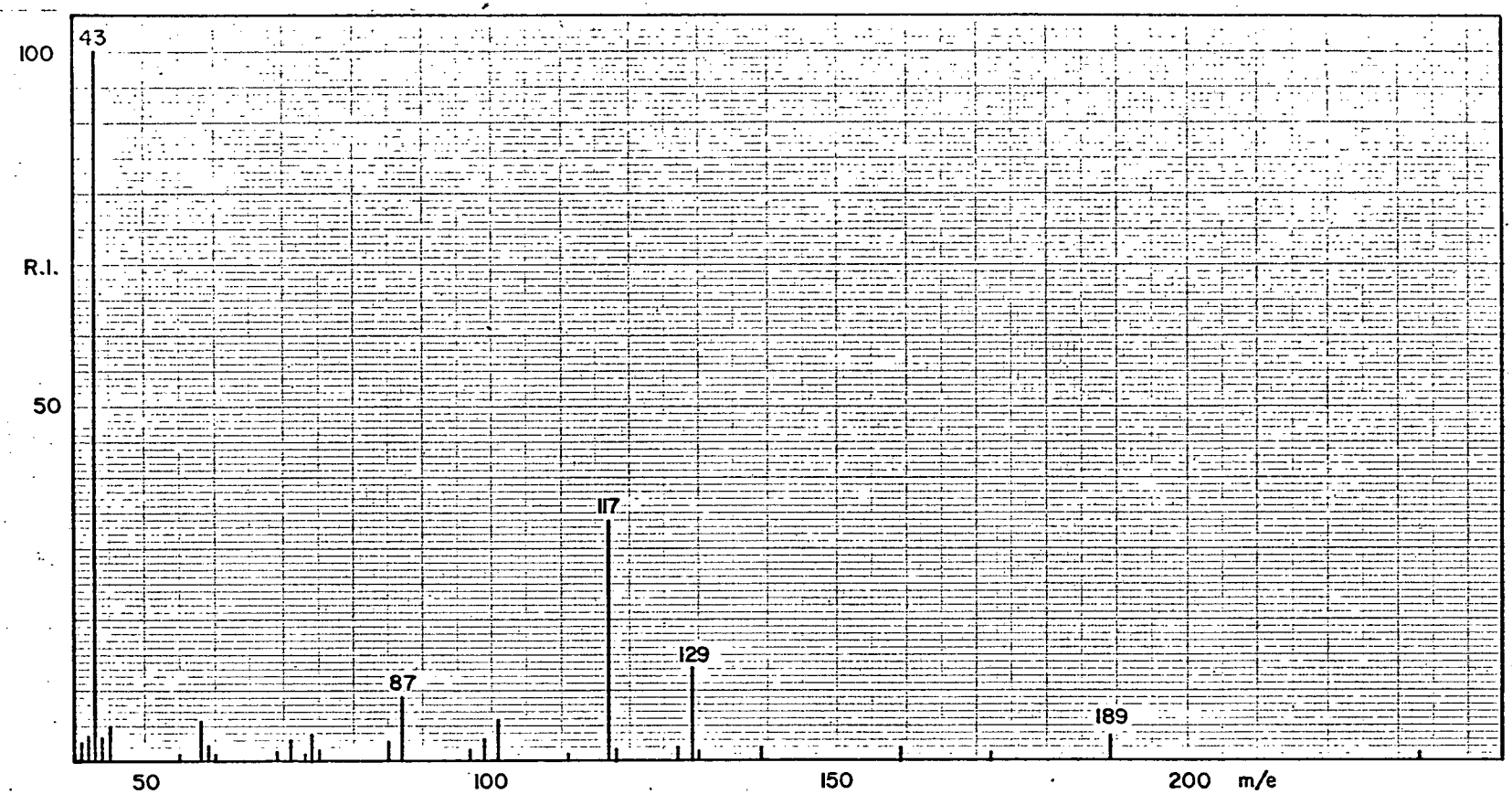


FIGURE 43

LEMON GUM	PEAK 10
1/10N H_2SO_4 LEMON GUM	PEAK 4
SMITH POLYALCOHOL LEMON GUM	PEAK 6
LEMON GUM SI	PEAK 6

[DIFFERENT SAMPLES SHOWED ONLY MINOR INTENSITY CHANGES]

