THE CONSTITUTION OF THE HEMICELLULOSES OF CORN LEAVES AND CORN STALKS

BY

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Date 21 st January, 1970

ABSTRACT

Chairman: Professor G.G.S. Dutton

The Constitution of the Hemicelluloses of Corn Leaves and Corn Stalks

Fractionation of the polysaccharides from corn leaves by extraction with dilute alkali furnishes a branched chain arabinoxylan containing D-glucuronic acid, small amounts of 4-O-methyl-D-glucuronic acid and D-glucose. Complete acid hydrolysis of the periodate oxidised polysaccharide gives ethylene glycol, glycerol, erythritol and D-xylose in a molar ratio of 1:34:8:18. Hydrolysis of the methylated hemicellulose has yielded a mixture of 2-O- and 3-O-methyl-D-xylose; 2,3-di-O-methyl-D-xylose; 2,3,4-tri-O-methyl-D-xylose; 2,3,5-tri-O-methyl-L-arabinose and 2-O-(2,3,4-tri-O-methyl-α-D-glucopyranosyluronic acid)-3-O-methyl-D-xylose in a molar ratio of 4.6:3:40:1:4.5:6. The side chains of the polysaccharide consist of single units of uronic acid and single units of L-arabofuranose joined to positions 2 and 3, respectively, of D-xylopyranose units of the xylan molecular framework.

A similar polysaccharide is present in corn stalks.

The variety used was Golden Bantam.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor G.G.S. Dutton for his invaluable guidance and encouragement throughout the period of this work.

Helpful suggestions offered by research workers of this laboratory are also gratefully acknowledged.

INTRODUCTION

Purpose:

Hemicelluloses may be considered as one of the major constituents of plant materials. The term hemicellulose is applied to those plant cell-wall polysaccharides associated with cellulose which are unextracted by water or ammonium oxalate but which are extracted by aqueous alkali.

Hemicelluloses are polymeric materials built up of a relatively limited number of sugar residues, the principal ones being D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, 4-O-methyl-D-glucuronic acid, D-galacturonic acid, D-glucuronic acid, and to a lesser extent, L-rhamnose, L-fucose and various O-methylated sugars. The structural chemistry of the hemicelluloses has been reported in many publications (1,2), and hence, detailed description of its historical background has been omitted from this chapter.

Although the structure of the hemicelluloses of corn cobs (3,4), corn fiber (5,6), and corn hulls (7,8) have been well investigated, no structural investigation of corn leaf hemicelluloses has yet been reported in the literature. Also hemicelluloses of corn stalk have not been studied to a great extent.

A preliminary investigation of corn stalk hemicellulose (9) has indicated that it consists mainly of D-xylose, L-arabinose, and D-glucuronic acid. In another report (10), corn stalk hemicelluloses were extracted from the plant tissue with different solvents and each extract

was analysed by periodate oxidation in order to identify the major glycosidic linkages of the anhydropentoses contained therein. Gramera and Whistler (11) have separated the corn stalk polysaccharides into three fractions, each of which was homogeneous in composition. One, a glucan, obtained in small amount, resembled cellulose; a second was an acidic tetraheteroglycan, and a third, examined in greatest detail was a neutral arabinoxylan being composed of D-xylose and L-arabinose units.

The general aim of the present work is to study the structure of the hemicelluloses of corn stalk and corn leaf in detail. There has been a very considerable development of methods in structural polysaccharide chemistry in recent years. Methylation, a conventional method for structural analysis of polysaccharides, has been made more convenient due to improvements in procedure (12) and gas-liquid chromatography has become a routine operation for separating partially methylated monosaccharide derivatives. Mass spectrometry has been successfully applied for the identification of these partially methylated monosaccharide derivatives (13,14,15,16). In a similar way, gas-liquid chromatography has been applied to estimate simultaneously polyhydric alcohols and sugars obtained by the application of the periodate oxidation to polysaccharides (17). Hence, the purpose of the present investigation is to analyse the structures of corn leaf and corn stalk polysaccharides applying these recent chemical methods and experimental techniques.

CORN LEAF HEMICELLULOSE

DISCUSSION

Extraction and Hydrolysis:

Corn leaves were extracted successively with boiling ethanol-benzene (1:2) to inactivate enzymes, with cold water which removed the water soluble pectin together with much protein, and with ethylene-diaminetetra-acetic acid disodium salt which removed the less soluble pectic material. Delignification and subsequent extaction of the plant material with alkali afforded hemicellulose fractions which in most cases were free from protein. Only one fraction (Table 2, fraction A) which represented ca. 1% of the total hemicellulose extracted, contained 25.62% of protein.

Hemicellulose fractions, after acid hydrolysis, were separated into neutral and acidic sugars by ion-exchange resins. Neutral sugars on chromatographic examination were found to contain xylose as the main product together with arabinose and traces of glucose.

All hemicellulose fractions had high negative specific rotation. This pronounced negative rotation is characteristic of xylans where xylose residues are linked in the β-D configuration.

Quantitative investigation of neutral sugars was carried out on a gas-liquid chromatograph with a thermal conductivity detector. Different sugars cause the detector to respond to a varying degree, thus for identical molar amounts of different compounds the peak areas are not the same. Molar response factors (m.r.f.) of polyols and sugars have been worked out in this laboratory by Dutton and coworkers (17). With the help of those figures (also verified by the present

author where necessary) the composition of different peaks together with their molar ratios were worked out and are quoted in Table 3.

The acidic sugars formed on acid hydrolysis of the polysaccharide were desorbed from Duolite A-4 resin by elution with water containing formic acid. Hemicellulose fractions contained ca. 10% of hexuronic acid residues and a very small amount of methoxyl groups (ca. 0.5%).

Paper chromatography of uronic acid fragments gave rise to spots corresponding to glucuronic, 4-O-methyl-D-glucuronic, aldobiouronic and aldotriouronic acids. The major portion was aldobiouronic acid. The methyl ester methyl glycosides of the uronic acids were reduced with lithium aluminium hydride and hydrolysed to give 4-O-methyl-D-glucose (traces only), D-xylose and D-glucose. This indicated that the original polysaccharide contained glucuronic acid as the major acidic constituent together with traces of 4-O-methyl-D-glucuronic acid. The presence of both D-glucuronic and 4-O-methyl-D-glucuronic acid has also been observed in the polysaccharides of sapote gum (18).

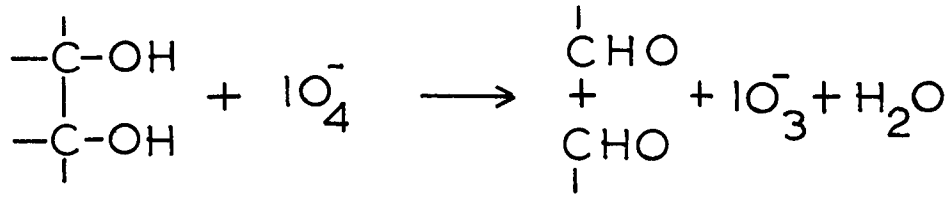
Periodate Oxidation:

In 1928, Malaprade found that periodic acid and its salts quantitatively cleaved the carbon-carbon bond of 1,2 diols (19):

$$R_2C(OH)CR_2OH + H_5IO_4 \longrightarrow 2R_2C{=}O + HIO_3 + 2H_2O$$

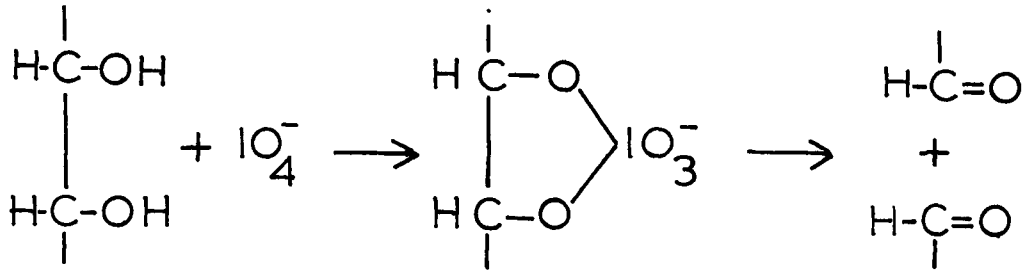
Later Fleury (20) showed that the hydroxyl groups had to be on

adjacent carbon atoms, and established periodic acid and its salts as useful analytical reagents.



Periodate oxidations are quantitative and reasonably fast at room temperature. They could be carried out over a wide range of pH. Periodate ion could be estimated in the presence of iodate ions and other reaction products, because it oxidized iodide ion to iodine in neutral or mildly alkaline solution. Periodate oxidation is a two-electron oxidation reaction, requiring one molecule of periodate in which the iodine atom is reduced from the +7 to the +5 valency state with the formation of iodate ions.

As early as 1933, a mechanism for periodate oxidation of glycols suggested by Criegee (21) involved the formation of a cyclic periodate complex. The evidence consists of direct spectrophotometric observations (22,23) on the complexes and more extensively, of kinetic evidence (24, 25,26). Also a study of the stereochemical requirements for diol cleavage by periodate has given powerful support to this hypothesis (27). The rate of periodate oxidation is strongly dependent on the dihedral angle between the two hydroxyl groups. Accordingly, compounds with vicinal cis-hydroxyl groups are oxidized more rapidly than those with trans-hydroxyl groups (27).



Periodate oxidation has been employed to a great extent in the structural determinations of polysaccharides. The procedure involves periodate oxidation of the polymer, reduction of the newly formed aldehyde groups to alcohols with sodium borohydride, acid hydrolysis of the resulting polyol, and identification of the products. This approach has been developed by Smith and coworkers (28). The periodate oxidation of a linear xylan is shown in Fig. 1. Ethylene glycol is obtained from the non-reducing end groups and glycerol from the reducing and the internal xylose residues. The proportion of ethylene glycol produced is therefore a direct measure of the number of non-reducing end groups present. When any interior residue is substituted at C-2 or C-3 in the (1 - 4)linked xylan backbone, this unit is immune to periodate attack. This accounts for the isolation of xylose in the periodate oxidation of a xylan. Erythritol is obtained from the glucan accompanying the polysaccharide.

Corn leaf polysaccharide was subjected to periodate oxidation. After 4 days 0.77 mole of periodate had been consumed per sugar residue. This slow rate of the periodate oxidation is probably due to the steric

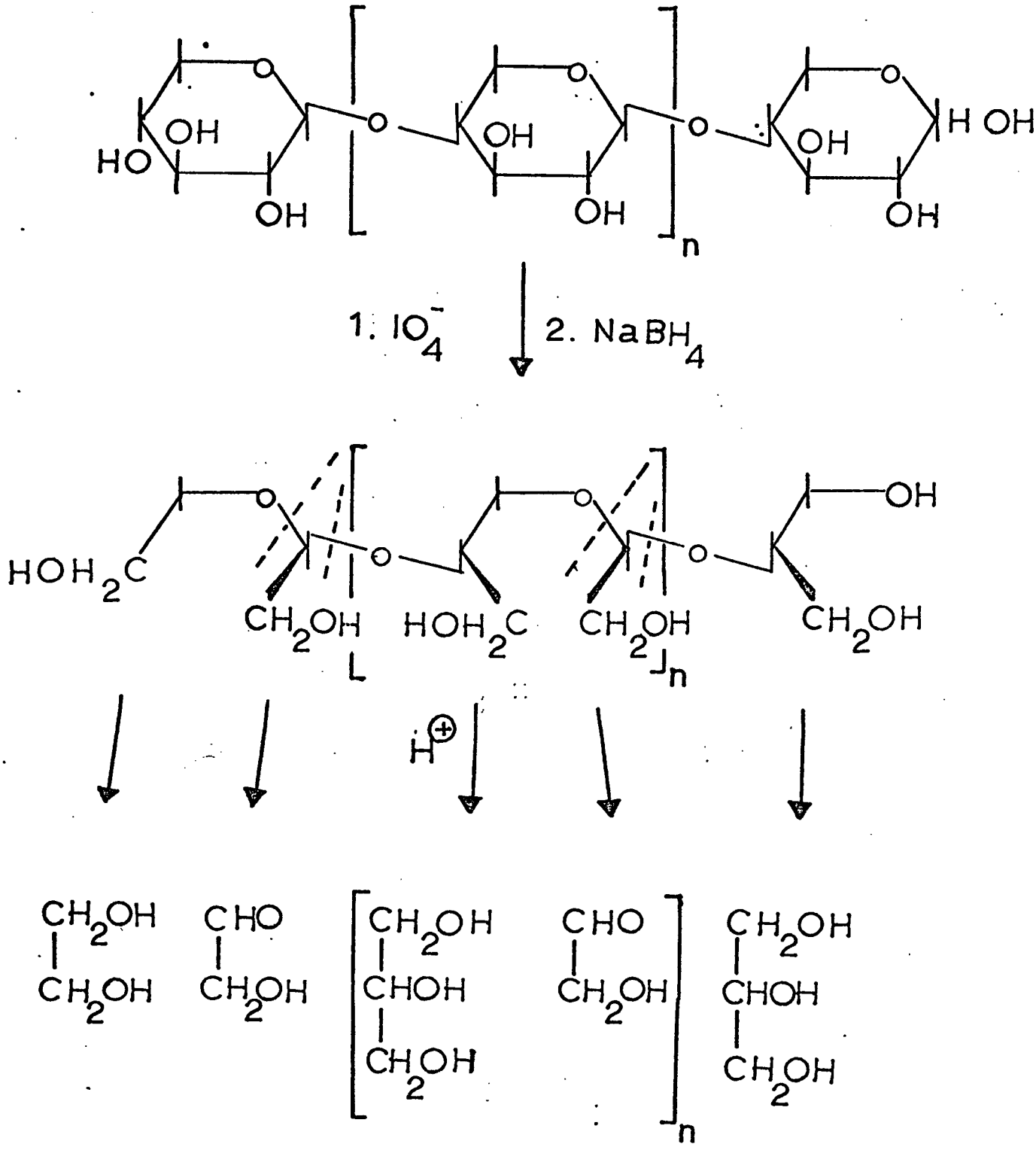


Figure 1. Periodate oxidation of the xylan framework of a polysaccharide

requirements of the vicinal trans-hydroxyl groups oxidized, as the polysaccharide under investigation contained D-xylose residues as the main constituent. After complete oxidation of the unreduced polysaccharide with periodate the resulting polyaldehyde was reduced with borohydride and subsequently hydrolysed completely with acid. The components were separated by gas-liquid chromatography as their trimethylsilyl derivatives on a column of SF 96. One of the separation curves is shown in Fig. 20. The average molar ratio between ethylene glycol, glycerol, erythritol, and D-xylose was 1:34:8:18. Molar ratios were calculated with the help of figures of molar response factors of polyols and sugars as calculated by Dutton and coworkers (17).

It is seen from Fig. 1 that glycol aldehyde is produced from the Smith degradation of a linear (1 - 4) linked xylan. The fate of glycol aldehyde in Smith degradation has not been quantitatively established. However, Dutton et al (loc. cit.) have concluded that glycol aldehyde was almost entirely destroyed in the hydrolysis of the polyalcohol and hence did not interfere with the quantitative estimations of polyhydric alcohols by gas-liquid chromatography. The present work is also in agreement with the fact that glycol aldehyde is not a serious factor in such analyses.

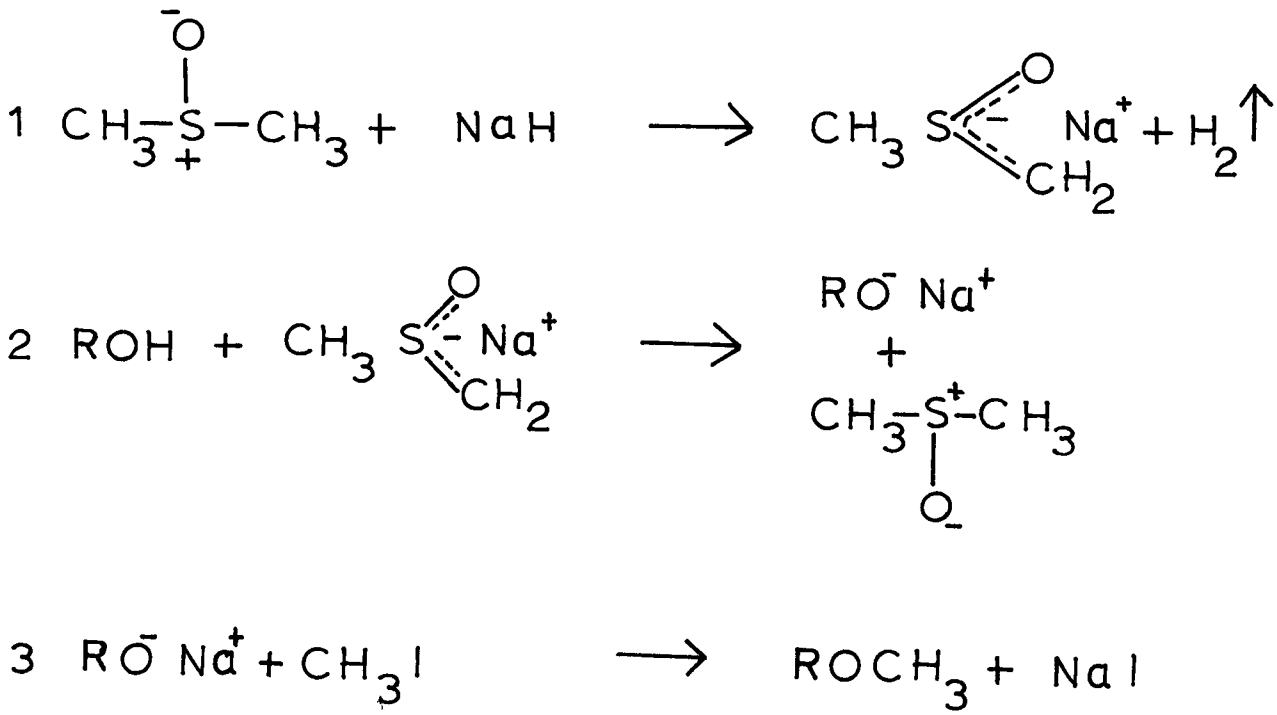
Methylation Studies:

The purpose of the methylation is to achieve an etherification of all of the free hydroxyl groups in the polysaccharide. The procedures for methyl ether formation are limited in scope. The methylating

agents used are either dimethyl sulphate or methyl iodide; the former usually in conjunction with a strong alkali, which serves to promote ionization of the pertinent hydroxyl function, and the latter with an agent such as silver oxide. However, these methylating agents are not always very effective, because the operation has to be repeated several times before a complete methylation is accomplished.

An effective and rapid methylation technique has been developed by Hakomori (12), wherein the methylsulphinyl anion (29,30) is used to generate the polysaccharide alkoxide prior to the addition of methyl iodide. Dimethyl sulphoxide is used as the solvent wherein methylation reactions proceed with favourable kinetic rates under relatively mild conditions. A high degree of methylation is achieved in one treatment.

Reaction sequences can be shown as follows;



Methylation of corn leaf polysaccharide was carried out according to the method described by Hakomori (12). Reaction was found to be complete in one treatment. Methylated polysaccharide was fractionated by extraction with a mixture of pet. ether-chloroform, the proportion of the latter being increased in stages. A series of fractions was obtained which varied not only in quantities but also in methoxyl contents (Table 5). Fraction 5 whose methoxyl content was in close agreement with the theoretical methoxyl content of a fully methylated pentosan, was used for quantitative analysis.

Methylated polysaccharide was hydrolysed according to the procedure of Lindberg et al (31), wherein the polymer was hydrolysed with 72% sulphuric acid at 0° for 30 minutes, followed by dilution (ten times) and hydrolysis at 100° for 4 hours. This procedure has been reported to produce no apparent demethylation which is a problem encountered with most other methods such as methanolysis, formolysis etc.

Mixtures of methylated sugars were separated into the acidic and neutral components by passage through ion-exchange resins. The acidic component was converted into its methyl ester methyl glycoside by refluxing with methanolic hydrogen chloride (3%) which on reduction with lithium aluminium hydride gave rise to a neutral disaccharide glycoside. When the disaccharide was hydrolysed two components were observed on a paper chromatogram. These were isolated from paper by elution with water. One of the components was 3-O-methyl-D-xylose, identified as crystalline sugar and the other was 2,3,4-tri-O-methyl-D-glucose, characterized by its crystalline β-methyl glycoside. The

crystalline β-methyl glycoside was further characterised by the fragmentation pattern of mass spectra (Fig. 16).

The isolation of 3-O-methyl-D-xylose from the partially methylated aldobiouronic acid suggested that the uronic acid moiety of the polysaccharide was linked with the xylose backbone through C-2 of xylose and C-1 of the uronic acid. Also, the high positive rotation of the methylated aldobiouronic acid ($+86.5^{\circ}$) indicates the presence of an α-D-linkage.

Preliminary investigation of the methylated neutral sugars on a paper chromatogram revealed 5 components (Table 6). The mixture of methylated sugars was separated on a column of hydrocellulose:cellulose (1:1) using methyl ethyl ketone-water azeotrope as eluent. Some of the early fractions were reseparated and these results are tabulated in Table 7.

The mixture was found to contain 2,3,4-tri-O-methyl-D-xylose; 2,3,5-tri-O-methyl-L-arabinose; 2,3-di-O-methyl-D-xylose and mono-O-methyl xyloses.

2,3,4-Tri-O-methyl-D-xylose was identified as its crystalline β- methyl glycoside.

2,3,5-Tri-O-methyl-L-arabinose was identified by qualitative chromatography and also by the fragmentation pattern of the mass spectrum of its methyl glycoside.

2,3-Di-O-methyl-D-xylose was identified by its optical rotation and also by conversion into its characteristic crystalline anilide derivative.