2,4-Me_2-GAL

1/1ON H_2SO_4 LEMON GUM

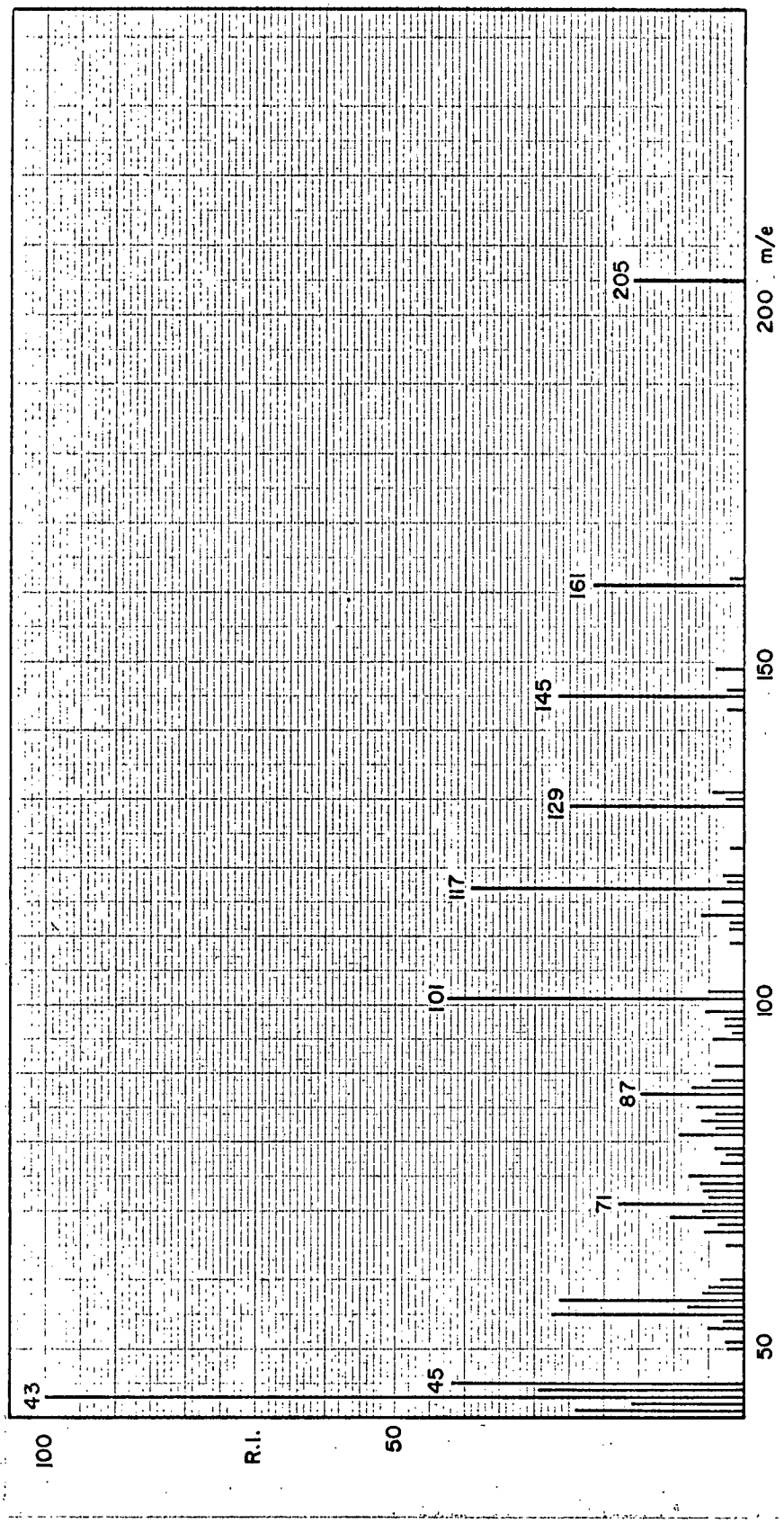


FIGURE 44: 1/1ON H_2SO_4 LEMON GUM
PEAK 1
2,3,4,6-Me_4-GAL

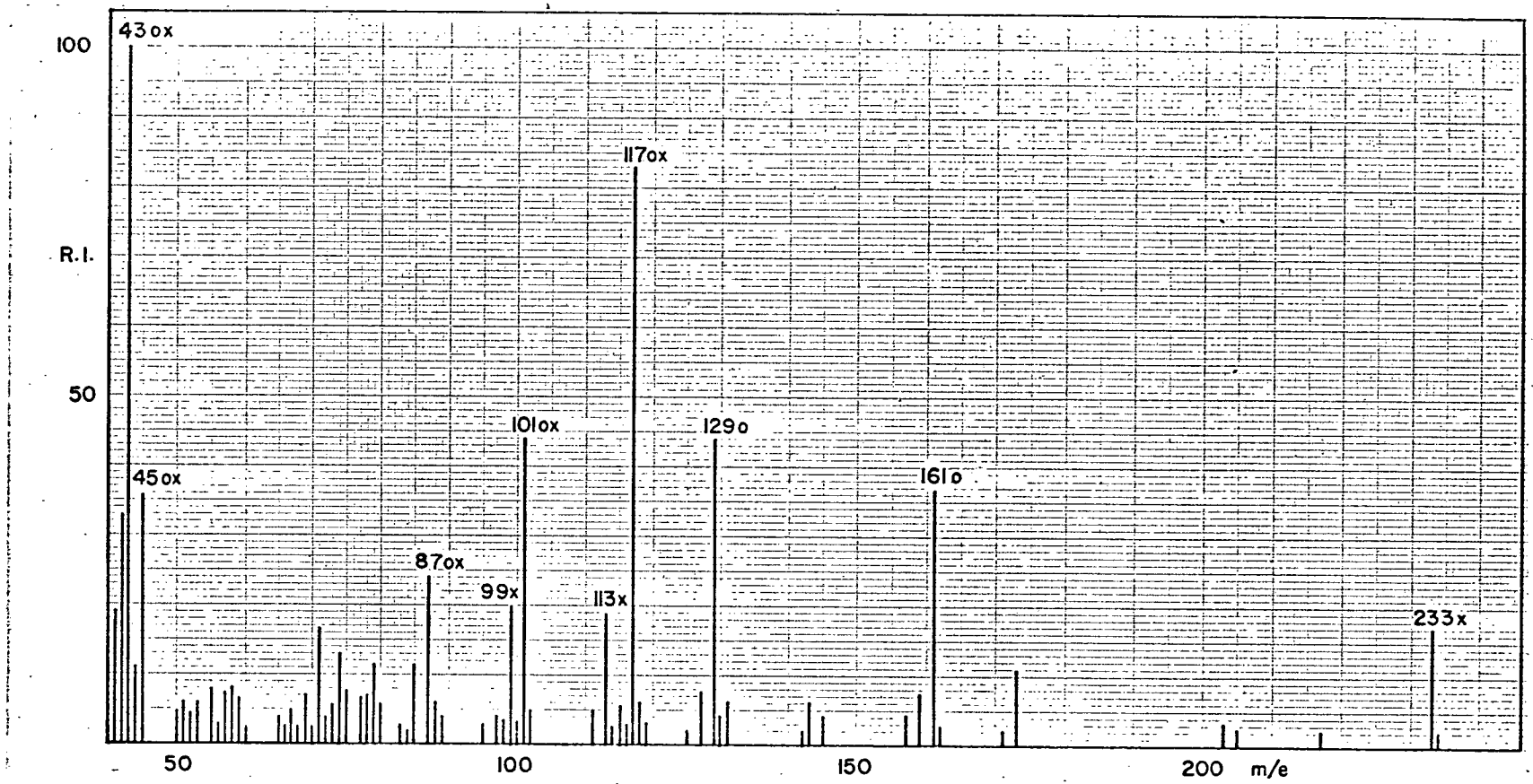