Mono-methyl xyloses were identified by qualitative paper chromatography.

Identification and quantitative estimation of partially methylated sugars obtained from hydrolysis of the methylated polysaccharide, were also carried out by gas-liquid chromatography. According to Lindberg (15), in methylation analysis of polysaccharides, the mass spectrum of a partially methylated alditol acetate obtained from a methylated sugar, in combination with the sugar composition of the original polysaccharide and T-values would, in most cases, give sufficient evidence for an unambiguous characterization of the methylated sugar.

Partially methylated neutral sugars were converted into corresponding alditol acetates and subsequently analysed by gas-liquid chromatography. Alditol acetates of partially methylated monosaccharide derivatives on gas-liquid chromatography give single peaks for each component. This is because of the fact that reduction of sugars to alditols with borohydride removes the possibility of anomerization and acetylation of free hydroxyl groups gives a volatile derivative capable of being resolved. The separation was carried out on a column consisting of a 3% liquid phase of an organosilicone polyester of ethylene glycol succinate chemically combined with a silicone of the cyanoethyl type. It was found that separations of different components were better achieved and solvent tailing effect was greatly reduced if the columns were held isothermally at 160° for 3 minutes and then programmed at 2° per minute to hold at 180°, rather than isothermally at 180°. The separation curves were found to be quite reproducible.

The mixture of partially methylated alditol acetates was found to contain 4 components: 2,3,5-tri-O-methyl arabinitol; 2,3,4-tri-O-methyl xylitol; 2,3-di-O-methyl xylitol and mono-O-methyl xylitols. The assignment of each of the 4 peaks was verified by comparison of retention times under identical conditions and also by the fact that each peak was superimposed upon that obtained from the authentic samples of partially methylated alditol acetates, when they were individually injected together with the unknown mixture into the gas-liquid chromatograph.

Although alditol acetates of 2,3,4-tri-O-methyl-D-xylose, 2,3,5-tri-O-methyl-L-arabinose and 2,3-di-O-methyl-D-xylose are well separated from one another, alditol acetates of 2-O-methyl, and 3-O-methyl-D-xyloses could not be separated since they have identical retention time on gas-liquid chromatographs. Identical retention times of these compounds have also been reported by Jones et al (32).

The time required for reduction of partially methylated sugars with sodium borohydride requires brief comment. Although Lindberg et al (33) have reported a reduction period of 2 hours for borohydride reduction of partially methylated sugars, it was found in our present work that a shorter reduction time gave rise to many peaks on the chromatogram which was probably due to incomplete reduction. Hence a longer reduction time (ca. 12 hours) was employed for the preparation of partially methylated alditol acetates. This observation is in agreement with a report of Bragg and Hough (34) on quantitative aspects of borohydride reduction of carbohydrates. The reduction of

an aldose to a glycitol would be preceded by ring opening of the cyclic modification to the aldose of the aldehydo-form in the acyclic staggered zig-zag conformation. A bulky substituent at C-3 of this conformation causes the approach of borohydride ion to the aldehyde group to be sterically hindered.

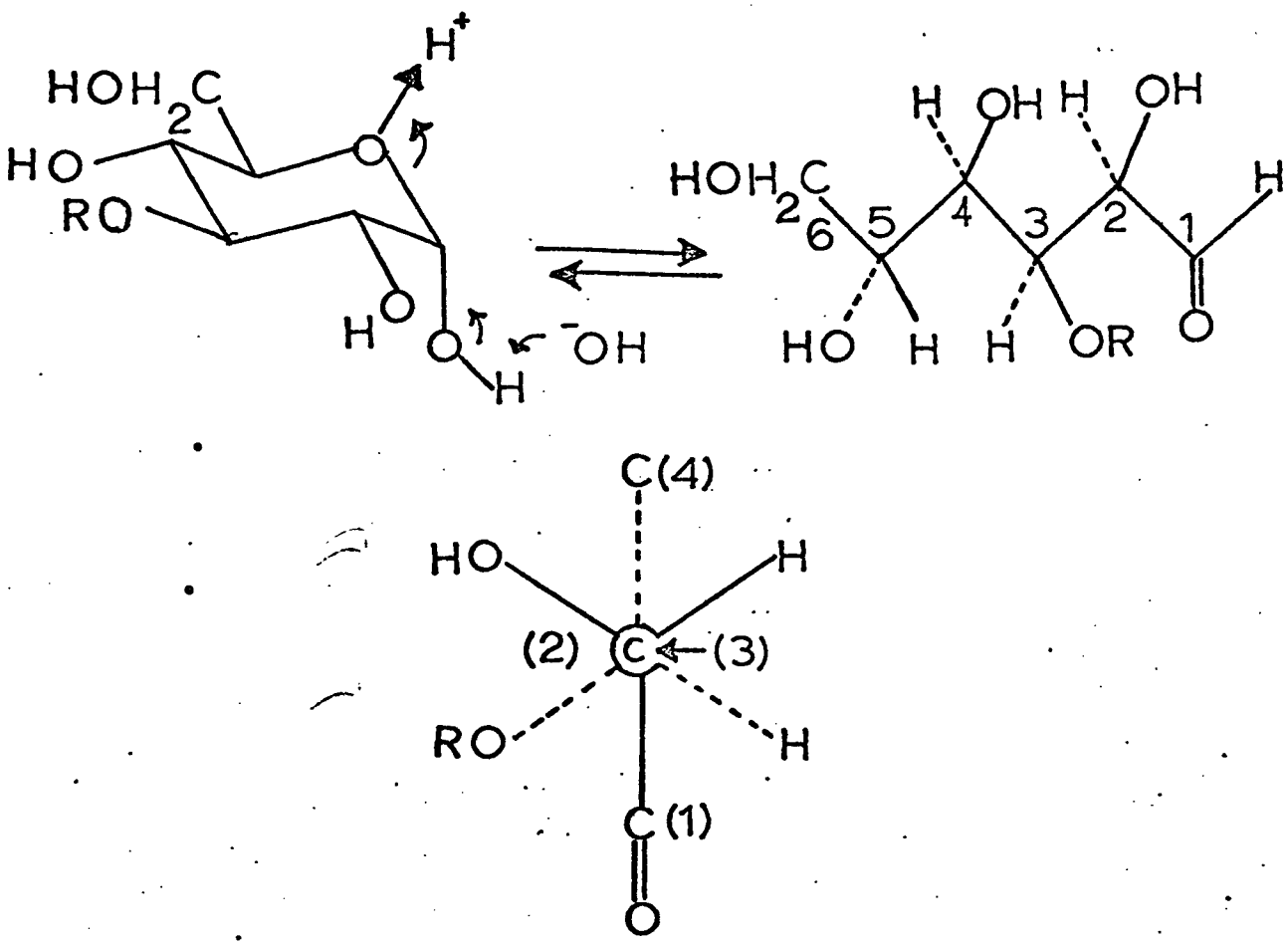


Fig. 2. Steric hindrance by substituent at C-3 in borohydride reduction.

Sephton (35) has rpeorted a separation of the hydrolysed products of a methylated xylan as their trimethylsilyl derivatives by gas-liquid chromatography. Although trimethylsilyl derivatization is simple and rapid, it suffers from certain shortcomings. In solution a sugar may exist as an equilibrium mixture of the anomers of the furanose and pyranose forms. Hence each monosaccharide can give rise to as many as four derivatives during its conversion to trimethylsilyl derivatives. These derivatives, which are formed through anomeric and ring isomerization, each produce a peak on the chromatogram. Thus a complex mixture of carbohydrates could give a multiplicity of bands which would make total resolution quite complicated.

However, in certain cases, trimethylsilyl derivatives of partially methylated sugars have one advantage over corresponding partially methylated alditol acetates. The anomeric pairs of 2-O-methyl and 3-O-methyl-trimethylsilyl derivatives of xyloses are well separable from one another when examined by gas-liquid chromatography. Hence a quantitative estimation of mono-O-methyl xyloses in a mixture can be accomplished by this procedure.

Anomeric peaks of trimethylsilyl derivatives of 2,3,4-tri-O-methyl-D-xylose; 2,3,5-tri-O-methyl-l-arabinose and 2,3-di-O-methyl-D-xylose were not well separated on a column of SE 52. This observation is also in agreement with that reported by Sephton (35).

Mass Spectrometry:

Mass spectrometry has now become an important technique for the structural analysis of carbohydrate derivatives. Improved methylation procedures and subsequent separation of partially methylated monosaccharide derivatives by gas-liquid chromatography have made mass-spectral technique a useful supplement to chemical methods for the analysis of polysaccharides.

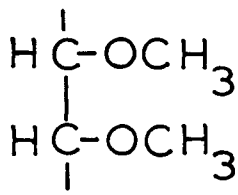
Carbohydrates being practically non volatile, mass-spectral studies on them have been carried out on their more volatile derivatives, such as, methyl ethers (13), acetates (14), alditol acetates (15), trimethylsilyl ethers (16,36), trifluoro acetates (37).

Although mass spectrometry has been used in organic chemistry for molecular weight determination, unfortunately, owing to the extreme instability of the carbohydrate molecule, the molecular ion (M^+) can hardly be traced in the mass spectra of most of the carbohydrate derivatives. Only recently Kochetkov et al (37) have found a molecular peak intense enough for direct molecular weight determination in the mass spectra of trifluoroacetyl derivatives of some monosaccharide derivatives. In this chapter, fragmentation patterns of some partially methylated monosaccharide derivatives encountered in the present investigation, will be briefly discussed.

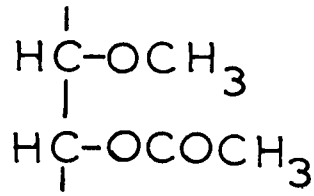
1. Partially Methylated Alditol Acetates:

From mass spectra one can distinguish between primary and secondary fragments of alditol acetates. Primary fragments arise by fission between two

carbon atoms in the alditol chain; either of the two fragments could carry the positive charge. The secondary fragments are observed from the primary fragments by single or consecutive elimination of acetic acid (60), ketene (42), methanol (32) or formaldehyde (30). The intensity of primary fragments increases with decreasing mass number. Fragmentation processes take place between carbon atoms in the partial structures 1 and 2 in preference to 3, and the positive ion is stabilized by a methoxyl grouping. Again, structure 1 is cleaved in preference to structure 2 (15).



1



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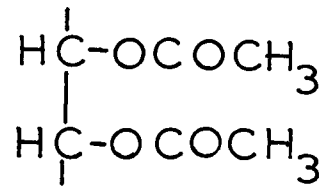


3

The present investigation includes analyses of mass spectra of the alditol acetates of 2,3-di-O-methyl xylose; 2,3,4-tri-O-methyl xylose; and 2,3,5-tri-O-methyl arabinose. Molecular ion (M^+) could not be traced in any of the three cases. The base peak in all cases was at m/e 43 which is due to acetylium ion. Peaks of low intensities (< 5% of the base peak) were not considered in interpreting mass spectra.

Some results are evident on inspection of the fragmentation patterns shown in Figs. 4, 6, and 8. Primary fragments, m/e 117 and m/e 161 are common to all compounds. However they differ in their

relative intensities. In the spectrum shown in Fig. 3, primary fragments arise preferentially from the cleavage of C-2, C-3 bond. In the spectra of Figs. 5 and 7, primary fragmentation takes place at C-2, C-3, C-4 bonds.

The prominance of peak at m/e 45 in Fig. 7 in comparison to other compounds can be accounted for by the presence of a methoxyl group at the terminal carbon atom. A peak of low intensity ($\sim$5%) at m/e 45 can also be found in mass spectra of other compounds and is probably a secondary fragment.

A peak of very high intensity is observed at m/e 145 in Fig. 5. This is a secondary fragment arising from the primary fragment at m/e 205. The primary fragment at m/e 205 which is not very stable, is expected to arise from alditols methylated at position 2, 3, and 4. This primary fragment by loss of acetic acid (60),gives rise to a sharp peak at 145. Hence prominence of secondary fragment at m/e 145 is characteristic of only 2,3,4-tri-O-methyl pentose derivatives.

The primary fragment at m/e 189 (Fig. 3) is characteristic of alditols methylated at position 3 but not at 1 and 2. Hence this should be a prominent peak only of 2,3-di-O-methyl xylitol triacetates as found in Fig. 3, absent in Figs. 5 and 7. The primary fragment at m/e 189 on loss of acetic acid (60) gives rise to a secondary fragment at m/e 129, which undergoes further fission as illustrated in Fig. 4.

Hence it is evident from the above discussion that assignments and interpretations of mass spectra of different partially methylated alditol acetates permit their identification.

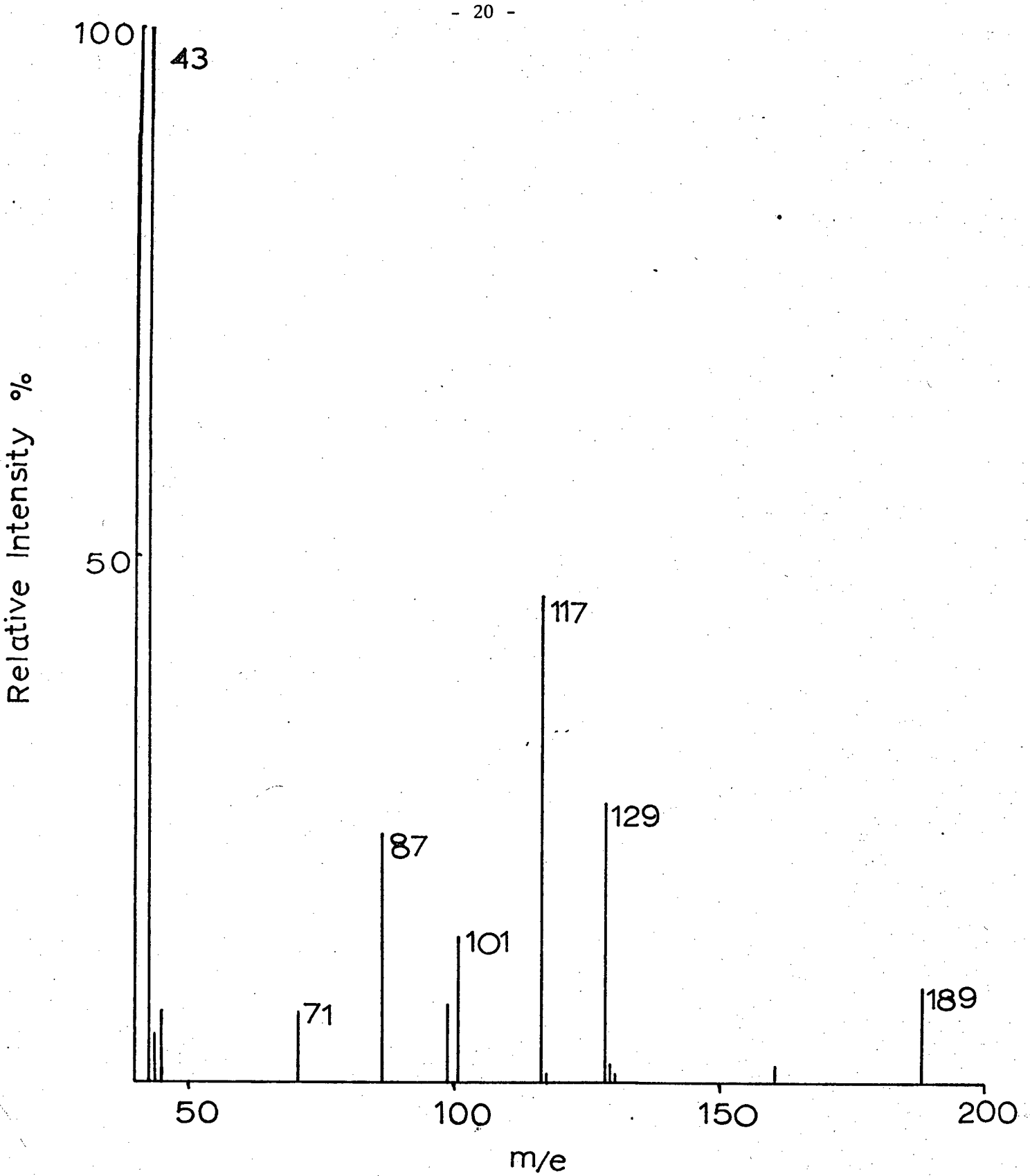


Figure 3. Mass spectrum of 1,4,5-tri-O-acetyl-2,3-di-O-methyl-D-xylitol

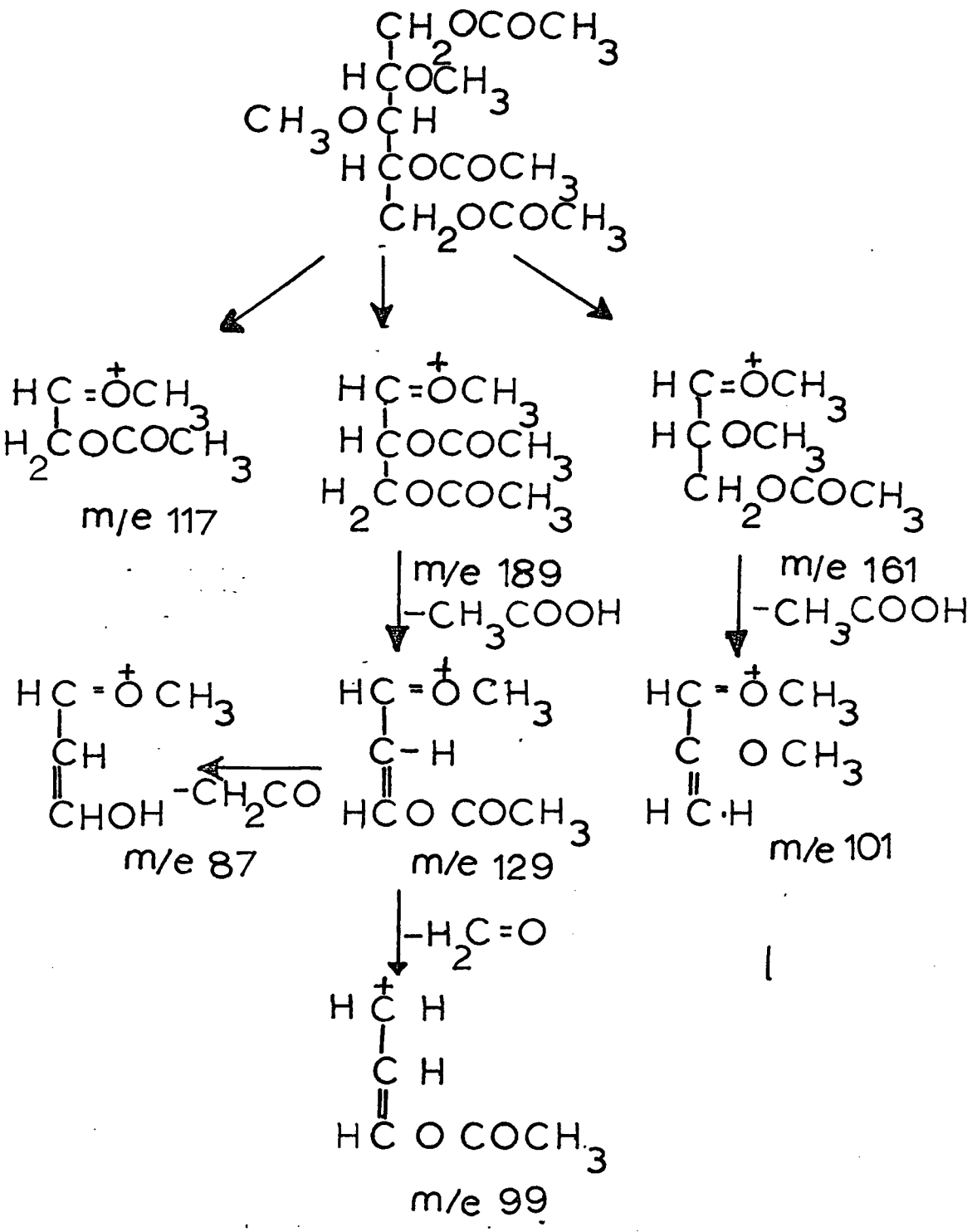


Figure 4. Source of the most important ions of 1,4,5-tri-O-acetyl-2,3-di-O-methyl-D-xylitol