FIGURE 45: 1/10N H_2SO_4 LEMON GUM

PEAK 2

o 2,4,6-Me_3-GAL

x 2,3,6-Me_3-GAL

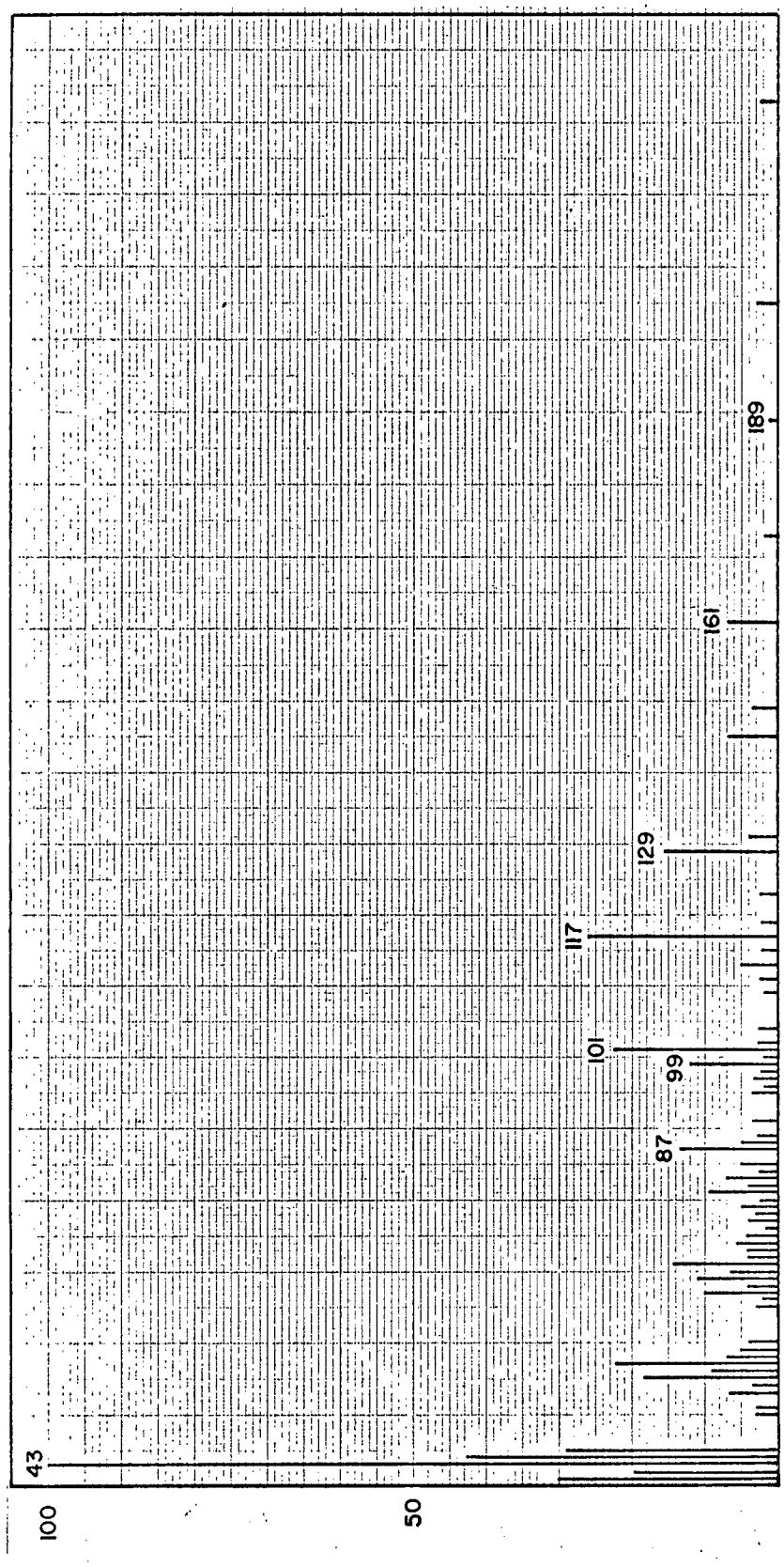


FIGURE 46: 1/10N H_2SO_4 LEMON GUM
PEAK 3
2,3,4-Me_3-GAL

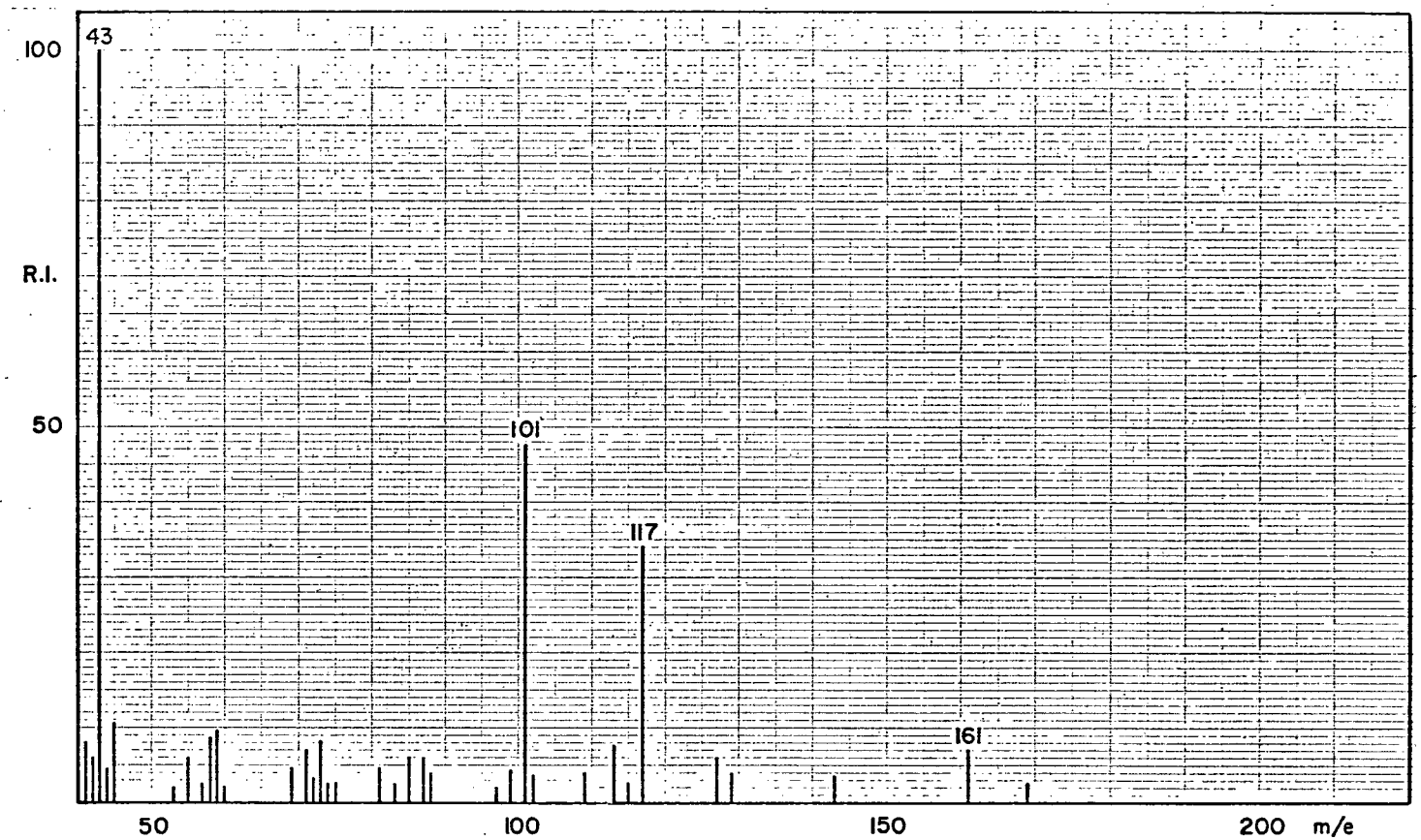


FIGURE 47: SMITH POLYALCOHOL LEMON GUM

PEAK 2

2,3,4-Me_3-ARAB

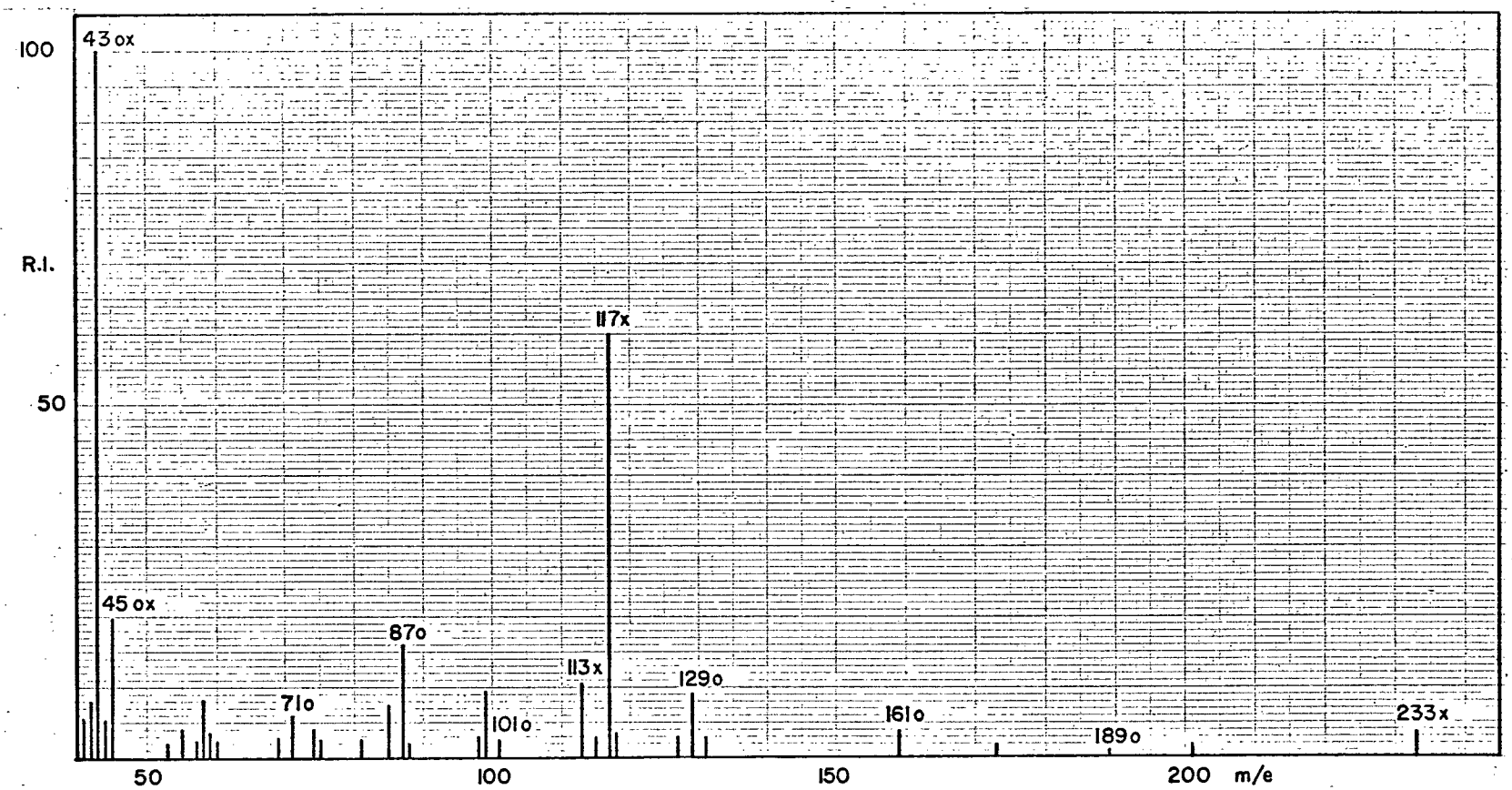


FIGURE 48: SMITH POLYALCOHOL LEMON GUM

PEAK 3

2,5-Me_2-ARAB [greater amount]