Figure 5. Mass spectrum of 1,5-di-O-acetyl-2,3,4-tri-O-methyl-D-xylitol

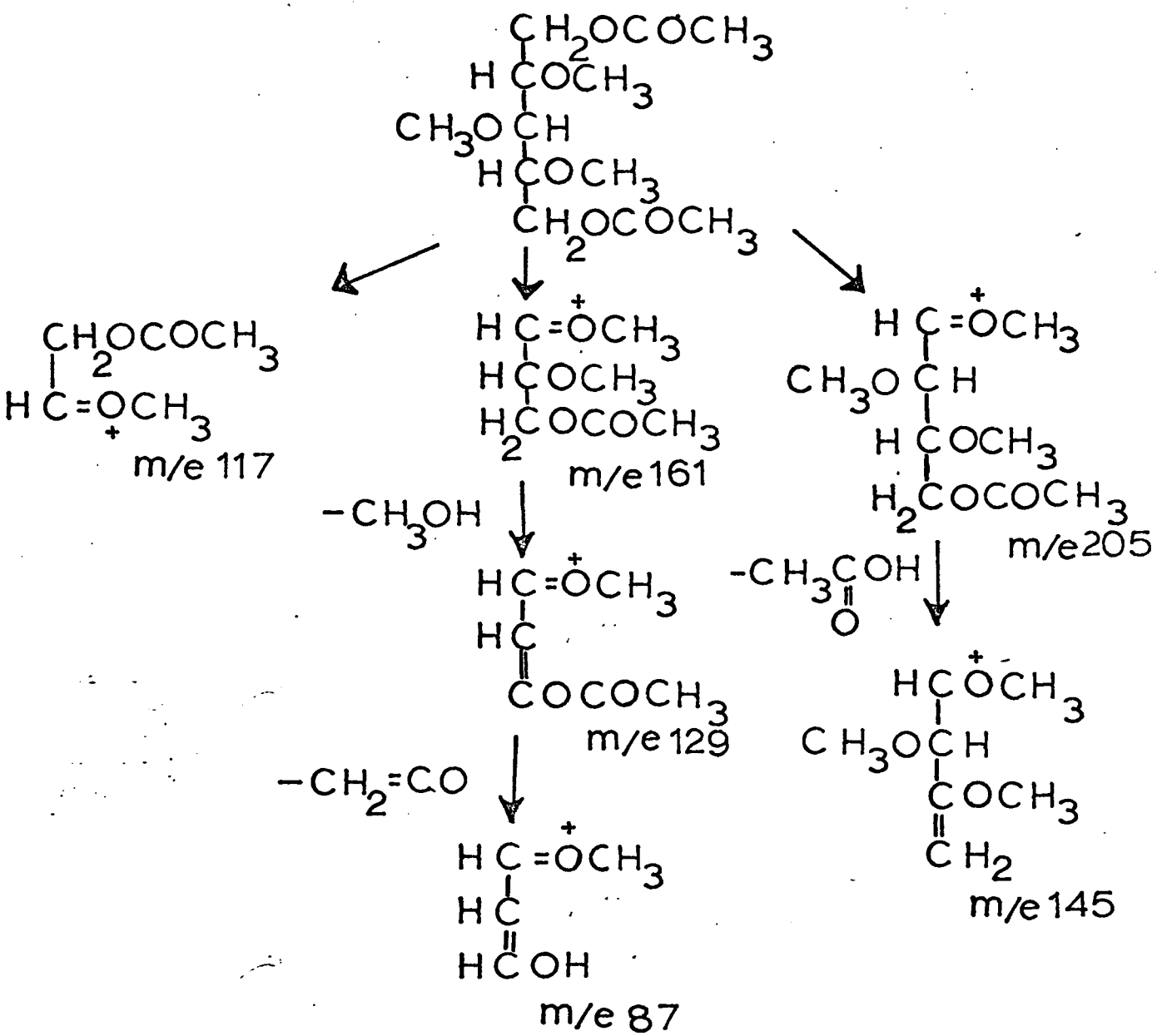


Figure 6. Source of the most important ions of 1,5-di-O-acetyl-2,3,4-tri-O-methyl-D-xylitol

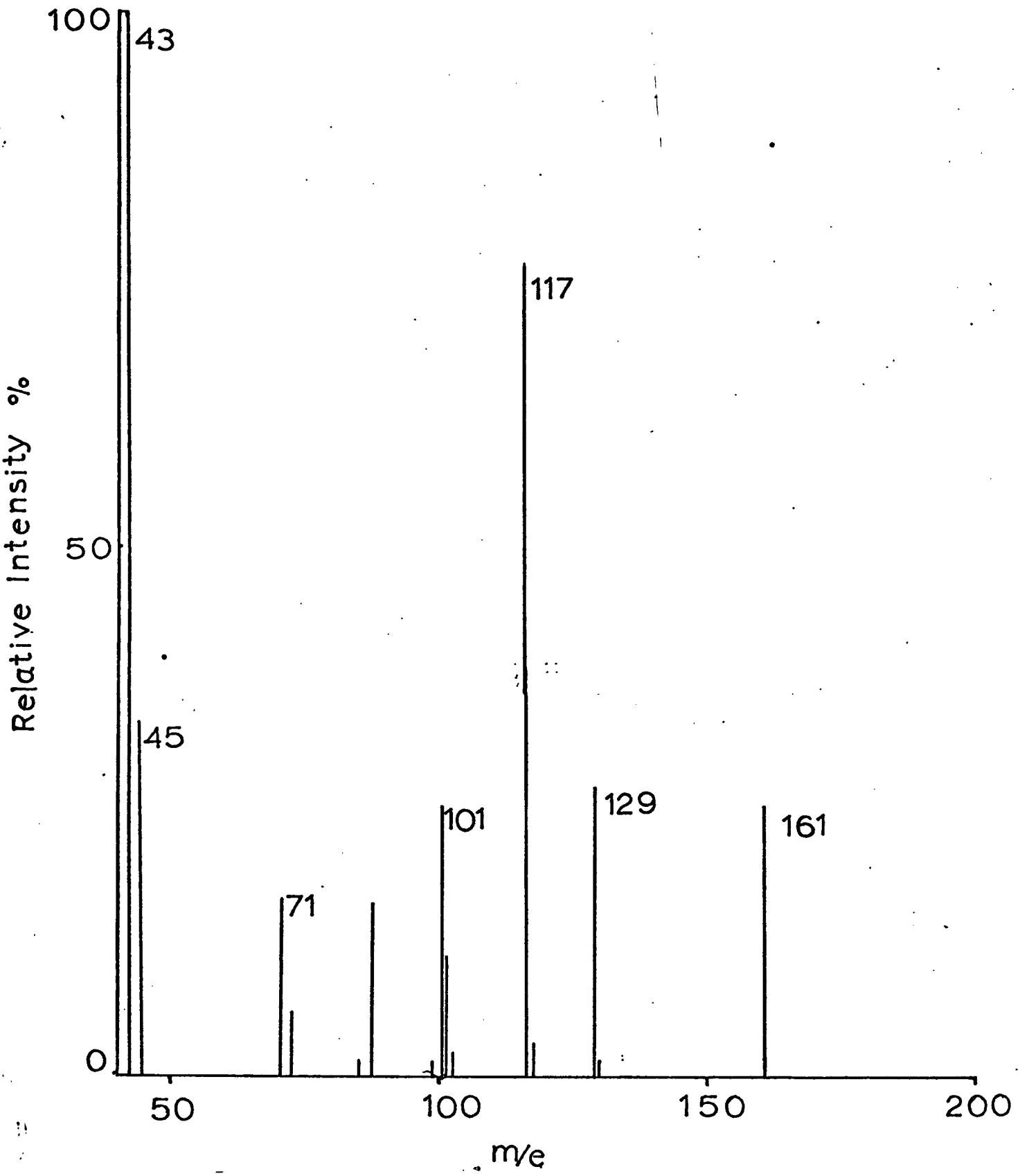


Figure 7. Mass spectrum of 1,4-di-O-acetyl-2,3,5-tri-O-methyl-L-arabinitol

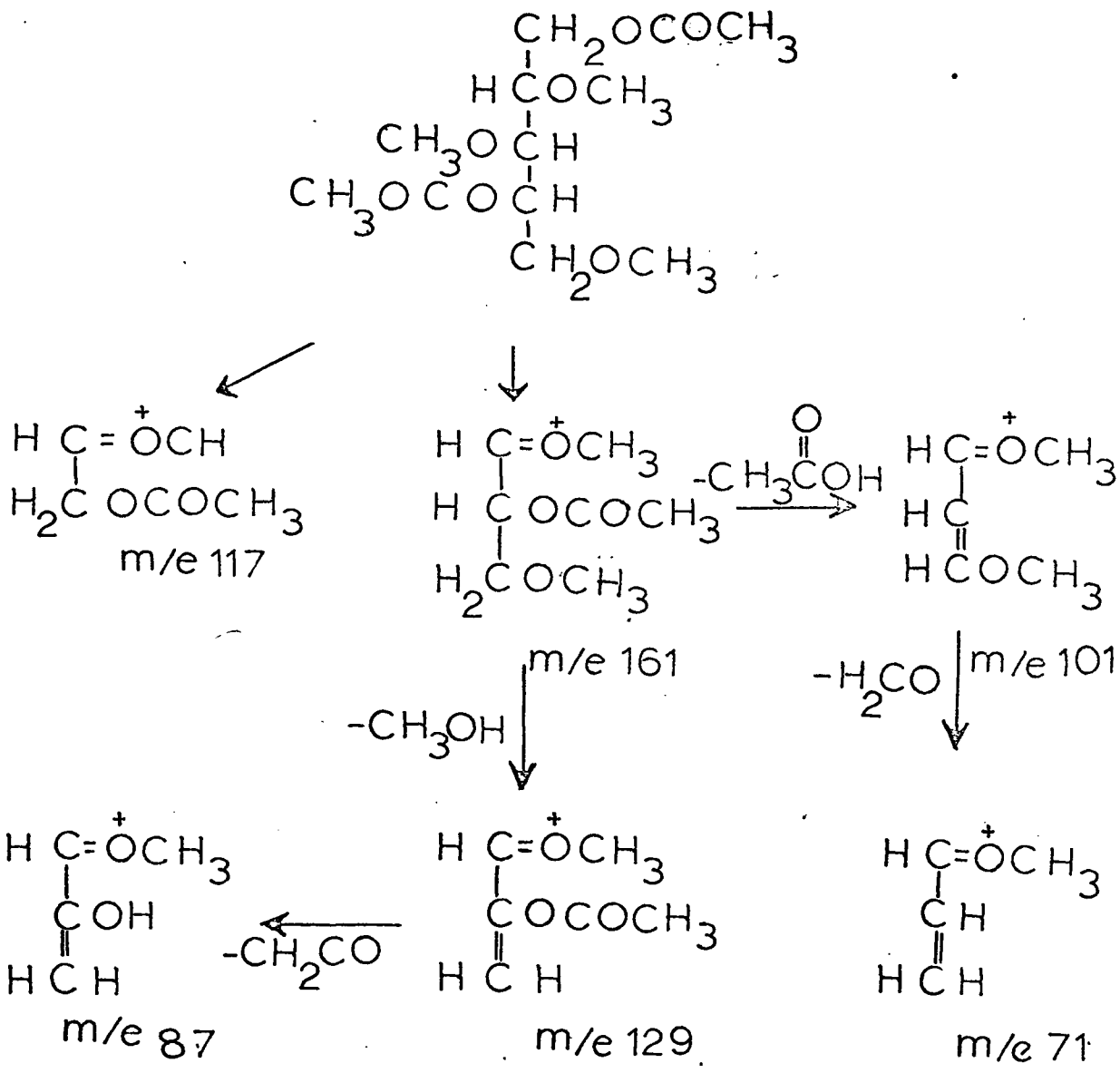


Figure 8. Source of the most important ions of 1,4-di-O-acetyl-2,3,5-tri-O-methyl-L-arabinitol

2. Mass Spectrometry of Methylated Methyl Glycosides:

Fragmentation patterns of methyl glycosides of 2,3,4-tri-O-methyl-D-glucose and 2,3,5-tri-O-methyl-L-arabinose have been worked out according to the scheme put forward by Kochetkov and Chizhov (13). These authors designate a series of ions as A, B, C, D, F, J, H, K etc. According to that scheme, 'A' series of ions are produced by the loss of substituents from C-1, with subsequent elimination of other substituents. 'B' series of ions are produced by elimination of C-5 and oxygen as formaldehyde. Ions of the C, D, F, and J. series are initiated by cleavage of the C-1 to C-2 bond and subsequent fragmentation and distribution of charges on the fragments. A conjugated electronic shift gives rise to ions of the H and K series, depending on the charge localisation. The origin of most of these ions are illustrated in Fig. 16 and 18.

3. Mass Spectrometry of Trimethylsilyl Derivatives of Methylated Aldopentoses:

Fragmentation patterns of most of the peaks can be explained by analogy with the fragmentation pattern of a methylated methyl glycoside. Several peaks corresponding to fragment ions typical for trimethylsilyl derivatives are in the low mass range. The peaks at m/e 45, 59, 73, 75, and 89 have shown to be characteristic of the trimethylsilyl function (38). The peak at m/e 73, corresponding to the trimethylsilyl ion, was intense in all spectra. Fragmentation patterns of trimethylsilyl derivatives of 2,3-di-O-methyl-D-xylose, 2-O-methyl-D-xylose and 3-O-methyl-D-xylose are shown in Fig. 9-14, respectively. Ions of the

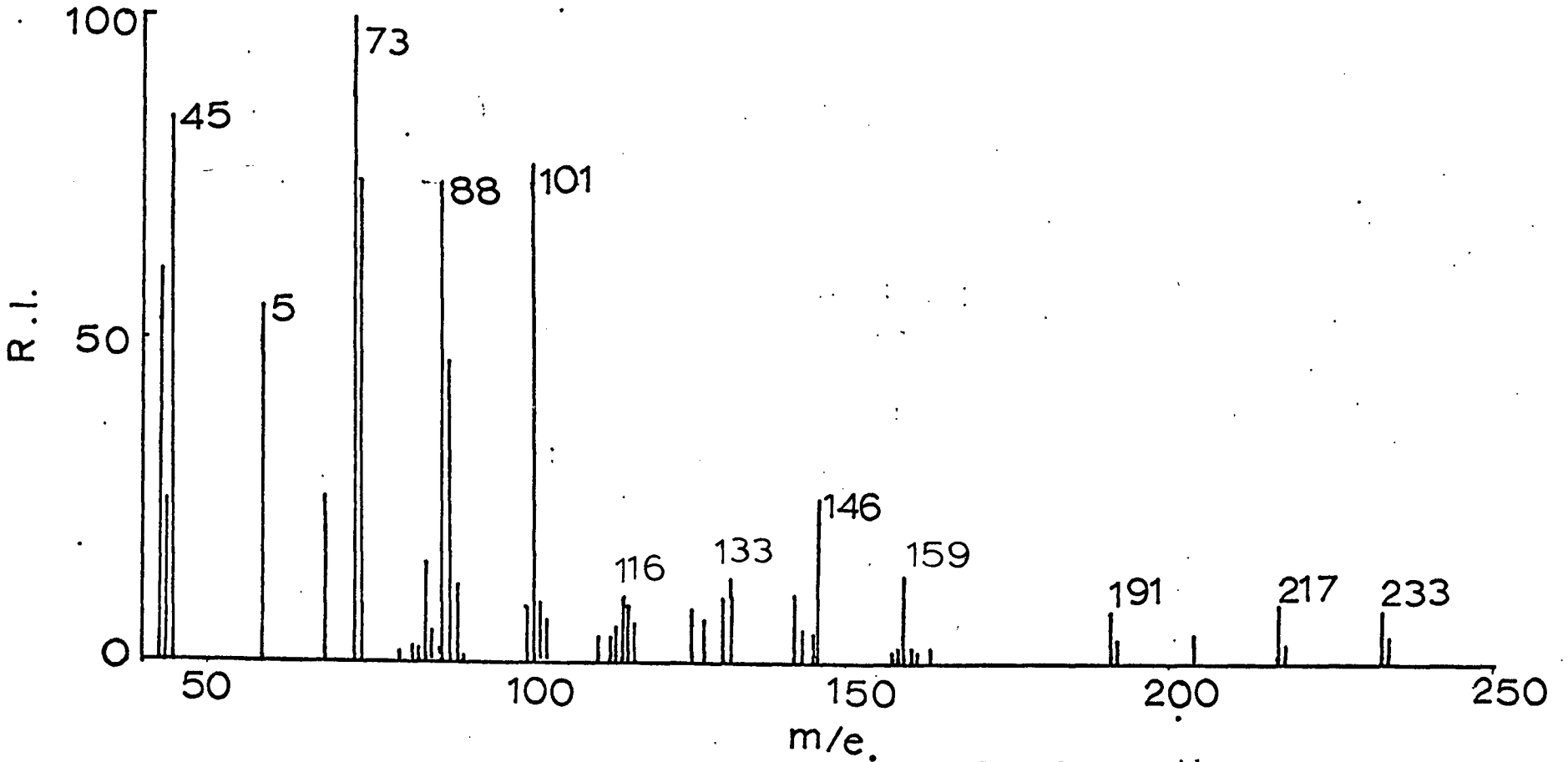


Figure 9. Mass spectrum of TMS 2,3-di-O-methyl-4-O-TMS-D-xylopyranoside

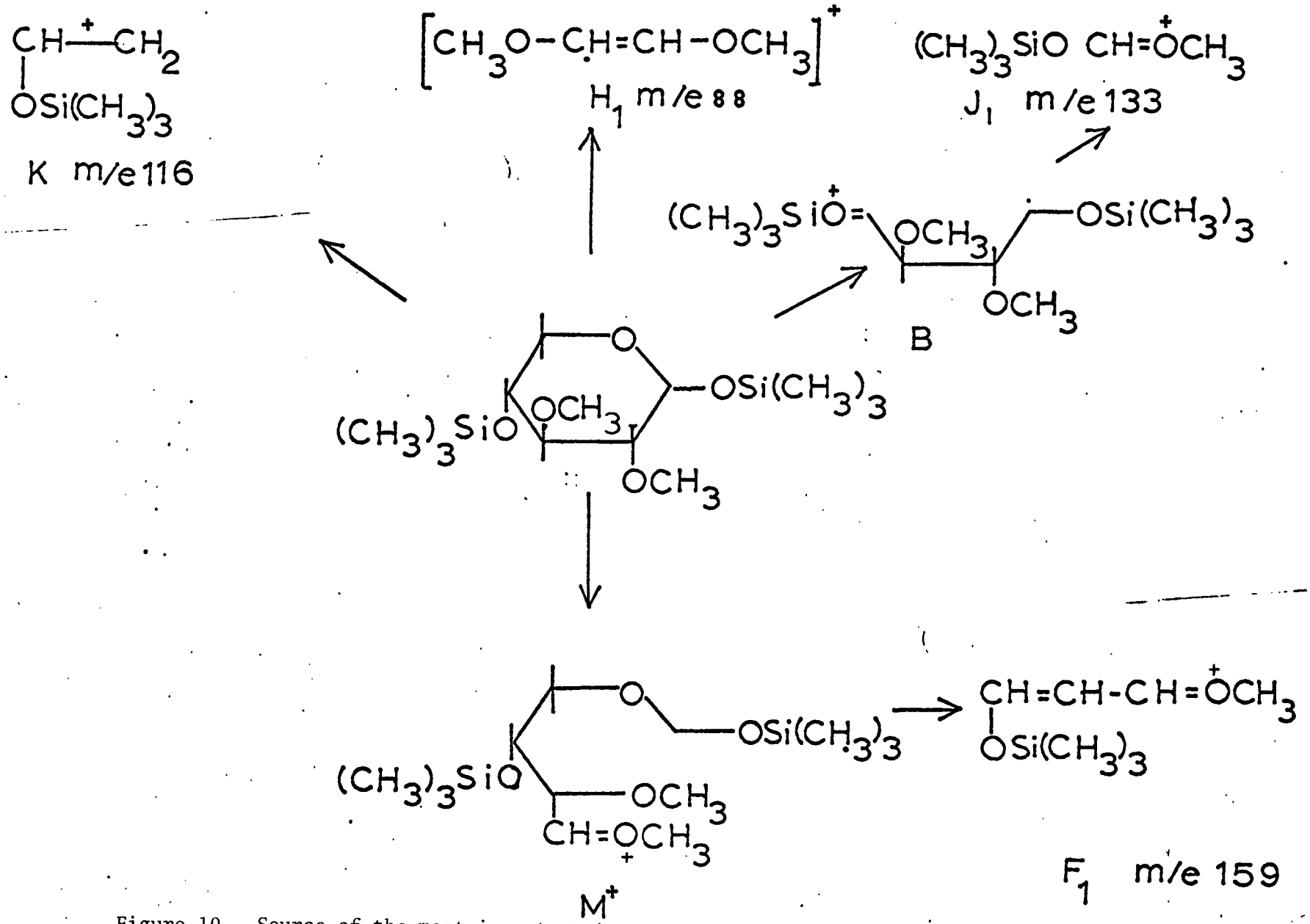


Figure 10. Source of the most important ions of TMS 2,3-di-O-methyl-4-O-TMS-D-xylopyranoside