3,5-Me_2-ARAB

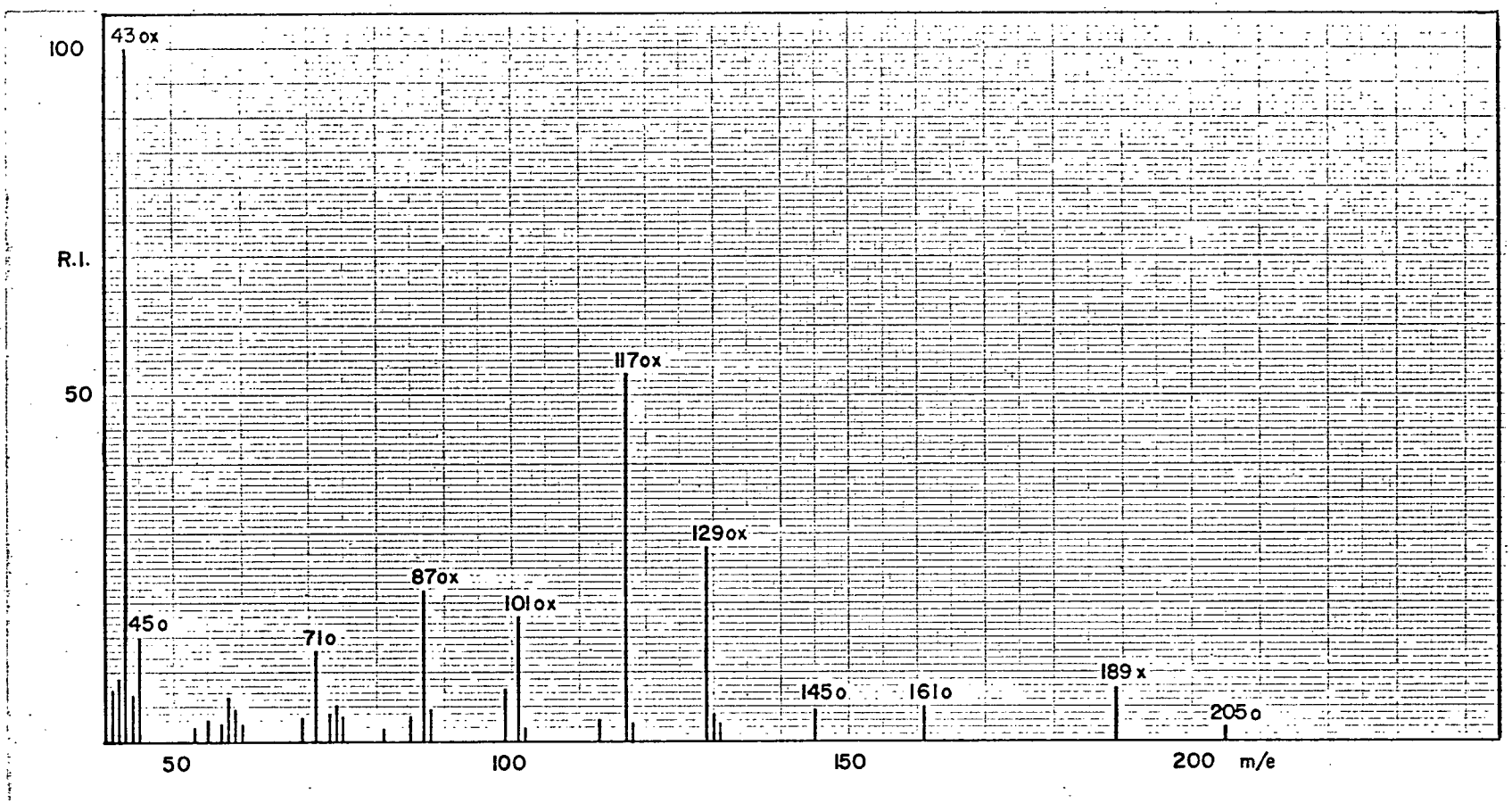


FIGURE 49: SMITH POLYALCOHOL LEMON GUM

PEAK 4

o 2,3,4,6-Me_4-GAL

x 2,3,-Me_2-ARAB

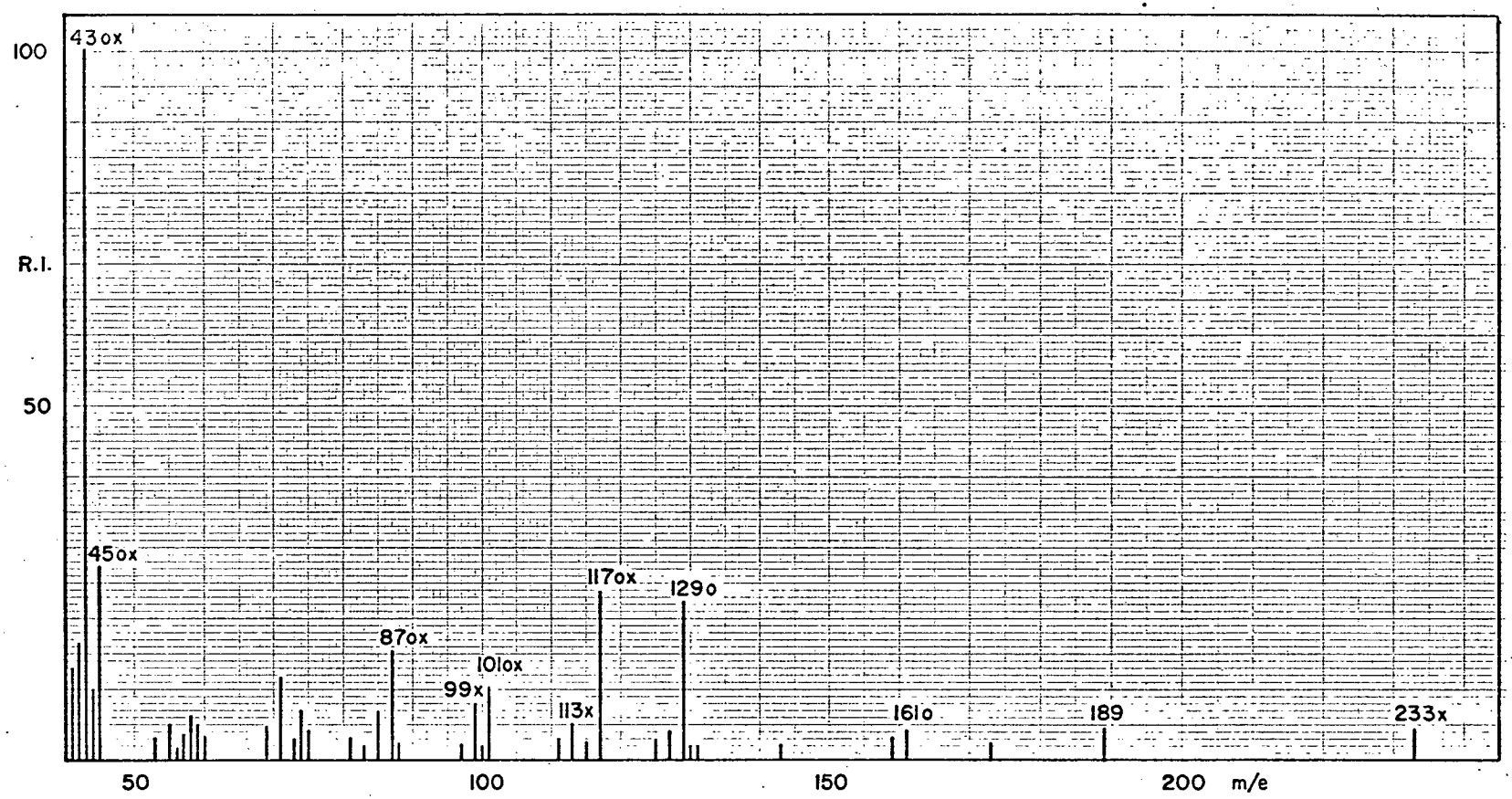