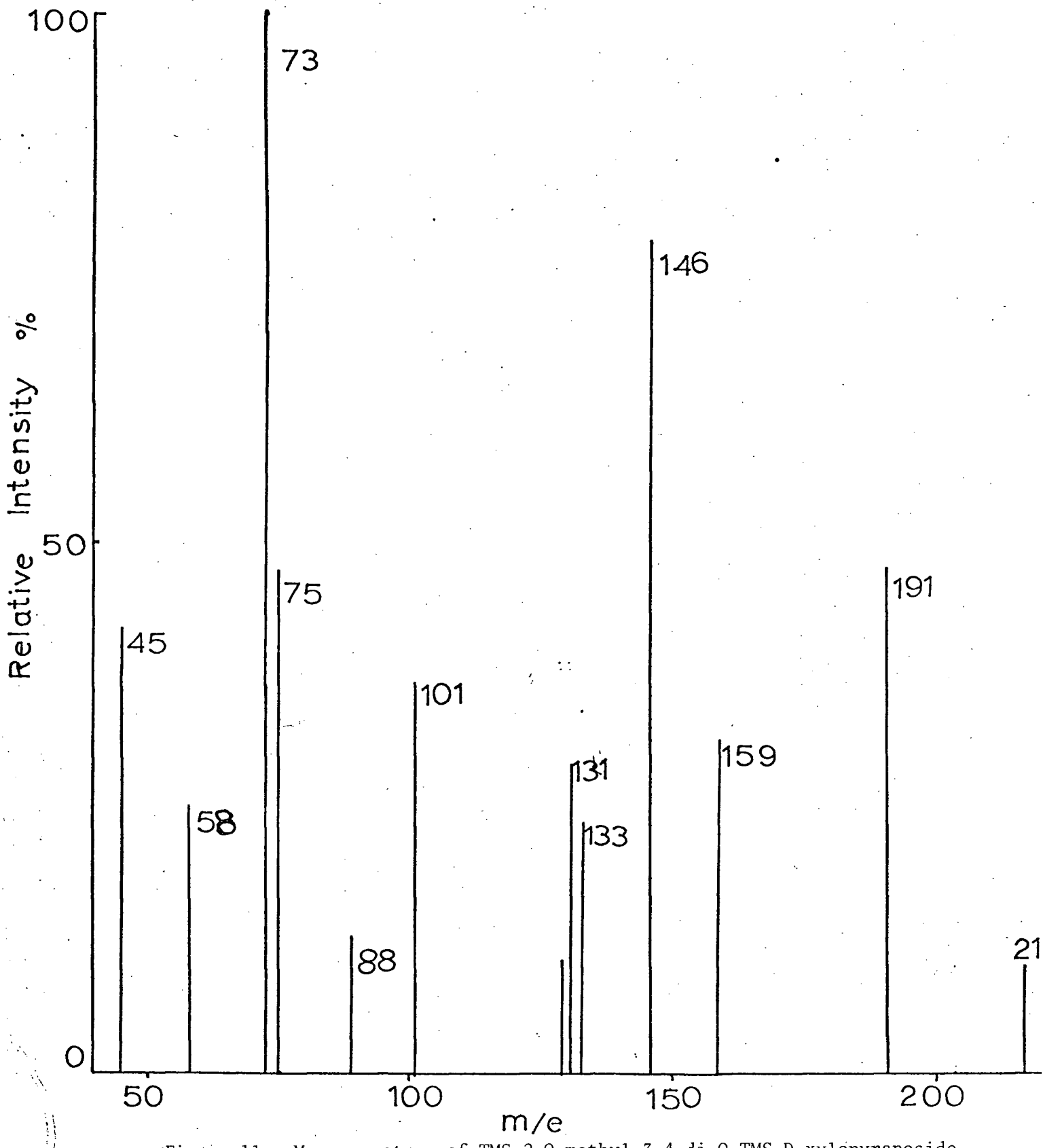


Figure 11. Mass spectrum of TMS 2-O-methyl-3,4-di-O-TMS-D-xylopyranoside

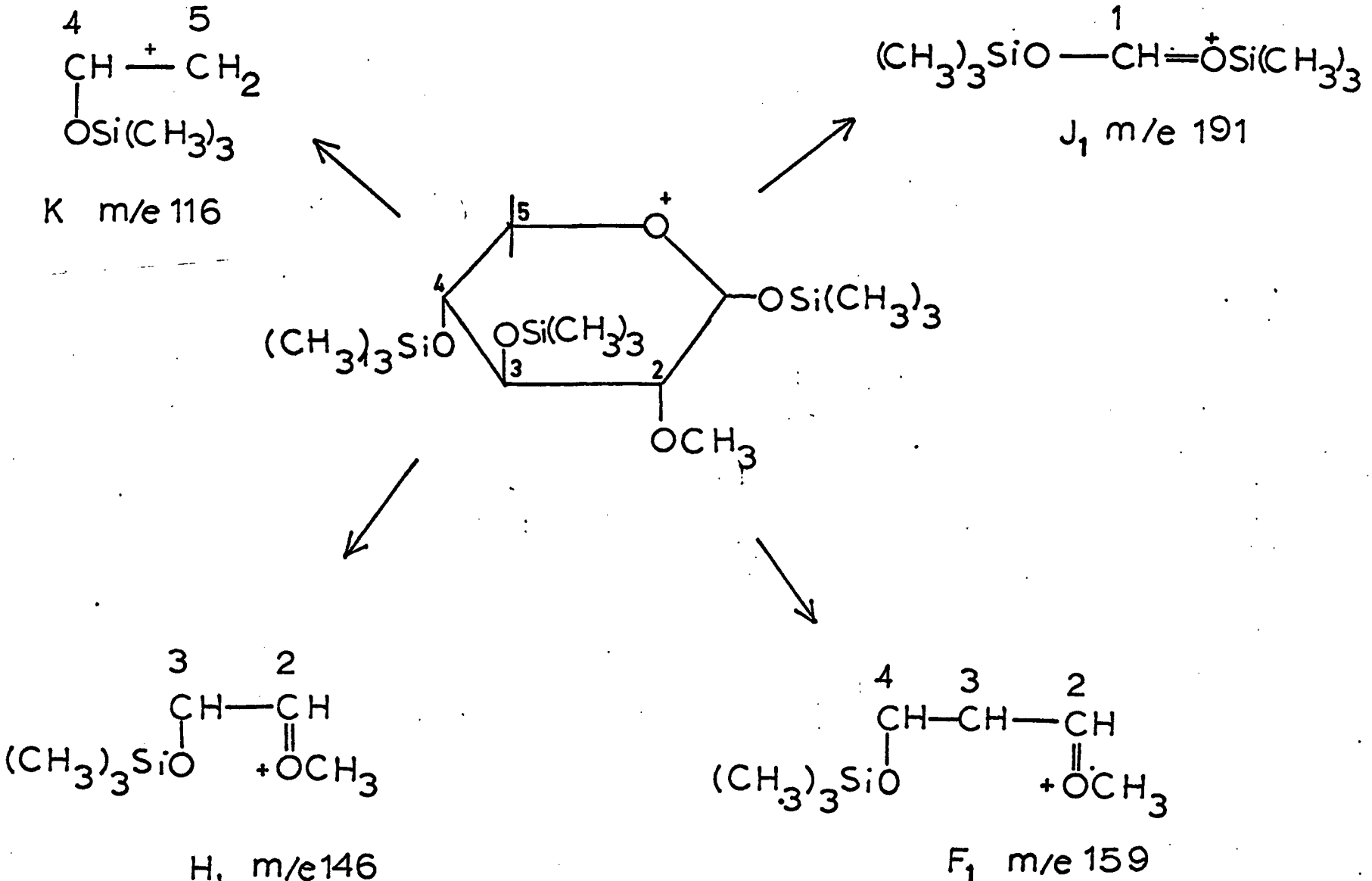


Figure 12. Origin of the most important ions of TMS 2-O-methyl-3,4-di-O-TMS-D-xylopyranoside

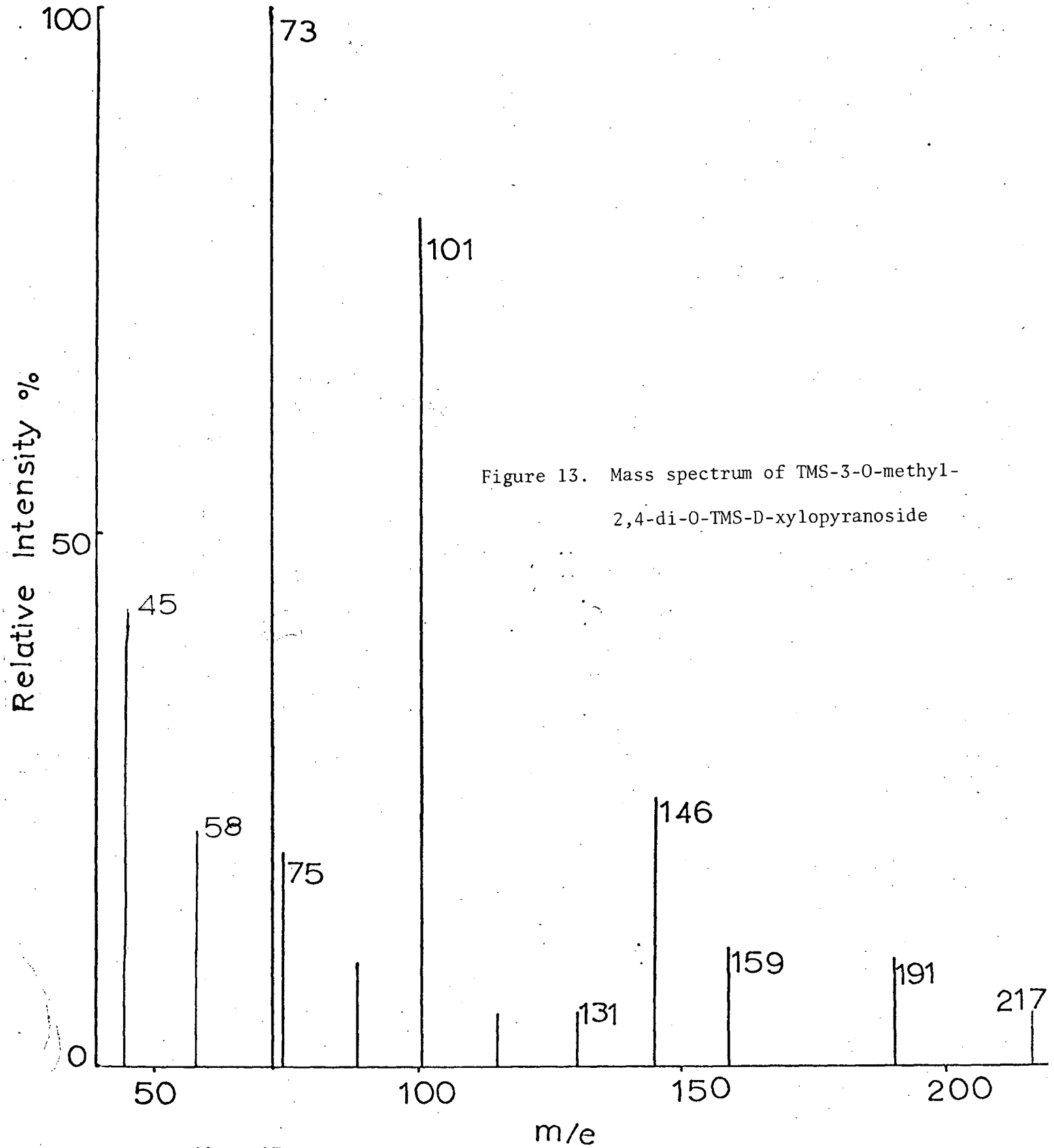


Figure 13. Mass spectrum of TMS-3-O-methyl-2,4-di-O-TMS-D-xylopyranoside

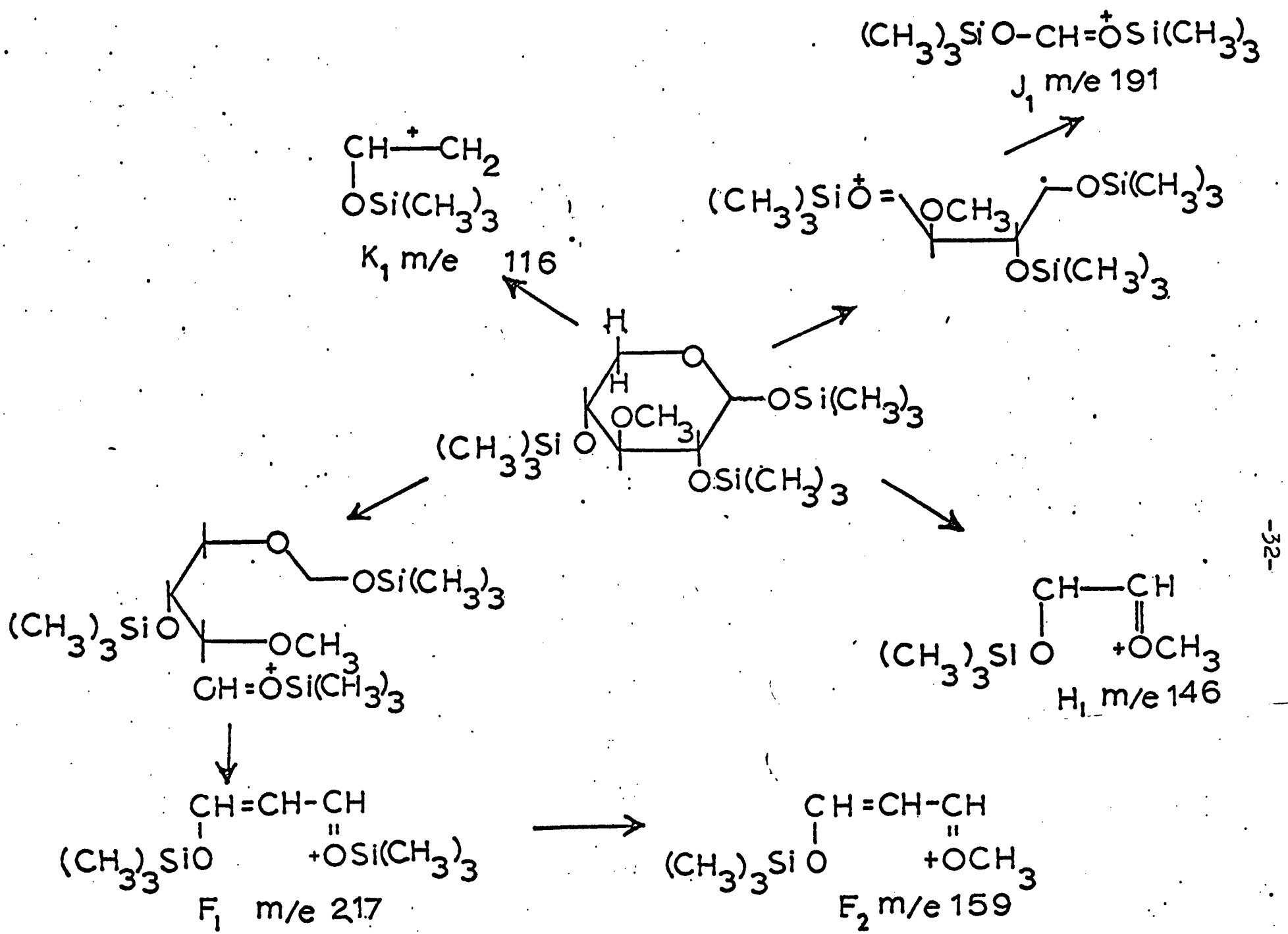


Figure 14. Source of the most important ions of TMS 3-O-methyl-2,4-di-O-TMS-D-xylopyranoside

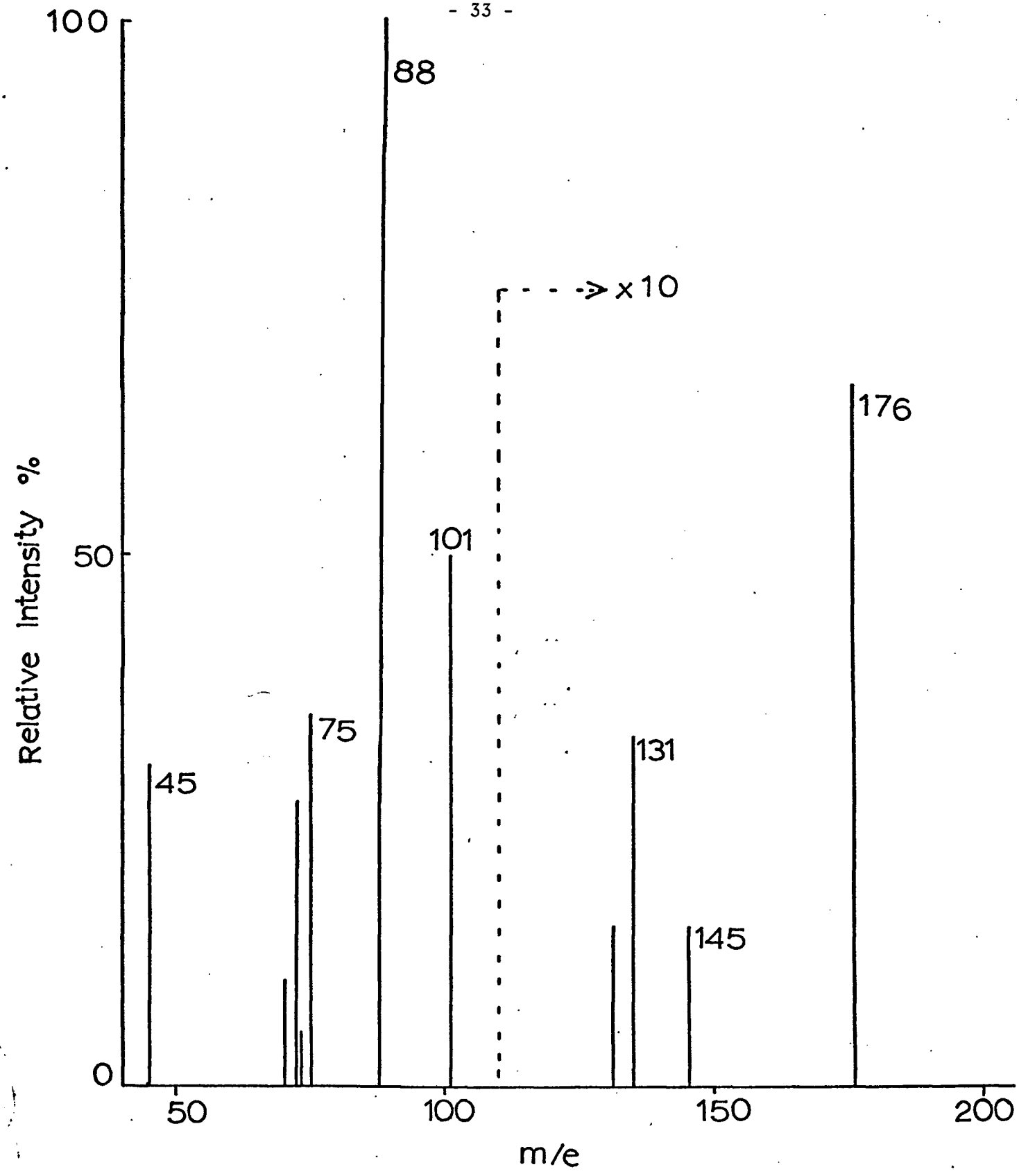
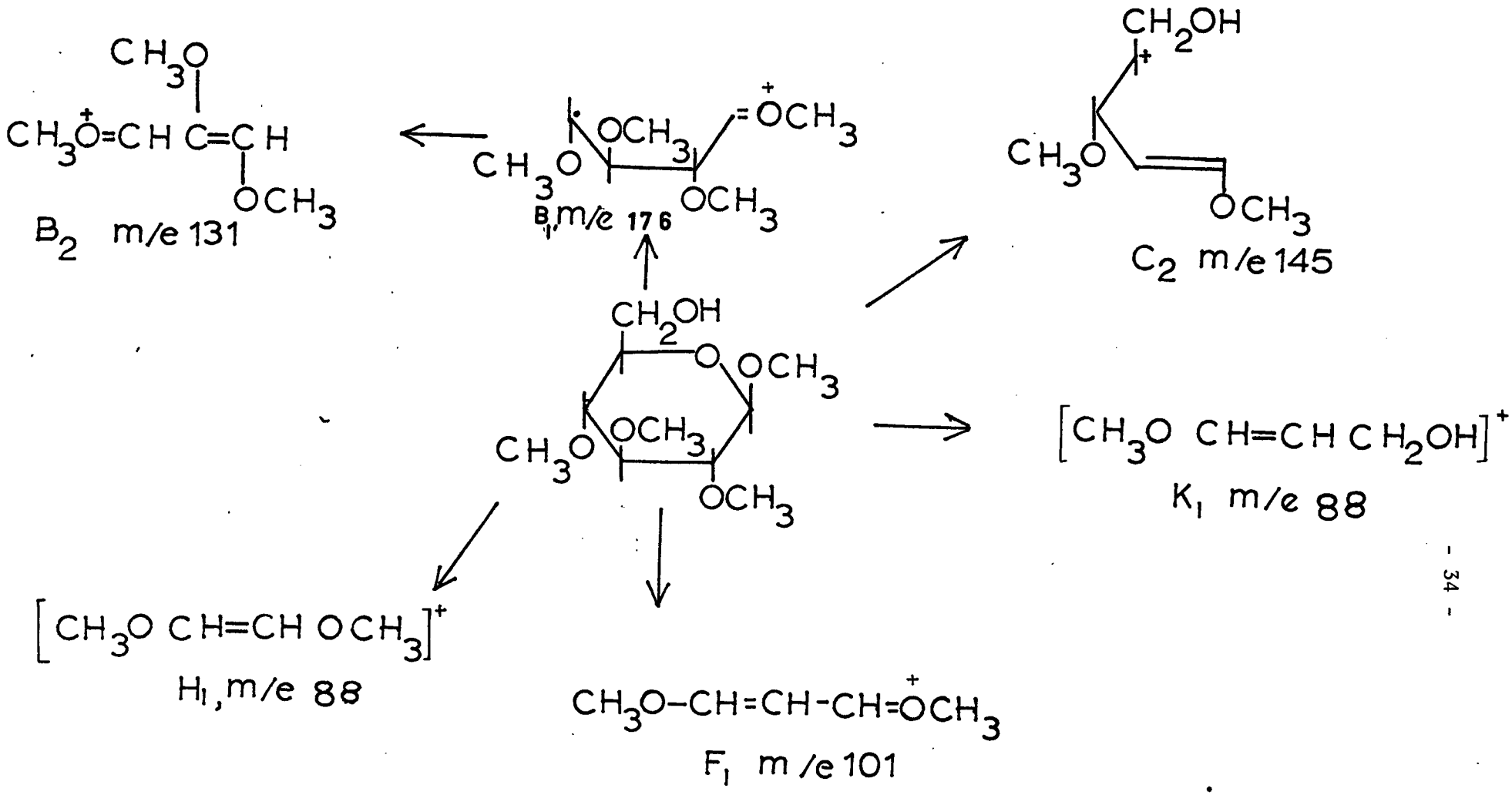


Figure 15. Mass spectrum of methyl 2,3,4-β-D-glucoside



Frg.16. Source of the ions of methyl 2 3 4 -tri-O-β-D-glucoside.