FIGURE 50: SMITH POLYALCOHOL LEMON GUM

PEAK 5

2,3,4-Me_3-GLU

x 2,3,6-Me_3-GAL

o 2,4,6-Me_3-GAL

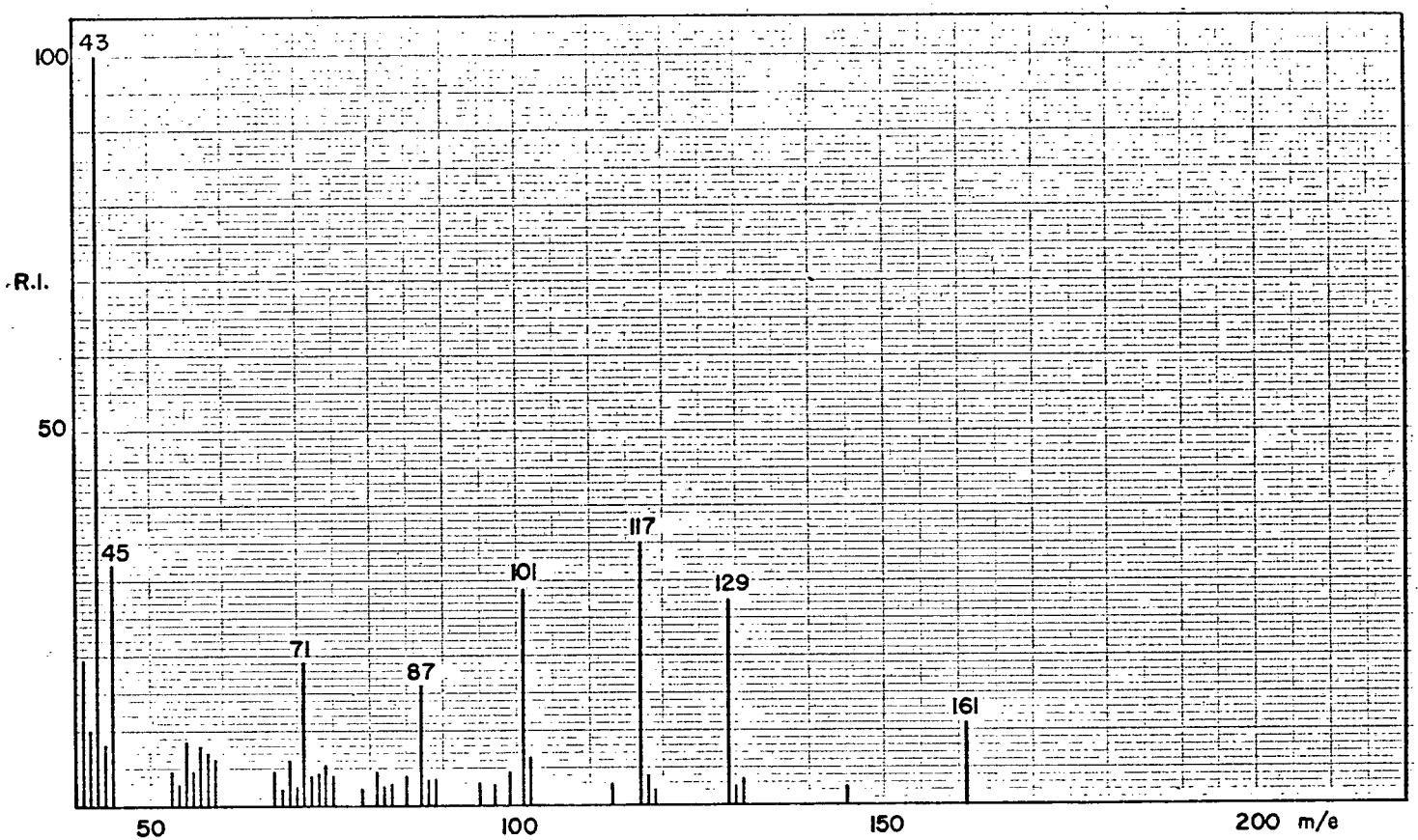


FIGURE 51: LEMON GUM SI

PEAK 1

2,3,5-Me_3-ARAB

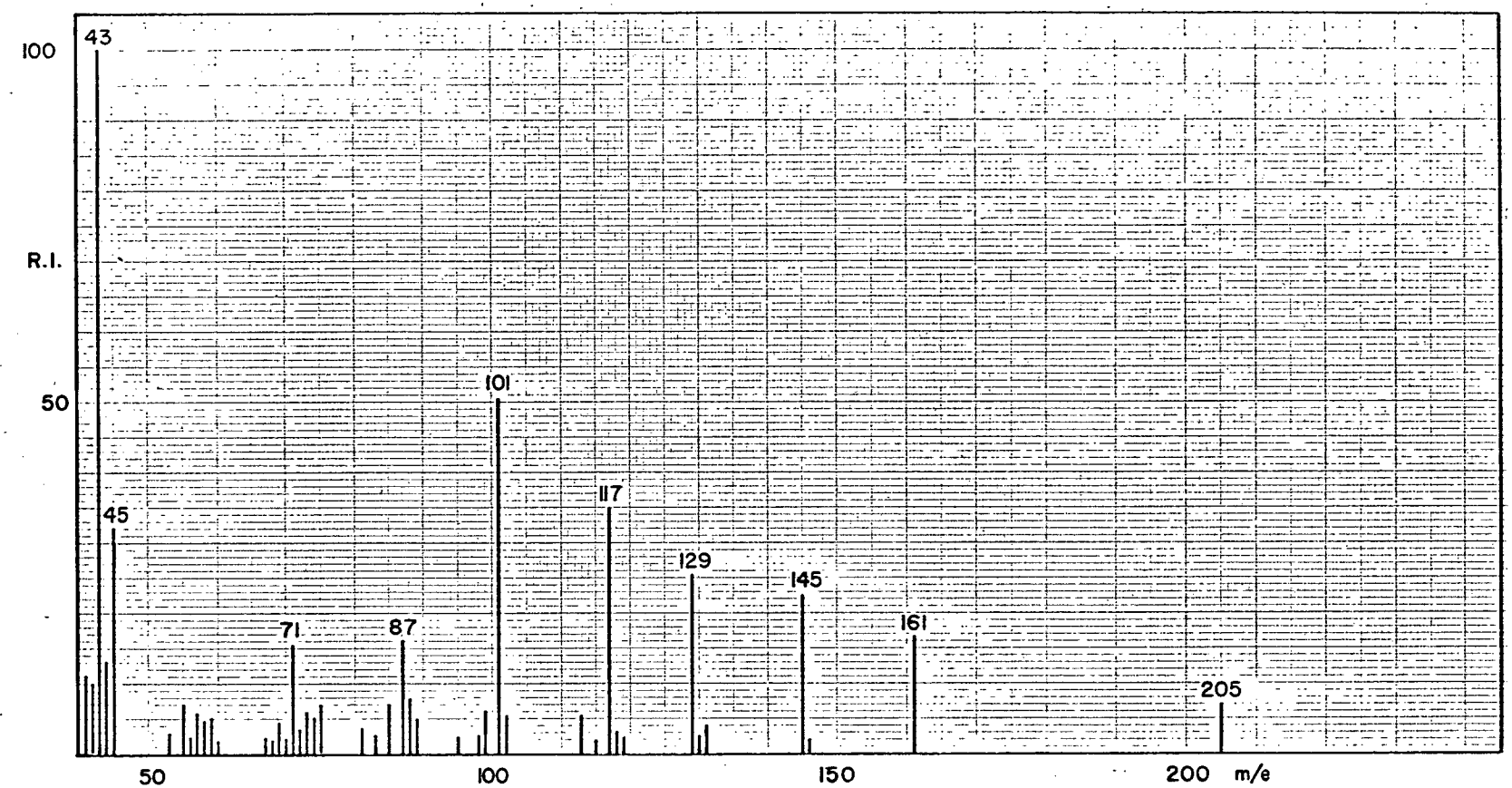


FIGURE 52: LEMON GUM SI

PEAK 2

2,3,4,6-Me_4-GAL

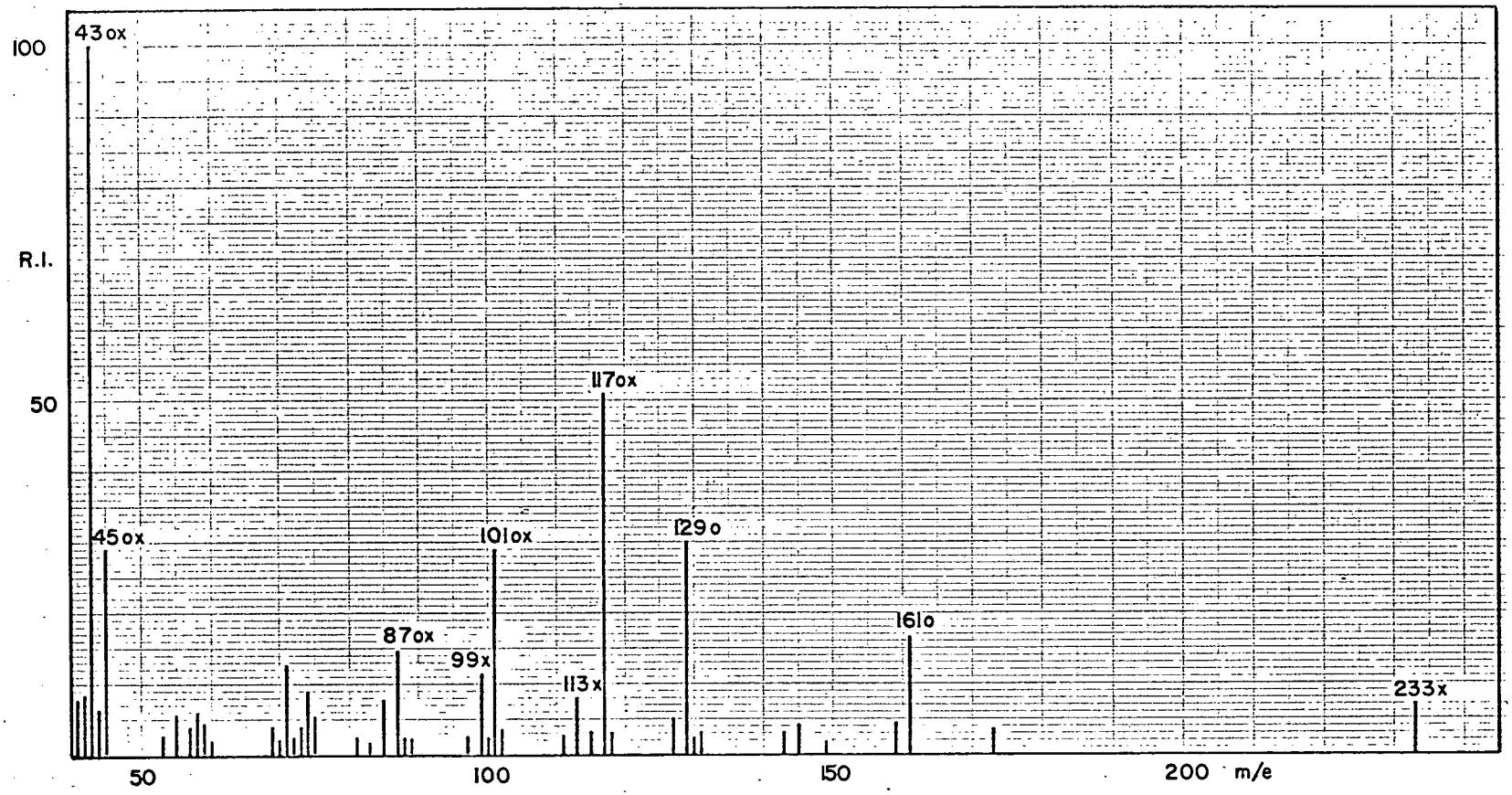


FIGURE 53: LEMON GUM SI

PEAK 3

o 2,4,6-Me_3-GAL

x 2,3,6-Me_3-GAL

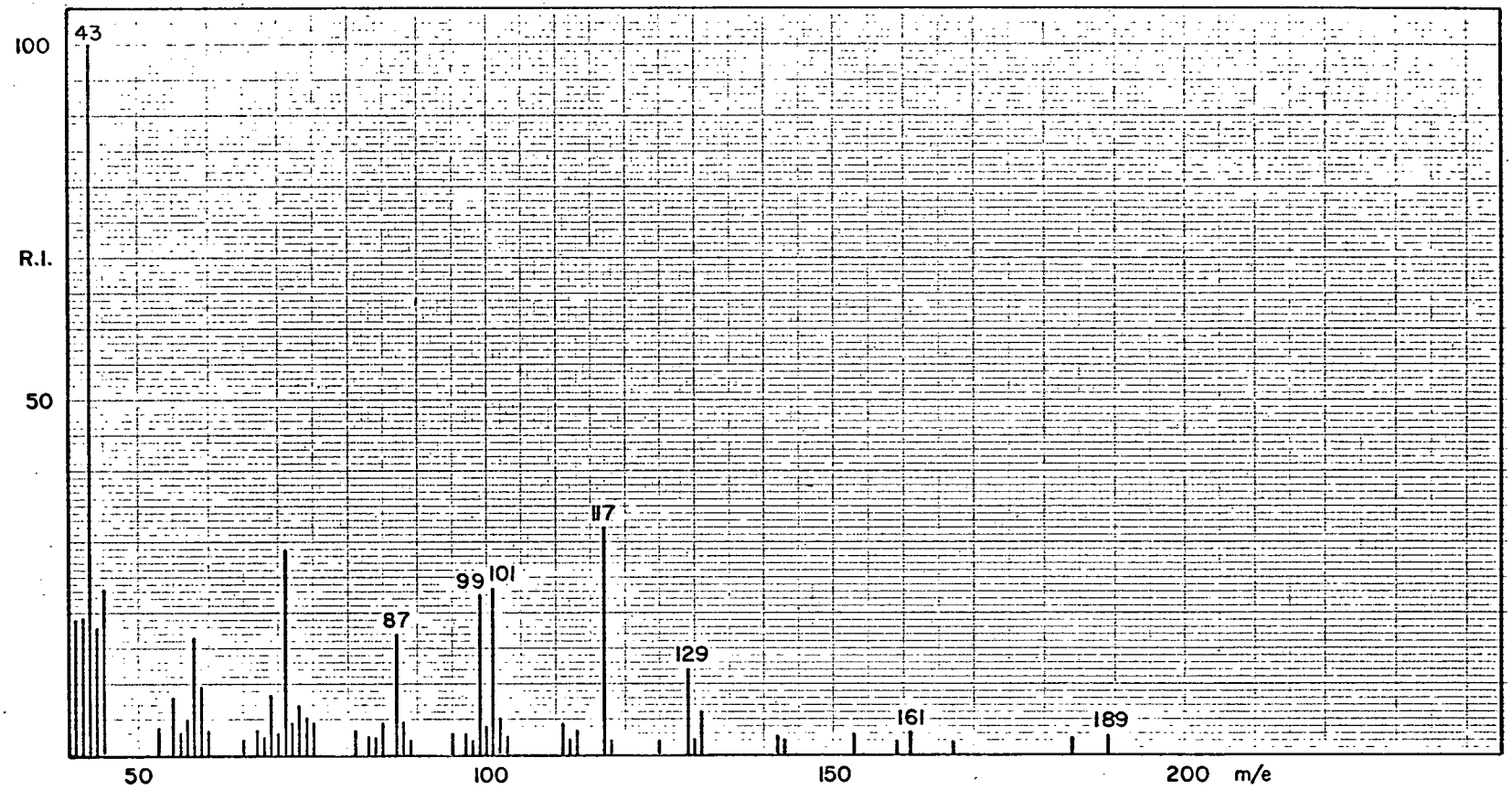