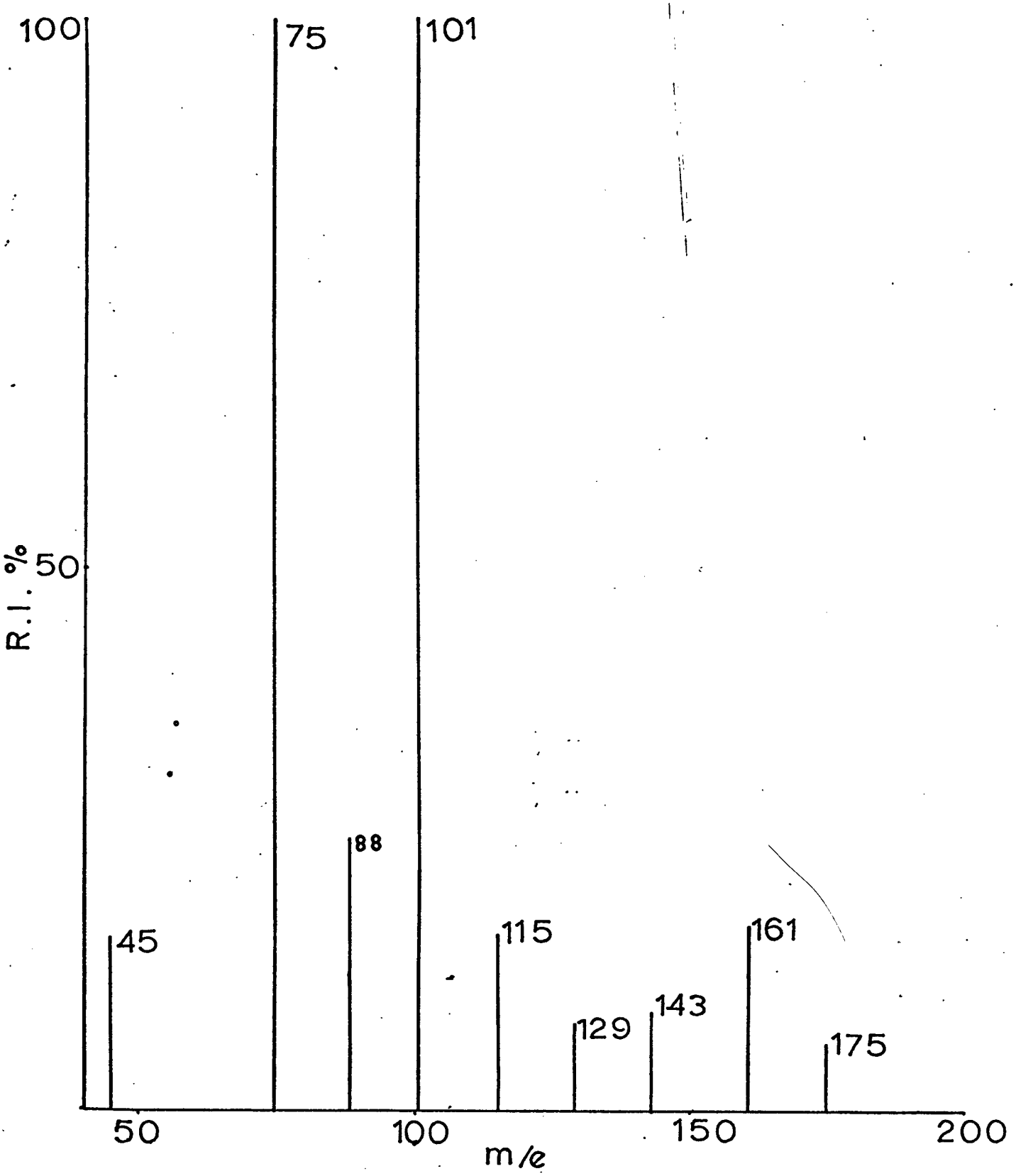


Figure 17. Mass spectrum of methyl 2,3,5-tri-O-methyl-L-arabino-furanoside.

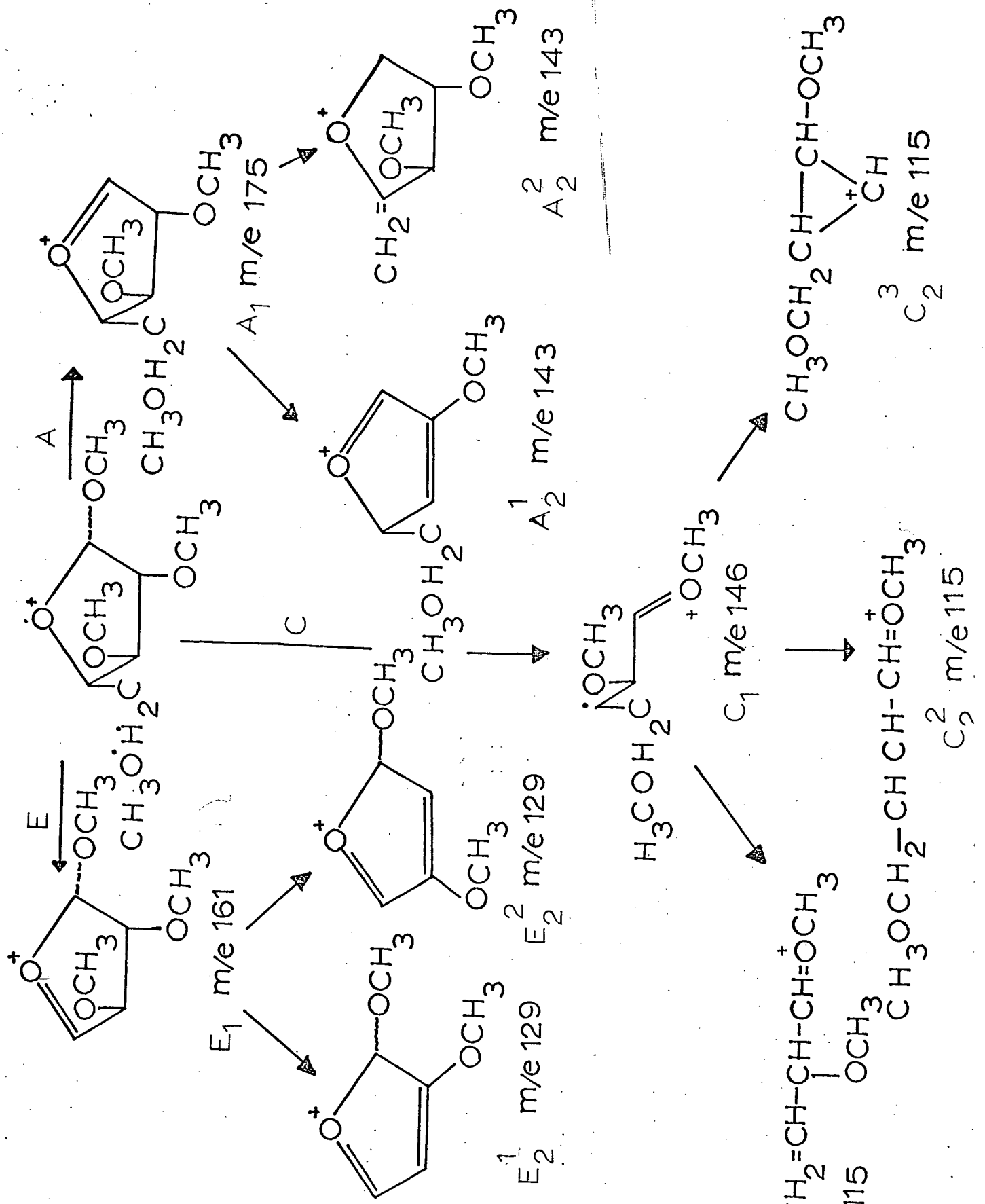


Figure 18. Source of the ions of methyl 2,3,5-tri-O-methyl-L-arabinofuranoside

series A, C, and F tend to eliminate the substituents from C-3 (13). Therefore, a characteristic difference between 2-O-methyl and 3-O-methyl-D-xyloses (trimethylsilyl derivatives) can be noticed. Thus the peak at m/e 159 is more intense for 2-O-methyl compound (Fig. 11) than that for 3-O-methyl compound (Fig. 13).

Conclusion:

From the above experimental evidence, certain structural features of corn leaf polysaccharide can be inferred. It is evident the 2,3,4-tri-O-methyl-D-xylose is derived from terminal D-xylopyranose residues in the polysaccharide. The large amount of 2,3-di-O-methyl-D-xylose together with the high negative rotation of both unmethylated and methylated polysaccharides would suggest that the main body of the hemicellulose consisted of D-xylose units of the pyranose type β-linked through positions 1 and 4. The 2-O-methyl-D-xylose is derived from units of xylose which form branch points in the molecule; these units are joined through position 3 in addition to position 1 and 4. In a similar manner, the 3-O-methyl-D-xylose represents a branch point with linkages at positions 1, 2 and 4.

It is apparent that 1 mole of 2,3,4-tri-O-methyl-D-xylose is obtained per 40 moles of 2,3-di-O-methyl-D-xylose. This ratio is in close agreement with the ratio of ethylene glycol and glycerol (1:34) obtained from the periodate oxidation of corn leaf polysaccharide (Table 1). Considering the mole ratios, it is found that 1 mole of the methylated aldobiouronic acid is obtained per 9 moles of methylated

TABLE 1

QUANTITATIVE ANALYSIS OF METHYLATED SUGARS ISOLATED FROM THE HYDROLYSATE OF THE METHYLATED CORN LEAF HEMICELLULOSE

Sugar Derivative	Wt., mg.	Mole ratio[a]	Mole ratio[b]
2,3,5-Tri-O-methyl-L-arabinose	59.9	4.5	4
2,3,4-Tri-O-methyl-D-xylose	13.2	1	1
2,3-Di-O-methyl-D-xylose	491.9	40	38
3-O-Methyl-D-xylose	34.7	3	3.6
2-O-Methyl-D-xylose	48.3	4.6	4.4
2-O-(2,3,4-Tri-O-methyl-D-glucuronosyl)-3-O-methyl-D-xylose	109.0	6	-

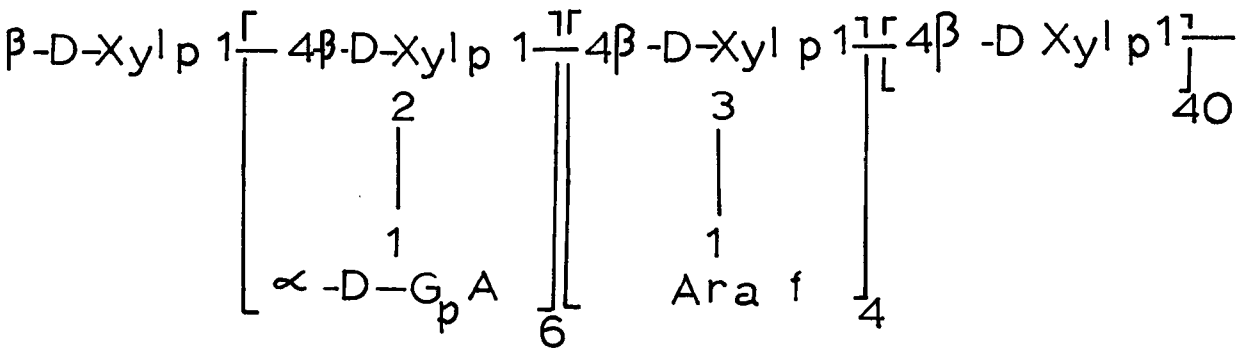
[a] Mole ratio from weight yields.

[b] Mole ratio from gas-liquid chromatography.

pentoses. This agrees reasonably well with the values found earlier for the number of anhydroxylose units per acid group. The high positive rotation of the methylated aldobiouronic acid indicates the presence of an α-D-linkage. The characterisation of the partially methylated disaccharide indicates that uronic acid residues are linked as single unit side chains to position 2 of D-xylose units of the main chain. It is also apparent that 2,3,5-tri-O-methyl-L-arabinose residues are linked to the D-xylose backbone at position 3 since the moles of 2,3,5-tri-O-methyl-L-arabinose obtained are almost equal to the number of moles of 2-O-methyl-D-xylose (Table 1).

Although D-xylose obtained from the hydrolyzate of the methylated polysaccharide is in trace amounts, it may have arisen out of demethylation, or incomplete methylation or due to multiple branching in a molecule.

From above facts, a simplified structure may be proposed as follows:



EXPERIMENTAL

Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers with the following solvent systems (v/v):

A. Methyl ethyl ketone - water azeotrope

B. Ethyl acetate-acetic acid-formic acid-water, 18:3:1:4

C. 1-Butanol-pyridine-water, 6:4:3

The descending technique was used for paper chromatography. Sugars were detected with p-anisidine trichloroacetate. Fraction D (Table 2) and Fraction D (Table 8) were used for structural analyses throughout this investigation.

Unless otherwise stated, all evaporations were carried out under pressure at a bath temperature of 40°C.

The melting points reported are uncorrected and the specific rotations quoted are equilibrium values.

Gas-liquid chromatography was carried out on an F and M 720 dual column instrument fitted with a thermal conductivity detector. Helium was used as the carrier gas. Peak areas were obtained with the help of an automatic digital integrator.

The mass spectra were recorded on an A.E.I. M.S. 7 mass spectrometer at an ionizing potential of 70 e.v.

A. Extraction of Hemicellulose

Samples of powdered corn leaves were extracted exhaustively with hot ethanol-benzene (1:2, v/v). Dried leaves (396 gm.) were extracted with water at room temperature for 96 hours. The plant material was

next extracted with 2% ethylenediaminetetra-acetic acid (EDTA), adjusted to pH 6.8, at 70°C for 2 hours. The process was repeated thrice, each time the material being extracted with 1.5 litres of 2% EDTA solution. The filtrate was concentrated to a volume of 2.3 litres and an equal volume of ethanol was added to it. The precipitated material was centrifuged, rinsed with acetone and ether, several times. Amount of EDTA extract was found to be 1.09 gm.

In order to delignify, plant material was digested with an aqueous solution (3 litres) of sodium chlorite (30 gm.) and acetic acid (10 ml.) at 75°C for a period of 3 hours. Fresh reagents were added after every 1 hour.

The delignified material was extracted successively with 0.2 N (3.2 litres), 1.0 N (2.0 litres) and 2.5 N (1.0 litre) of potassium hydroxide solutions. In each case acidification of the alkaline extracts to pH 4-5 afforded precipitates and further precipitates were obtained by addition of ethanol (1 vol.). Precipitated polysaccharides of different fractions were dried by rinsing several times with acetone and petroleum ether (30-60°). Table 2 indicates the yields of protein, methoxyl, uronic acid, ash content and specific rotations of different fractions.

B. Hydrolysis of Corn Leaf Hemicellulose:

Samples of different fractions (ca. 0.50 g.) were hydrolysed with sulphuric acid (1 N, 20 ml.) in sealed tubes for 8 hrs. at 100°C. Residues were removed by filtration and the filtrates were neutralized with barium carbonate. The filtrates were passed through Amberlite IR-120 (H^+) and Duolite A-4 resin. The neutral eluates were evaporated to

TABLE 2

FRACTIONS OF POLYSACCHARIDES FROM CORN LEAF*

Fraction	Method of Extraction	Yield,gm.	Uronic acid	Specific rotation	Methoxyl	Protein
			%	$[\alpha]_D^{22}$	%	%
A	0.2N KOH (acid) Ext.	0.29	11.72	-51.21°	0.65	25.62
B	0.2N KOH (ethanol) Ext.	0.54	16.56	-47.93°	0.85	-
C	1.0N KOH (acid) Ext.	7.81	10.48	-49.91°	0.47	-
D	1.0N KOH (ethanol) Ext.	10.89	11.42	-64.06°	0.89	-
E	2.5N KOH (acid) Ext.	0.94	6.72	-45.71°	0.42	-
F	2.5N KOH (ethanol) Ext.	9.54	8.80	-57.69°	0.54	-

* All figures are based on ash free solid.

syrup. Acidic sugars were eluted from the anion exchange resin with 10% formic acid and evaporated to syrup. Paper chromatographic examination of neutral sugars in solvent systems A, B, and C showed spots corresponding to arabinose, xylose and traces of glucose.

C. Identification of Neutral Sugars:

Routinely 10 mg. samples of neutral sugars were silylated. The procedure described is for this amount but the size of the reaction could be altered readily.

Neutral sugars (10 mg.) were dissolved in anhydrous pyridine kept over potassium hydroxide pellets (1 ml.). Hexamethyldisilazane (0.2 ml.) and trimethylchlorosilane (0.1 ml.) were added to the sugar solution. The mixture was shaken vigorously at room temperature for 30 seconds and then allowed to stand for 5 minutes. The mixture was evaporated to dryness until there was a faint smell of pyridine. Cyclohexane (4 ml.) was added to the evaporated sample and it was agitated to dissolve trimethyl silyl ethers. Small amounts of sample were injected into gas-liquid chromatograph.

Separations of sugars were carried out on a copper column (8' x 1/4"), packed with 20% SF 96 on 60-80 mesh Diatoport S. Experiment was carried out isothermally at 190°C for 3 minutes and then programmed at 3° per min. to hold at 230°C. The injection port was 270°C, the detector block 295° and the helium flow 88 ml per min.

D. Identification of Acidic Sugars:

The acidic fraction isolated from the hydrolysate of the hemicellulose was examined chromatographically on paper in solvent system B for

TABLE 3

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF CORN LEAF

NEUTRAL SUGARS

Fraction	Method of Extraction	Arabinose	Xylose %	Glucose
B	0.2N KOH (ethanol) Ext.	25.0	72.5	2.5
C	1.0N KOH (acid) Ext.	15.3	84.7	-
D	1.0N KOH (ethanol) Ext.	17.6	75.7	6.7
E	2.5N KOH (acid) Ext.	19.6	80.4	-
F	2.5N KOH (ethanol) Ext.	13.8	79.5	6.7

16 hours. Spots corresponding to a mixture of aldobiouronic acids (R_x 1.00, 0.56), traces of free monouronic acids (R_x 1.20, 0.65) and higher oligouronic acid (R_x 0.19) were observed. The spot corresponding to the component with R_x 0.56 was the most prominent among all.

A portion of the syrupy acidic constituent (20 mg.) was dissolved in 4% methanolic hydrogen chloride (4 ml.) and the solution was refluxed over a steam bath for 6 hours. Neutralization with silver carbonate and subsequent removal of silver ions by H_2S gave rise to syrup (24 mg.) which did not reduce Fehlings solution.

The ester was dissolved in tetrahydrofuran (2 ml., dried by distillation from lithium aluminium hydride). A solution of lithium aluminium hydride (200 mg.) was prepared by adding the finely crushed hydride to dry ether (10 ml.) and refluxing for 1 hour, by which time the majority of the solid had dissolved. The solution of the ester was added gradually with swirling to the hydride solution at room temperature. The solution was refluxed for 4 hours. Excess hydride was destroyed by addition of ethereal ethyl acetate followed by dilute aqueous acetic acid until the solution was acidic. The reaction mixture was evaporated to dryness and the reduced material was heated with 1 N sulphuric acid (6 ml.) for 12 hours. The solution was neutralized with barium carbonate and subsequently deionised by passage through Amberlite IR-120 (H^+) resin. Evaporation of the effluent yielded a syrup (12 mg.) which upon chromatographic examination on paper in solvent system B showed the presence of three plots corresponding to D-xylose, D-glucose and 4-O-methyl-D-glucose, the last one being present in traces.

E. Periodate Oxidation of Corn Leaf Polysaccharide:

Corn leaf polysaccharide (2.74 g.) was dissolved in 1 N sodium hydroxide (50 ml.). The solution was made slightly acidic by neutralising excess alkali with dilute acetic acid. The solution was kept at 4°C for 1 hour. Periodic acid (0.5 M,50 ml.) was added to the polysaccharide solution and final volume was adjusted to 250 ml. The oxidation was allowed to proceed in the dark at 4°C until the periodate consumption became constant.

1. Determination of Periodate Consumption:

To an aliquot of periodate oxidized material (2 ml.), sodium bicarbonate (1 g.) was added. The solution was shaken so that sodium bicarbonate dissolved quickly. Sodium arsenite (0.1 N, 5 ml.) and potassium iodide (20%, 1 ml.), prepared in saturated sodium bicarbonate solution, were added to the above mixture. After standing 15 min., excess sodium arsenite was titrated with 0.1 N iodine solution using starch as indicator. The titration was carried out until the periodate uptake was constant.

2. Reduction with Sodium Borohydride:

To the periodate oxidized solution, a slurry of barium carbonate was added to precipitate periodate and iodate. To the clear filtrate was added sodium borohydride (1 g.) and the solution left overnight. The solution was neutralized, deionised by passage through IR-120 (H^+) resin and evaporated to dryness. Borate was removed from the polyol by several treatments with methanol containing 1% hydrogen chloride.

TABLE 4

PERIODATE UPTAKE OF CORN LEAF POLYSACCHARIDE

Time (hour)	Moles of periodate/Moles of sugar
1	0.04
4	0.14
8	0.27
12	0.46
18	0.56
42	0.67
66	0.73
96	0.77

3. Acid Hydrolysis of Polyol:

A portion of the periodate oxidized corn leaf polysaccharide (110 mg.) was hydrolysed with sulphuric acid (1 N, 5 ml.) at 100°C, for 8 hours. After neutralization with barium carbonate, the hydrolysate was resolved into a neutral and an acidic fraction by passage through cation and anion exchange resin columns.

4. Characterization of the Neutral Fraction Obtained on Periodate Oxidation by Gas-Liquid Chromatography:

A portion of the neutral hydrolysate (10 mg.) was dissolved in dry pyridine (2 ml.) and converted to trimethylsilyl derivatives by

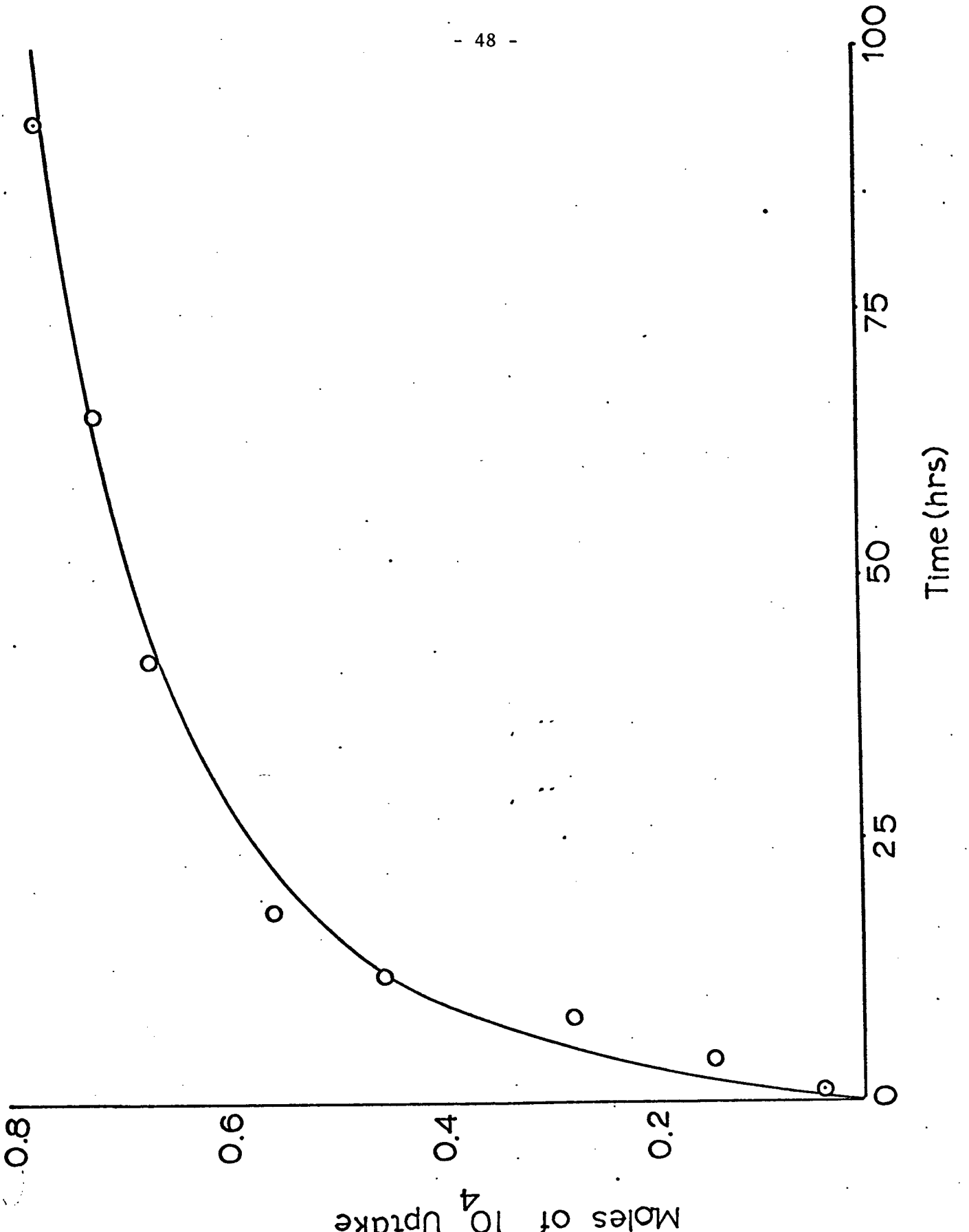


Figure 19. Periodate oxidation of corn leaf polsaccyaride.

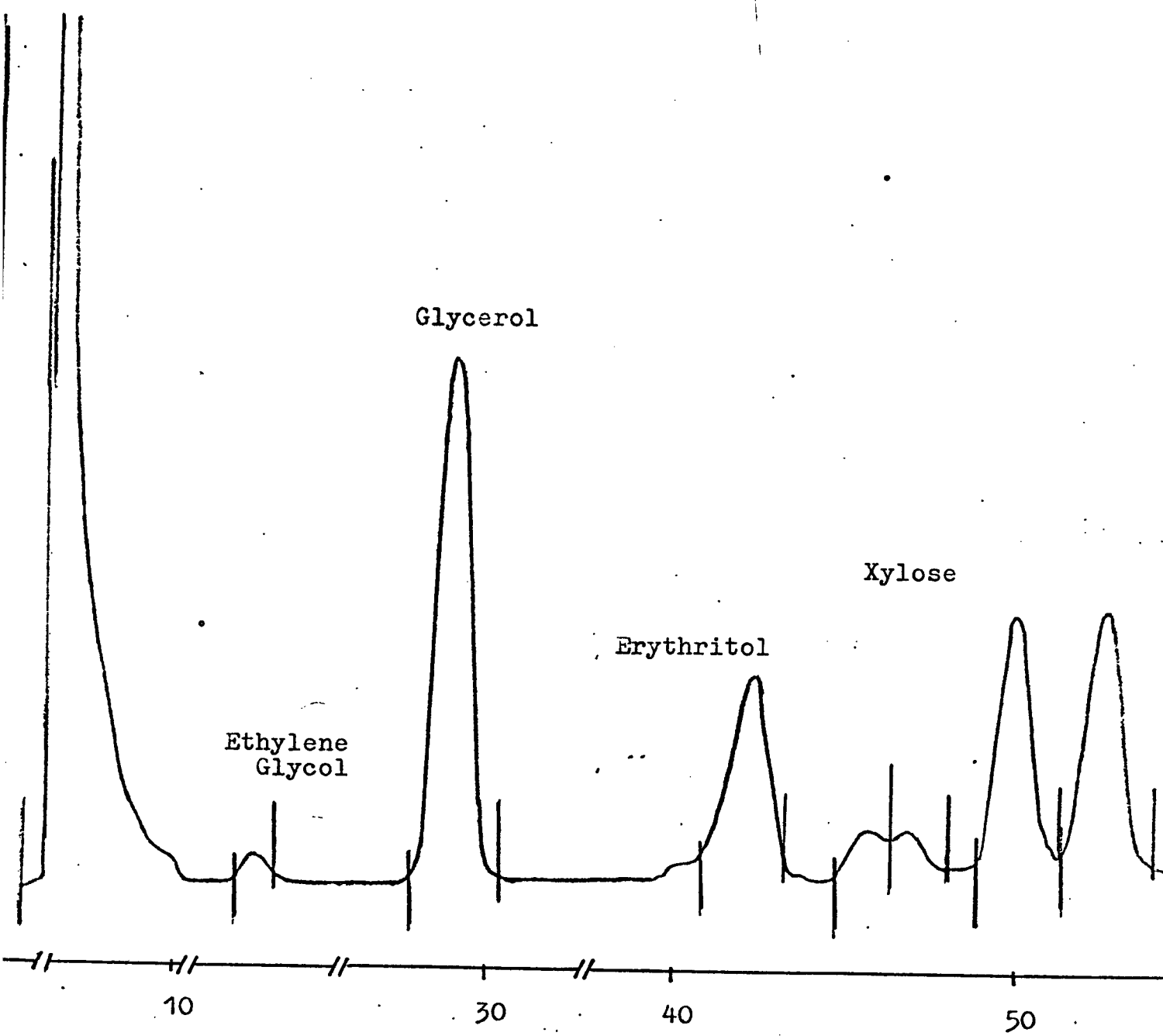


Figure 20. Separation of products as trimethylsilyl derivatives from corn leaf polysaccharides.

adding hexamethyldisilazane (1 ml.) and trimethylchlorosilane (0.5 ml.). The solution was used for gas-liquid chromatography.

Separations of trimethylsilyl derivatives of polyhydric alcohols and sugars were carried out on a pair of stainless steel columns (8 ft. x 0.25 in.) packed with equal weights of 20% SF 96 on 60-80 mesh Diatoport S. The columns were held isothermally at 90°C for 3 minutes and then programmed at 3°C per min. to hold at 220°C.

Average molar ratio of ethylene glycol, glycerol, erythritol and xylose was 1:34:8:18.

F. Methylation of Corn Leaf Polysaccharides:

1. Preparation of Methylsulphinyl Anion:

Into a dry, 250-ml. three-necked round-bottom flask fitted with serum caps and containing a magnetic stirring bar was weighed 2.5 g.of sodium hydride,50% oil dispersion. The sodium hydride was washed three times by stirring with dry petroleum ether (30°-60°C) and decanting the wash. After the third wash, the residual dry petroleum ether was evacuated with a vacuum pump through an 18-gauge needle inserted into the serum cap. Dimethyl sulphoxide (20 ml.), distilled from calcium hydride under reduced pressure and stored over dried molecular sieves, was transferred into the flask. The mixture was stirred at 50°C until the solution became clear, green and evolution of hydrogen gas ceased (ca. 2 hours).

2. Generation of the Polysaccharide Alkoxide:

Corn leaf polysaccharide was first passed through a 200-mesh sieve and dried for a period of 6 hours at 60°C under reduced pressure.

Dried material (1.32 g.) was added to 65 ml. of dry dimethyl sulphoxide in a 250-ml. three-necked round-bottom flask containing a magnetic stirring bar and fitted with serum caps through which reagents were introduced and nitrogen gas was passed continuously. The suspension was heated at 60°C and stirred with a magnetic bar until all of the polysaccharide dissolved to form a pale brown coloured solution. After cooling the solution to room temperature, methylsulphinyl anion (15 ml.) was added to the polysaccharide solution. The resulting solution became thick and was stirred for 6 hours when the mixture appeared homogeneous.

3. Methylation Reaction:

The polysaccharide alkoxide solution was cooled to 20°C in an ice-water bath and freshly distilled methyl iodide (5 ml.) was added to the stirred solution at a very slow rate such that the temperature did not rise above 20°C (30 min.). Within a few minutes after addition of methyl iodide, the solution became clear and the viscosity was markedly reduced. The mixture was stirred at room temperature for an additional period of 6 hours. Water (60 ml.) was added to the brown coloured solution. Immediate precipitation took place. The reaction mixture was dialyzed overnight vs. running tap water and extracted continuously with chloroform. The chloroform extract was dried over anhydrous sodium sulphate and was finally evaporated to dryness at 40°C under reduced pressure. The methylation reaction was complete as no OH band was found in the infrared spectrum. The yield of the crude methylated product was 1.48g.

4. Fractionation of the Methylated Corn Leaf Polysaccharide:

Methylated corn leaf polysaccharide was treated with a mixture of light petroleum ether (30-60°C)-chloroform, the amount of the latter solvent being increased in stages. The mixture was refluxed on a steam bath for 2 hours for each extraction; the insoluble material being allowed to settle and the clear liquid was decanted. The solvent was removed under reduced pressure and the residue was dried at 60°C in vacuo over phosphorus pentoxide to constant weight.

The results are shown in Table 5.

TABLE 5

FRACTIONATION OF METHYLATED CORN LEAF POLYSACCHARIDE

Fraction	Chloroform: Pet. Ether	Weight gm.	$[\alpha]_D^{22}$ ($CHCl_3$)	Methoxyl %
1	0:100	0.0052	-	-
2	10:90	0.0285	-	-
3	15:85	0.0503	-52.6	15.8
4	20:80	0.1249	-51.2	31.4
5	25:75	1.0334	-47.7	37.8
6	30:70	0.0336	-41.8	37.3

G. Hydrolysis of the Methylated Corn Leaf Polysaccharide:

Fraction 5 (1.01 g.) was dissolved in sulphuric acid (72%, 10 ml.) in a round-bottomed flask externally cooled with ice-water. The solution was kept at room temperature for 1 hour to solubilize the polysaccharide. Water was then added to bring the acid concentration to 8% and the hydrolysis mixture was heated at 100°C for 4 hours, then cooled to room temperature, and neutralised with barium carbonate. The precipitated barium sulphate was separated by filtration, solids being washed several times with water and ethanol. The filtrate and the extracts were combined and evaporated to a syrup at 35°C in vacuo.

H. Separation of Neutral and Acidic O-Methyl Sugars:

The mixture of methylated sugars (1.00 g.) was dissolved in water, passed through a column of Amberlite IR-120 (H^+) resin, and the resin washed with water until it gave a negative Molisch test.

The effluent from the cation exchange resin was passed through a column of Duolite A-4 resin which selectively removed the acidic component. The colunn was washed with water until it gave a negative Molisch test. The washings were evaporated to a syrup of constant weight (740.6 mg.). The acidic component was isolated from the column by eluting with formic acid (10%, 5 ml.). Evaporation of the effluent furnished the acidic component.

The mixture of methylated neutral sugars was examined by paper chromatography using solvent system A. The results are tabulated in Table 6.

TABLE 6

CHROMATOGRAPHIC EXAMINATION OF NEUTRAL METHYLATED SUGARS

Component number	Appearance	R_f value
1	mauve	0.80
2	pink	0.78
3	dark brown	0.53
4	reddish brown	0.23
5	faint brown	0.12

I. Separation of the Neutral Components of Methylated Corn Leaf Polysaccharide by Cellulose-Column Chromatography:

A mixture of methylated neutral sugars (740 mg.) was dissolved in a small amount of methyl ethyl ketone-water azeotrope (1.5 ml.) and placed on a column (40 x 3 cm.) packed with a mixture of hydrocellulose and cellulose (1:1). The same solvent was used for elution and the effluent was collected at 15 min. intervals for 115 tubes and then at 30 min. intervals for the rest of the tubes. The distribution of the sugars was determined by placing 5 drops of the solution of each tube on paper and spraying with p-anisidine trichloroacetate. Certain tubes were concentrated and examined chromatographically before the contents of the tubes were collected together. The syrups were redissolved in methanol and evaporated to constant weight. The recovery was 94.1%. The fractions were reexamined by paper chromatography. Fraction A

(Table 7) was found to contain more than one component and was put back on the cellulose-hydrocellulose column for reseparation. The effluent was collected at 10 min. intervals for 130 tubes.

J. Identification of the Components:

(a) Component 1

The syrup (73.1 mg.), $[\alpha]_D^{22}$ -28.24° (c, 1.5 in water) when examined on a paper chromatogram using methyl ethyl ketone-water azeotrope, showed the presence of two overlapping spots, both having an R_f value close to 0.80. Comparison with authentic samples indicated that this was consistent with the behaviour of 2,3,5-tri-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-xylose. The rotations of these compounds are, respectively, -38.5° and +18.5° (39), which shows component 1 to be a mixture of 18% tri-O-methyl-D-xylose (13.2 mg.) and 82% tri-O-methyl-L-arabinose (59.9 mg.).

A portion of the syrup (30.1 mg.) was refluxed with methanolic hydrogen chloride (3%, 1 ml.) for 6 hours. The acid was neutralised with silver carbonate, and the filtrate and methanol washings were evaporated to a syrup which was examined on a gas-liquid chromatograph using a column packed 5% by weight of butane-1,4-diol succinate polyester on 80-100 mesh Diatoport S, and the column (4' x 1/4") was run isothermally at 120°. Three peaks were observed and samples corresponding to each peak were collected in capillary glass tubes. Contents of the tube corresponding to the first peak and were found to have m.p. 48-50°, which agreed to that of methyl 2,3,4-tri-O-methyl-β-D-xylopyranoside. The reported value is 49-50° (40).

Comparison of retention times of authentic methyl glycosides of 2,3,5-tri-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-xylose with methyl glycosides of component 1 indicated that the second peak was a mixture of anomers of 2,3,5-tri-O-methyl-L-arabinosides and 2,3,4-tri-O-methyl-D-xylosides. The third peak corresponded to one of the anomers of 2,3,5-tri-O-methyl-L-arabinoside. A part of the material corresponding to the third peak was subjected to mass spectrometric investigation, the fragmentation of which was identical with that of methyl glycoside of 2,3,5-tri-O-methyl-L-arabinose. A portion of the material corresponding to the third peak was hydrolysed with 1 N sulphuric acid to generate free reducing sugar which on paper was chromatographically identical with 2,3,5-tri-O-methyl-L-arabinose.

(b) Component 2:

This component was shown to be chromatographically pure and to correspond with 2,3-di-O-methyl-D-xylose, $[\alpha]_D^{22}$ +21.4° (c, 1.00 in water).

A portion of the material (47.9 mg.) was dissolved in absolute ethanol (1.5 ml.) and freshly distilled aniline (0.3 ml.) added. The solution was refluxed for 3 1/2 hours. Evaporation of the solvent under reduced pressure gave a product which crystallized on standing. On recrystallization, the product had m.p. 124-126°. The literature quotes 125-126° (41). Ethylacetate-pet. ether was used for recrystallization.

(c) Component 3:

This component obtained in very small quantity (15.5 mg.) gave a spot, on paper chromatography in solvent system A, with R_f value 0.40.

TABLE 7

SEPARATION OF NEUTRAL SUGARS OF METHYLATED CORN LEAF POLYSACCHARIDE

Tube Number	Component Number	Weight, mg.	Identity
26-90	1,2	382.0	2,3,5-tri-O-methyl arabinose, 2,3,4-tri-O-methyl-D-xylose, and 2,3-di-O-methyl-D-xylose
91-110	2	203.0	2,3-di-O-methyl-D-xylose
111-125	2 (+ traces of 3)	17.8	2,3-di-O-methyl-D-xylose
126-140	3	15.5	Unknown
141-180	4	83.0	Mono-O-methyl-D-xyloses
181-200	5	12.1	D-xylose
RESEPARATION OF THE CONTENTS OF TUBES NUMBERING 26-90			
30-60	1	73.1	2,3,5-tri-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-xylose
61-79	2	98.1	2,3-di-O-methyl-D-xylose
80-130	2	190.8	2,3-di-O-methyl-D-xylose

No further investigation was carried out.

(d) Component 4:

This component appeared to be homogeneous on paper chromatography with a R_f value of 0.22 identical to that of mono-O-methyl xyloses. From the rotation of the mixture $[\alpha]_D^{22}$+27.5° (c, 1.5 in water), it was judged to contain ca. 56% 2-O-methyl-D-xylose and 44% 3-O-methyl-D-xylose. The rotation of these pure compounds are, respectively, +35.9° and +17° (42,43,44).

(e) Component 5:

Concentration of the eluates containing this component yielded a small amount of syrup (12.1 mg.) which did not crystallize. This was shown chromatographically to contain only xylose (R_f 0.06).

K. Gas-Liquid Chromatography of Methylated Neutral Sugars of Corn Leaf Polysaccharide:

1. Partially Methylated Alditol Acetates:

Methylated neutral monosaccharides (20 mg.), obtained from the acid hydrolysis of methylated polysaccharide, were reduced in water (100 ml.) with sodium borohydride (400 mg.) for a period of 12 hours. The solution was next treated with Amberlite IR-120 (H^+) resin and evaporated to dryness. Boric acid was removed by condensation with methanol and the product was treated with acetic anhydride-pyridine (1:1, 40 ml.). The acetylation reaction was continued for 1 hour. The product was diluted with water, evaporated to dryness and dissolved in chloroform. The solution was used for gas-liquid chromatography.

Separation of partially methylated alditol acetates was carried out on a column (8 ft. x 0.25 in.) containing 3% (w/w) of ECNSS-M on Gas Chrom Q (100-120 mesh) using a temperature programme 160-180°C. The columns were held isothermally at 160°C for 3 minutes and then programmed at 2°C per min. to hold at 180°C.

One of the separation curves is shown in Fig. 21.

To assign identities to the peaks, authentic samples of 2,3,5-tri-O-methyl-L-arabinose, 2,3,4-tri-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose and 3-O-methyl-D-xylose were converted separately into corresponding acetylated alditols by reduction with sodium borohydride and subsequent acetylation with acetic anhydride-pyridine. The products were injected into the gas-liquid chromatograph using 3% (w/w) of ECNSS-M column with the identical programme as used for the separation of partially methylated alditol acetates of neutral sugars of corn leaf polysaccharide. By comparing retention times with authentic samples, it was possible to conclude that peaks 1,2,3, and 4 of Fig. 21, represent alditol acetates of 2,3,5-tri-O-methyl-L-arabinose, 2,3,4-tri-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose and monomethyl xyloses respectively.