FIGURE 54: LEMON GUM SI
PEAK 4
2,3,4-Me_3-GAL

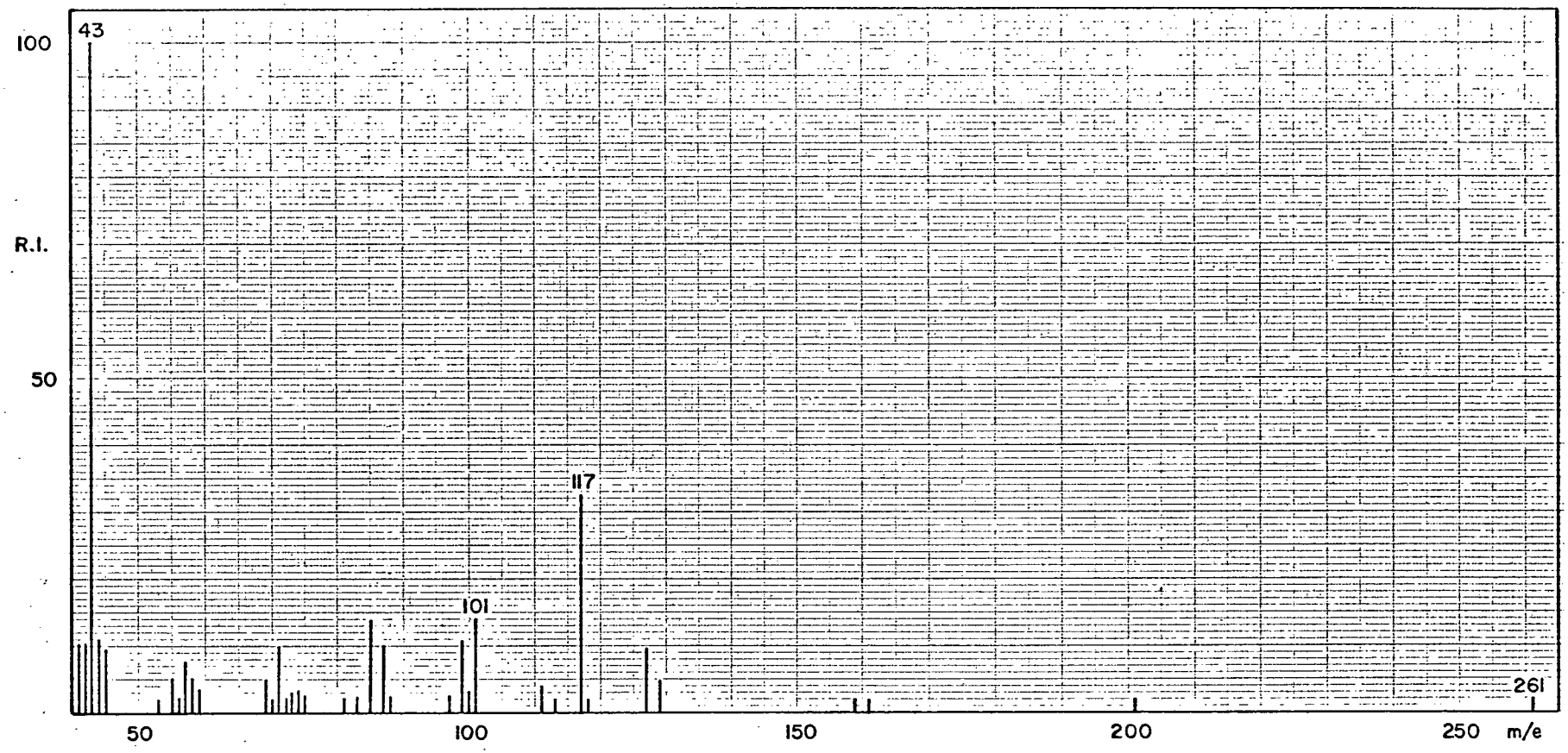


FIGURE 55: LEMON GUM SI

PEAK 5

2,3-Me_2-GLUC

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