2. Trimethylsilyl Derivatives of Methylated Aldopentoses:

Methylated neutral sugars (20 mg.), obtained from the hydrolysis products of the fully methylated corn leaf polysaccharide, were converted into trimethylsilyl derivatives, as before, using hexamethyl-disilazane (0.5 ml.) and trimethylchlorosilane (0.25 ml.). Trimethylsilyl

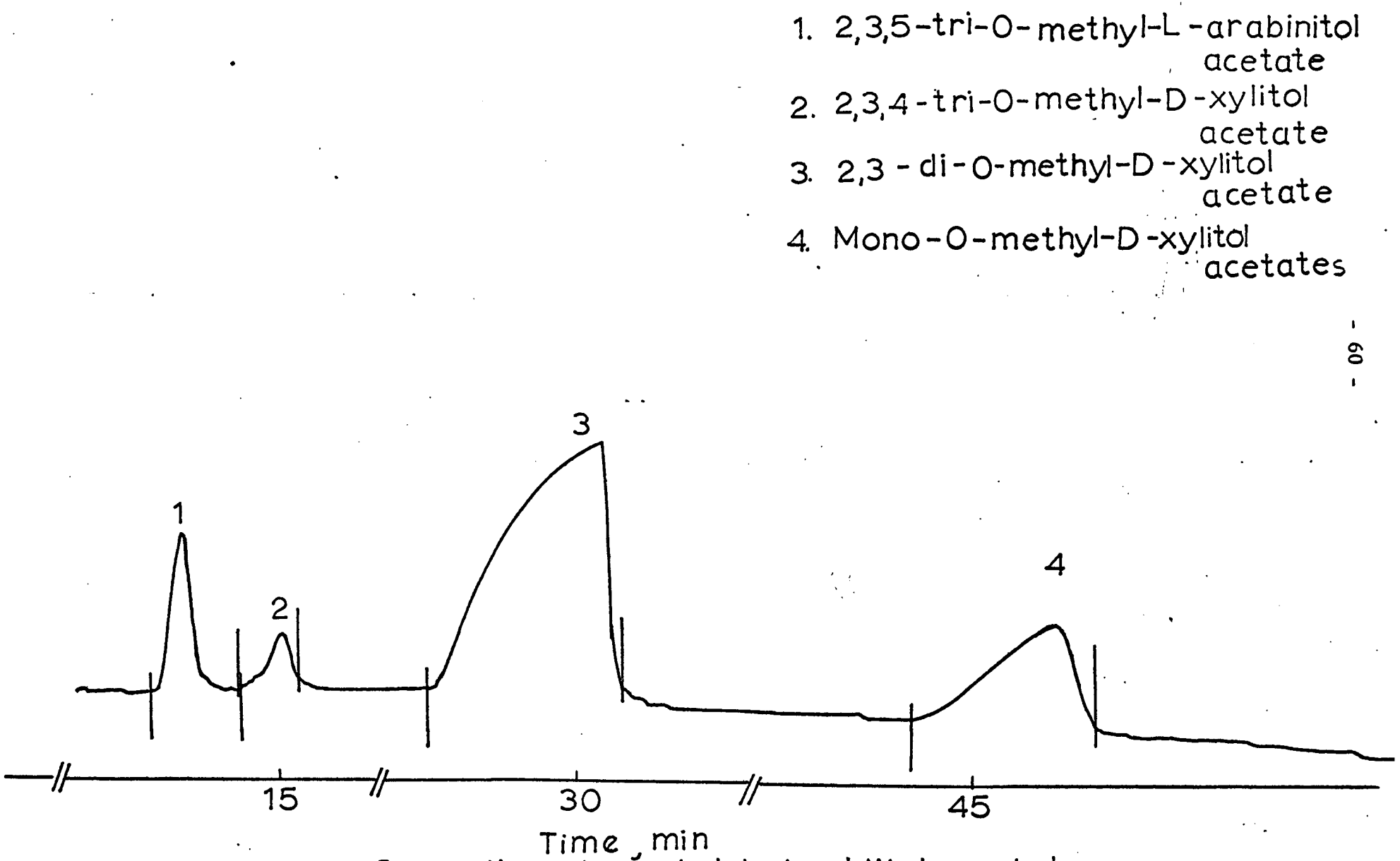


Fig. 21. Separation of methylated alditol acetates

derivatives of partially methylated sugars were separated by gas-liquid chromatography on a coiled copper column packed with 8% by weight of SE 52 on 60-80 mesh Diatoport S. The column (8 ft. x 0.25 in.) was held isothermally at 110°C for 3 min and then programmed at 3° per min to hold at 140°. The flow rate was 75 ml. of helium per min.

One of the separation curves is shown in Fig. 22.

The assignment of different peaks was verified by injecting authentic individual specimens on to the column with the unknown mixture and finding that they co-chromatographed with the corresponding individual sugars obtained from the hydrolysed products of corn leaf polysaccharide.

Trimethylsilyl derivatives of methylated sugars corresponding to different peaks were collected and subjected to mass spectrometric studies.

L. Identification of the Methylated Aldobiouronic Acid

The acidic fraction (109 mg.) from the hydrolysis of methylated polysaccharide was refluxed with methanolic hydrogen chloride (3%, 10 ml.) for 5 hours. The resulting syrup, after neutralization with silver carbonate and subsequent removal of excess silver ions by H_2S, was reduced with lithium aluminium hydride (200 mg.) in tetrahydrofuran (7 ml.); the solution being refluxed for 1 hour. After destruction of excess of hydride by adding dilute aqueous acetic acid, the reaction mixture was evaporated to dryness. The reduced material was heated with 1 N sulphuric acid at 100° for 8 hours. The solution was neutralized

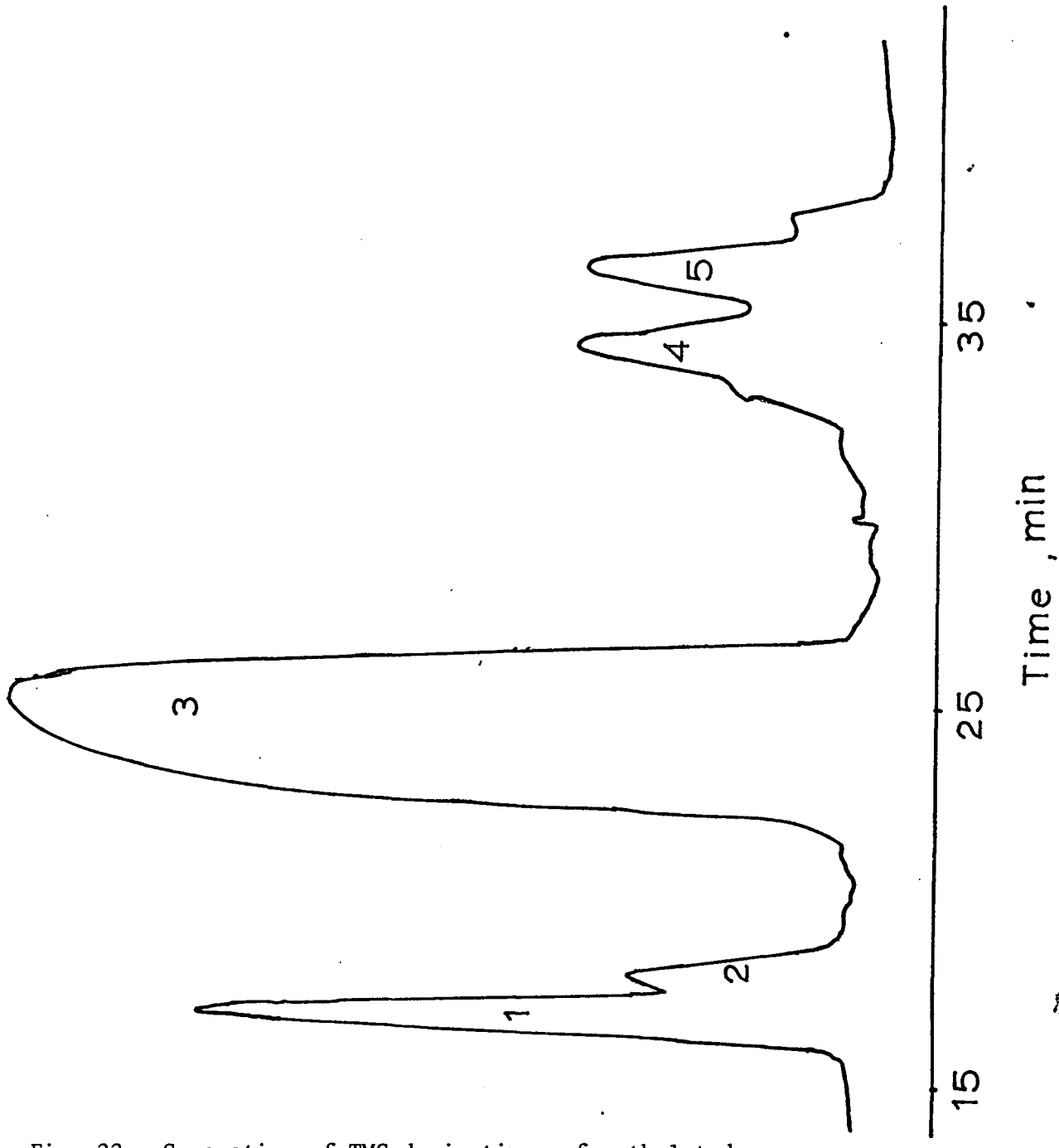


Fig. 22. Separation of TMS derivatives of methylated sugars. 1. 2,3,5-tri-O-methyl-L-arabinose, 2. 2,3,4-tri-O-methyl-D-xylose, 3. 2,3-di-O-methyl-D-xylose, 4. 3-O-methyl-D-xylose, 5. 2-O-methyl-D-xylose.

with barium carbonate, deionised with Amberlite IR-120 (H^+) resin and concentrated to a syrup (95 mg.). Hydrolysed products, on chromatographic examination on paper in solvent system A, were found to have identical R_f values with those of 3-O-methyl-D-xylose and 2,3,4-tri-O-methyl-D-glucose. The mixture of sugars was resolved into two main fractions by preparative paper chromatography in solvent system A over a period of 4 hours. The components were located by spraying half-inch strips out from edges of the sheet with p-anisidine trichloroacetate reagent. Bands corresponding to R_g (where g = 2,3,4,6-tetra-O-methyl-D-glucose) 0.64 and 0.24 were cut out and eluted with water.

1. Identification of 2,3,4-tri-O-methyl-D-glucose

Concentration of the eluates corresponding to R_g 0.64 gave rise to a syrup (38 mg.), $[\alpha]_D^{23}$ +78.5 (c, 1.0 in water). It was converted into its methyl glycoside as usual (P. 55) and examined on gas-liquid chromatography using a column (4 ft. x 0.25 in.) packed with 5% by weight of butane-1,4-diol succinate polyester on 80-100 mesh Diatoport S, at 120°C. β-Methyl glycoside of 2,3,4-tri-O-methyl glucose crystallized spontaneously, m.p. 92-94°C. The literature quotes 93-94°C (45). The β-methyl glycoside was further subjected to mass spectrometric investigation.

2. Identification of 3-O-methyl-D-xylose

Concentration of the eluates corresponding to R_g 0.24 gave rise to a syrup (29 mg.) which crystallised on standing, m.p. 91-93°. The literature quotes 95-96° (46).

CORN STALK HEMICELLULOSE

DISCUSSION

A similar series of extractions was carried out on corn stalks. But no hemicellulose fractions were precipitated when alkaline extracts (0.2 N and 1 N KOH) were acidified. Like corn leaf polysaccharides, D-xylose was found to be the main constituent of neutral sugars. All fractions of corn stalk polysaccharides contained very small amounts of methoxyl groups. Thus acidic sugars on being esterified, reduced and hydrolysed gave D-xylose, D-glucose and small amounts of 4-O-methyl-D-glucose. This indicated that the original polysaccharide contained mainly D-glucuronic acid with traces of 4-O-methyl-D-glucuronic acid.

Periodate oxidation, subsequent reduction of the polyaldehyde and complete acid hydrolysis gave ethylene glycol, glycerol, erythritol, and xylose in the molar ratio 1:48.3:5.4:16.8. Thus the ratio of the non-reducing end groups to the internal xylose residues is higher in corn stalk polysaccharides than that of corn leaf polysaccharide (1:34).

Methylation of corn stalk polysaccharide gave a methylated polysaccharide which was similar to the methylated corn polysaccharide. Both the methylated and unmethylated polysaccharide had high negative rotation indicating that D-xylose residues were linked in β-D configuration.

The methylated neutral sugars indicated the presence of 2,3,5-tri-O-methyl-L-arabinose; 2,3,4-tri-O-methyl-D-xylose; 2,3-di-O-methyl-D-xylose; 2-O-methyl-D-xylose and 3-O-methyl-D-xylose in the mole ratio 4:1:55:3.5:4.5.

Like corn leaf polysaccharide, the partially methylated aldobiouronic acid had a high positive rotation (+92.1°) characteristic of an α-D-linkage. The partially methylated aldobiouronic acid on being esterified, reduced and hydrolysed gave 2,3,4-tri-O-methyl-D-glucose and 3-O-methyl-D-xylose.

These results, therefore, indicate no characteristic difference between the polysaccharides from the leaves and stalks.

EXPERIMENTAL

Methods applied for the determination of structure of corn stalk polysaccharides were similar to those applied for corn leaf polysaccharides. Hence a detailed description of experimental procedures has been omitted from this chapter.

Samples of dried corn stalks (460 gm.), were extracted in a similar way as described for corn leaves, and afforded 4 different fractions. Amount of each fraction extracted together with figures of specific rotation, uronic acid and methoxyl content are tabulated in Table 8.

Different fractions were hydrolysed with 1 N sulphuric acid at 100° for 8 hours, neutralised with barium carbonate and separated into the neutral and acidic fractions by ion-exchange resins.

Identification of different sugar residues and their composition were determined by gas-liquid chromatography on a column of SF 96 as their trimethylsilyl derivatives (Table 9).

Acidic sugars were isolated from ion-exchange resin (Duolite A-4) by eluting with formic acid (10%, 5 ml.). Paper chromatography in solvent system B indicated the presence of aldobiouronic acid as the main component with traces of mono and aldotriouronic acid. Acidic sugars (20 mg.) were converted into the methyl ester methyl glycosides, reduced with lithium aluminium hydride in boiling tetrahydrofuran and hydrolysed with 1 N sulphuric acid. Chromatography of the resulting syrup (16 mg.) in solvent system B gave rise to D-xylose, D-glucose and traces of 4-O-methyl-D-glucose.

TABLE 8

FRACTIONS OF POLYSACCHARIDES FROM CORN STALK

Fraction	Method of Extraction	Yield, gm.	Uronic acid %	Specific rotation $[\alpha]_D^{22}$	Methoxyl %	Protein %
A	0.2N KOH (acid) Ext.	-	-	-	-	-
B	0.2N KOH (ethanol) Ext.	30.5	15.01	-58.48°	1.12	-
C	1.0N KOH (acid) Ext.	-	-	-	-	-
D	1.0N KOH (ethanol) Ext.	32.0	13.78	-59.70°	0.42	-
E	2.5N KOH (acid) Ext.	1.5	8.11	-70.20°	0.52	-
F	2.5N KOH (ethanol) Ext.	21.0	10.94	-64.35°	0.92	-

A quantity of corn stalk polysaccharide (2.22 gm.) was dissolved in alkali (0.2 N sodium hydroxide), acidified with acetic acid and the volume adjusted to 200 ml. To the cooled (5°) solution was added 0.5 M periodic acid (50 ml.) and the oxidation was allowed to proceed at 5° in the dark. Periodate consumption was determined periodically as before (Table 10), and after 7 days 0.65 moles of periodate had been consumed per mole of sugar with no further change upon longer standing. Upon removal of the periodate and iodate ions by precipitation (barium carbonate) the polyaldehyde was reduced with sodium borohydride (1.2 g.) overnight. The solution was neutralised, evaporated and borate removed from the polyol by several treatments with methanolic hydrogen chloride (3%). The residue was hydrolysed with 1 N sulphuric acid at 100° for 8 hours. The ratio of polyhydric alcohols and sugars were determined by gas-liquid chromatography as their trimethylsilyl derivatives. The molar ratio of ethylene glycol, glycerol, erythritol, and xylose (calculated as before) was found to be 1:48.5:5.4:16.8.

Corn stalk polysaccharide (1.00 gm.) was methylated according to the procedure described by Hakomori (12). Different reagents, required for the reaction, were applied in the same ratios as described for the methylation of corn leaf polysaccharide. The reaction was found to be complete by one step methylation, as no hydroxyl band was observed in infrared spectrum. Crude methylated polysaccharide (1.23 gm.) was fractionated by gradual addition of various mixtures of chloroform: petroleum ether. Results of fractionation are tabulated in Table 11.

TABLE 9

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF CORN STALK NEUTRAL SUGARS

Fraction	Method of Extraction	Arabinose	Xylose %	Glucose
B	0.2N KOH (ethanol) Ext.	14.1	81.9	4.0
D	1.0N KOH (ethanol) Ext.	16.4	78.6	5.2
E	2.5N KOH (acid) Ext.	12.8	82.6	4.6
F	2.5N KOH (ethanol) Ext.	15.7	80.2	4.1

TABLE 10

PERIODATE UPTAKE OF CORN STALK POLYSACCHARIDE

Time (hour)	Moles of Periodate/Moles of Sugar
1	0.05
2	0.08
4	0.13
5	0.15
12	0.23
24	0.40
48	0.50
72	0.56
96	0.59
144	0.66
168	0.65

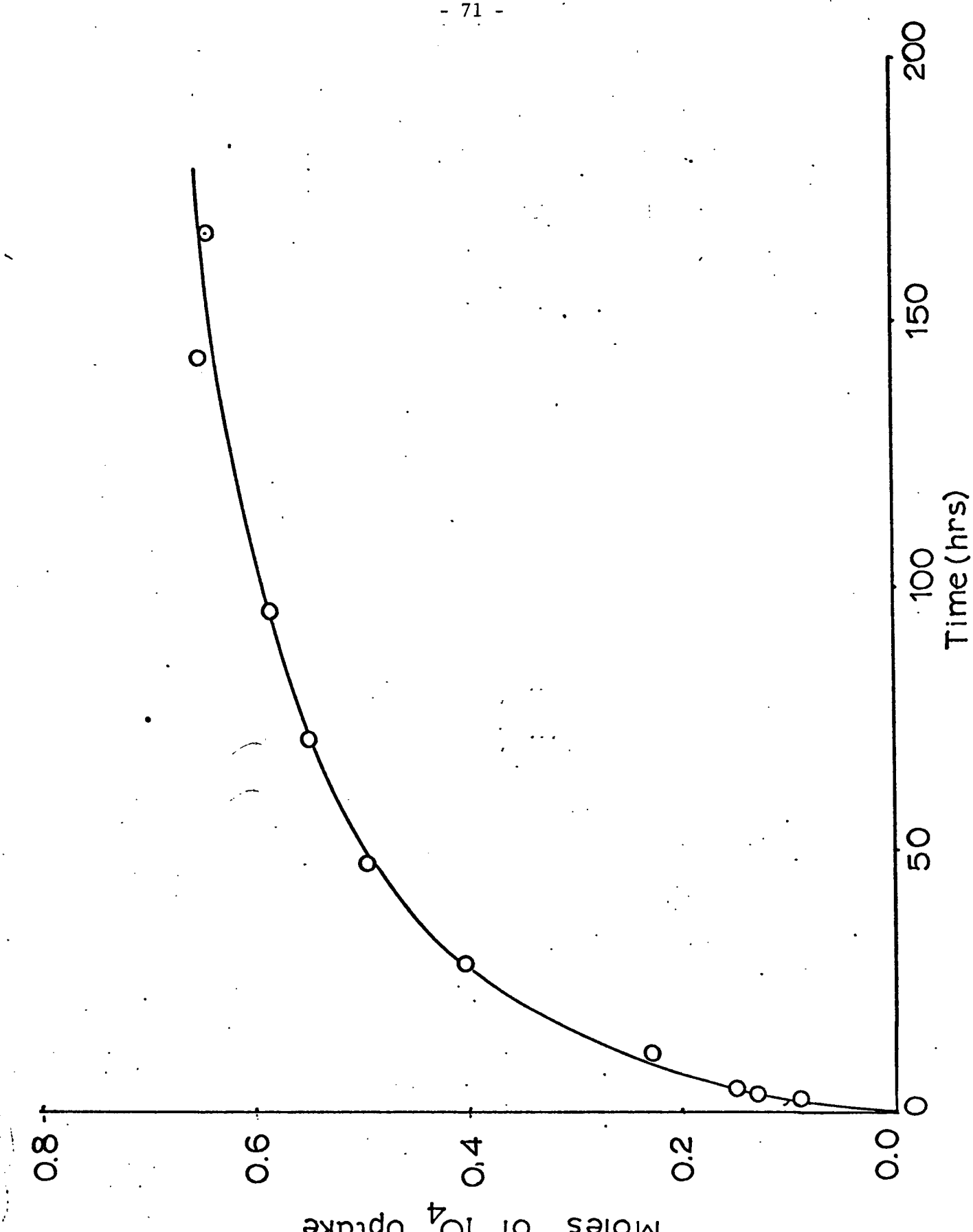


Figure 23. Periodate oxidation of corn stalk polysaccharide

TABLE 11

FRACTIONATION OF METHYLATED CORN STALK POLYSACCHARIDE

Fraction	Chloroform: Pet. Ether	Weight gm.	$[\alpha]_D^{22}$ ($CHCl_3$)	Methoxyl %
1	0:100	0.0024	-	-
2	10:90	0.0381	-	-
3	15.85	0.0595	-36.66	24.02
4	20:80	0.0510	-53.92	32.46
5	25:75	0.8925	-57.76	39.49
6	30:70	0.0913	-68.75	37.62

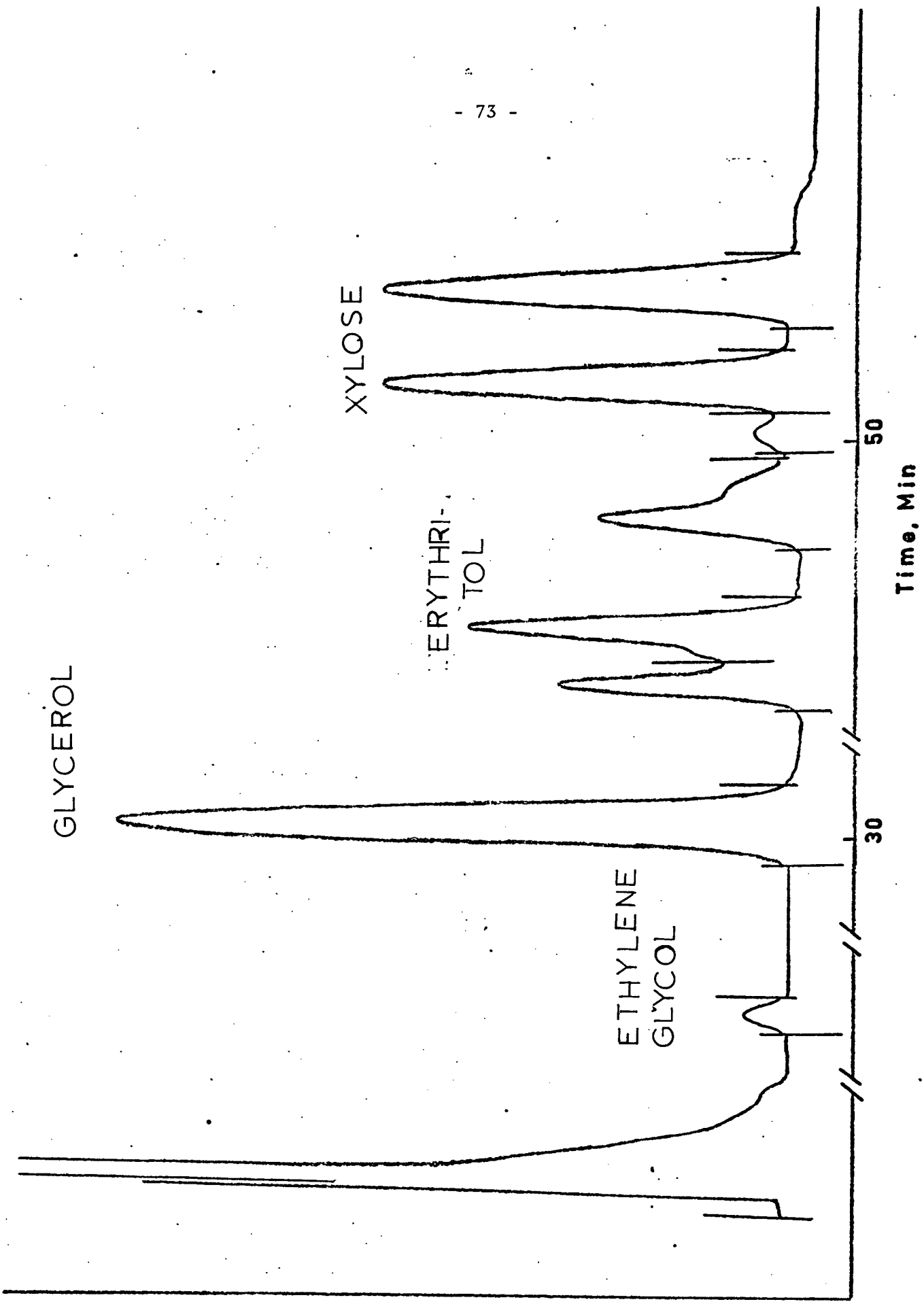


Figure 24. Separation of products as trimethylsilyl derivatives from corn stalk polysaccharide

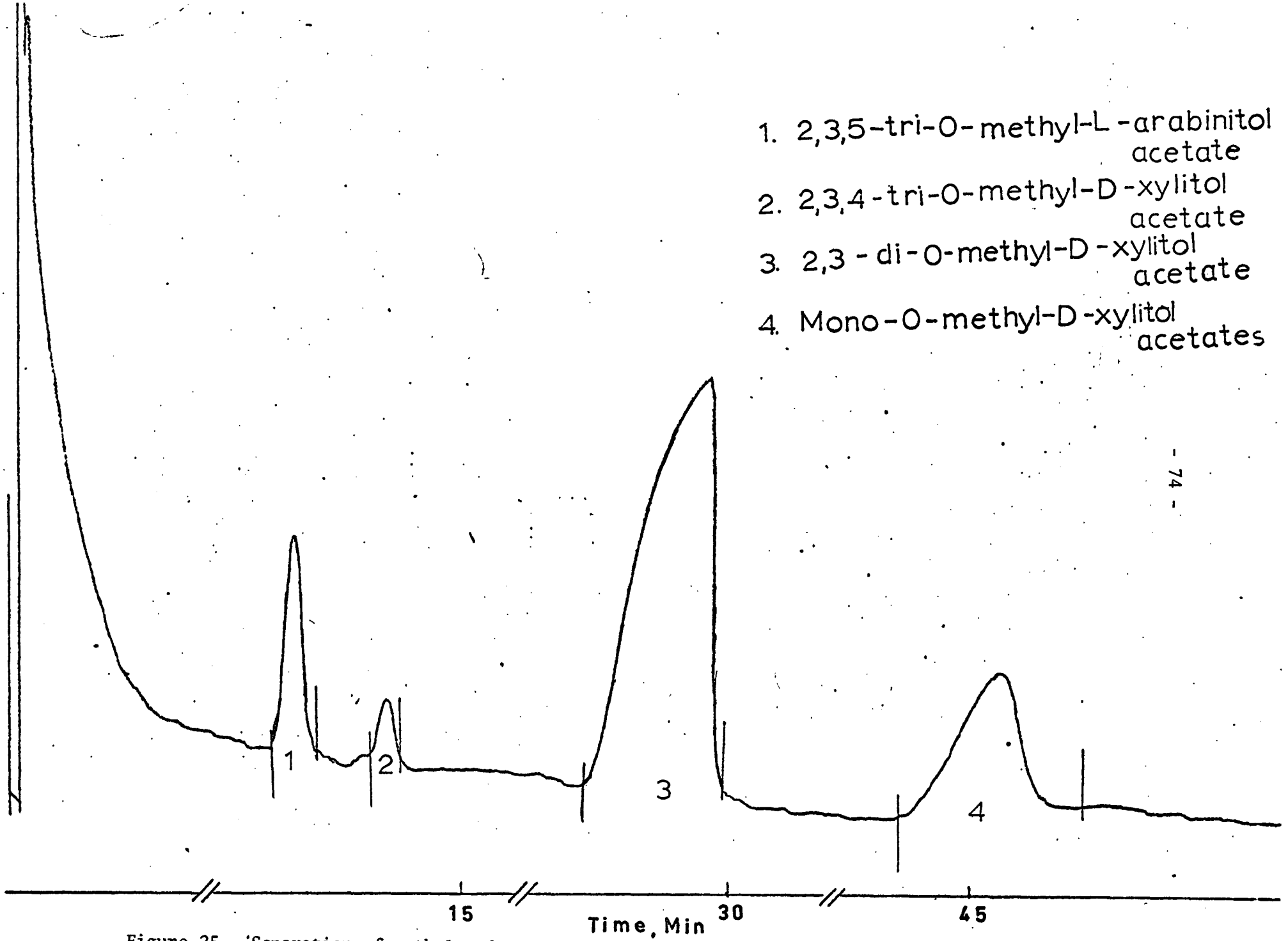


Figure 25. 'Separation of methylated alditol acetates of corn stalk polysaccharide

Fraction 5 (Table 11) was subsequently used for structural investigation.

The methylated polysaccharide (0.85 g.) was hydrolysed according to the procedure described by Lindberg (30). The aqueous phase was neutralised with barium carbonate and passed through Amberlite IR-120 (H^+) to remove barium ions and acidic sugars were absorbed on Duolite A-4 resins.

A mixture of neutral sugars (32 mg.) was converted into alditol acetates by reduction with sodium borohydride (12 hours) and subsequent acetylation with acetic anhydride. Gas-liquid chromatography of partially methylated alditol acetates showed the presence of components with retention times identical to those of alditol acetates of 2,3,5-tri-O-methyl-L-arabinose; 2,3,4-tri-O-methyl-D-xylose; 2,3-di-O-methyl-D-xylose and mono-O-methyl-D-xyloses. Each component was collected and subjected to mass spectrometric investigations.

Methylated neutral sugars from corn stalk polysaccharides (28 mg.) were converted into trimethylsilyl derivatives. Gas-liquid chromatographic examination on a column of SE 52 indicated the presence of trimethylsilyl derivatives of 2,3,5-tri-O-methyl-L-arabinose, 2,3,4-tri-O-methyl-D-xylose; 2,3-di-O-methyl-D-xylose; 2-O-methyl-D-xylose; and 3-O-methyl-D-xylose. Anomeric peaks of mono-O-methyl sugars were well separated and analysed by mass spectrometry.

Partially methylated acidic sugars (100 mg.) were eluted with formic acid (10%, 5 ml.) from Duolite A-4 resins. The acidic partially methylated sugars (100 mg.) were esterified by refluxing with methanolic

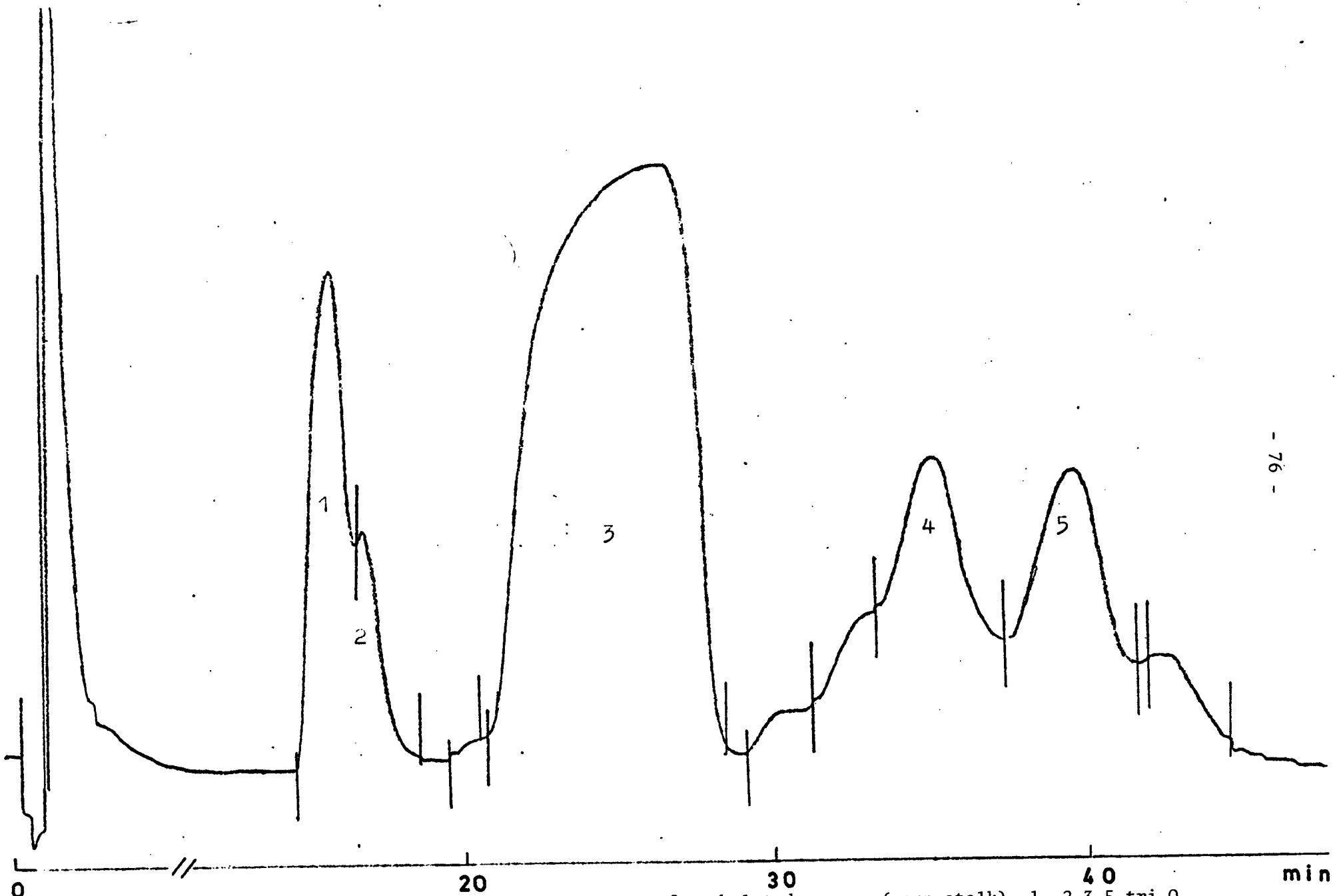


Figure 26. Separation of trimethylsilyl derivatives of methylated sugars (corn stalk). 1. 2,3,5-tri-O-methyl-L-arabinose; 2. 2,3,4-tri-O-methyl-D-xylose; 3. 2,3-di-O-methyl-D-xylose, 4. 3-O-methyl-D-xylose, 5. 2-O-methyl-D-xylose.

hydrogen chloride (3%) and the ester was reduced with lithium aluminium hydride in tetrahydrofuran. Hydrolysis of the partially methylated dissaccharide (94 mg.) and examination of the products on paper chromatogram using solvent system A indicated the presence of two components having identical R_f values as those of 2,3,4-tri-O-methyl-D-glucose and 3-O-methyl-D-xylose.

APPENDIX

PRECIPITATION OF THE HEMICELLULOSES OF CORN LEAVES AND CORN STALKS IN ETHANOL-WATER MIXTURES

Biological polysaccharides often occur in mixtures. In order to study these compounds, it is often desirable to separate the mixtures into chemically homogeneous polysaccharides. Fractionation in the presence of ethanol has been one of the common methods for the separation of polysaccharides. The success of this method depends on the type of the polysaccharide and also on the relative solubility of the different components present in the mixture. By using this method, Gramera and Whistler (11) have claimed to isolate an acidic polysaccharide free from glucan from the hemicelluloses of corn stalk.

In some cases, fractionation has also been achieved by precipitation with ethanol in the presence of inorganic salts such as potassium (47), manganous (48) and calcium (49). This method used is particularly useful for the isolation of acidic polysaccharides (49). The presence of cations, probably, affects the solubility of the polysaccharides containing anionic groups (e.g., COO^-) in aqueous ethanol due to the formation of ion pairs. The degree of formation of ion pairs is nearly independent of the polymer and salt concentration and is only determined by the properties of the polymer and the type of the counter ion (50,51). However, in many cases, addition of inorganic ions either does not lead to precipitation or causes the formation of gels occupying most of the volume, thus preventing fractionation.

In the present work attempts were made to isolate an acidic polymer free from glucan by fractional precipitation of the hemicelluloses of corn leaves and corn stalks in ethanol-water mixtures. The fractional precipitation was also carried out in the presence of calcium ions. However, it was not possible to isolate an acidic polymer free from glucan. No positive effect of calcium ions on the precipitation of

the polysaccharide was observed in this present investigation.

EXPERIMENTAL

Precipitation of the Hemicelluloses of Corn Leaves:

Corn leaf hemicellulose (0.50 g.) was dissolved in sodium hydroxide (1 N, 20 ml.), excess alkali being neutralised by adding acetic acid (1 N, 20 ml.). The final pH was adjusted to 7.0 and the final volume of the solution was adjusted to 50 ml. The hemicellulose solution (50 ml.) was divided into two equal portions. To one half inorganic ion (1×10^{-2} gm. equivalent of $CaCl_2$/litre) was added. No salt was added to the other half of the hemicellulose solution.

The hemicellulose solutions were fractionally precipitated at pH 7.0 by gradual addition of ethanol (95%), the mixtures being stirred continuously at the time of each addition. The fractions were removed by centrifugation when distinct precipitates were formed, washed several times with acetone and pet. ether (30-60°C). Salts were removed by dissolving the fractions in water (ca. 50 ml.) which were subsequently deionised by passage through cation-exchange resin Amberlite IR-120 (H^+) and recovered by freeze drying the aqueous solution. A portion of each fraction (10 mg.) was hydrolysed with sulphuric acid (1 N, 5 ml.) for a period of 8 hrs. at 100°C. The hydrolysate was neutralized with barium carbonate, deionised by passage through a cation exchange resin Amberlite IR-120 (H^+) and examined on a paper chromatogram using solvent system B for 18 hrs. Spots corresponding to xylose, arabinose and glucose (in traces) were observed in the paper chromatogram of each fraction. The uronic acid content of the major fraction (Fraction 2, Table 12) was found to be 12.2% which is

TABLE 12

FRACTIONATION OF CORN LEAF POLYSACCHARIDES IN ETHANOL-WATER MIXTURES

Fraction	Ethanol Added (%)	Amount of the Hemicellulose Precipitated %	
		With Ca^{++}	Without Ca^{++}
1	28	12	16
2	37	50	36
3	45	5	4
4	50	16	18
5	55	6	12
6	65	6	4

A typical precipitation curves is shown in Fig. 27.

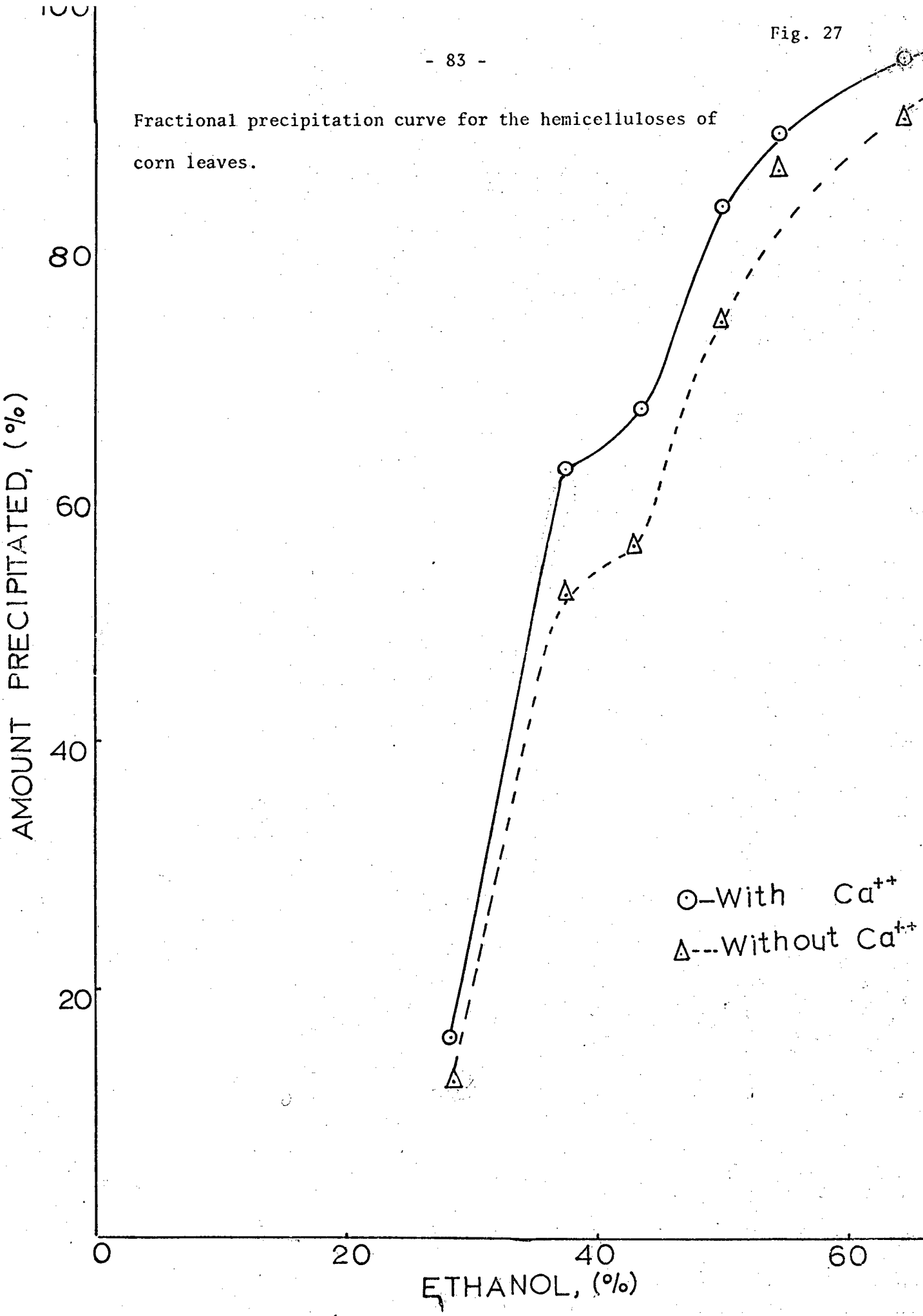
almost the same as that of the original polysaccharide (Table 2). The ratio among xylose, arabinose and glucose of Fraction 2 (Table 12) determined by gas-liquid chromatography as before (p. 43), was found to be 80:15:5. The fraction had $[\alpha]_D^{22}$ -58.0°.

Precipitation of the Hemicelluloses of Corn Stalks

Corn stalk polysaccharide (5.0 g.) was dissolved in sodium hydroxide (1 N, 50 ml.). Excess alkali was neutralised by the addition of acetic acid. The final volume was adjusted to 150 ml., the final pH being 7.0. The hemicellulose solution was divided into two equal

Fig. 27

Fractional precipitation curve for the hemicelluloses of corn leaves.



halves (75 ml. each). To one half only calcium ion (0.01 gm. equiv./litre) was added.

The hemicellulose solutions were fractionated by the gradual addition of 95% ethanol, and each fraction was removed and dried in the usual way. A small quantity of each fraction (ca. 10 mg.) was hydrolyzed with sulphuric acid (1 N, 5 ml.) at 100°C for 8 hrs. The hydrolysate after being neutralised and deionised, as before, was examined on a paper chromatogram using solvent System B for 18 hrs. Each fraction contained mainly xylose, arabinose and traces of glucose. The uronic acid content of the major fraction (Fraction 2, Table 14) was found to be 14.0% which is close to that of the original polysaccharide (Table 8).

TABLE 13

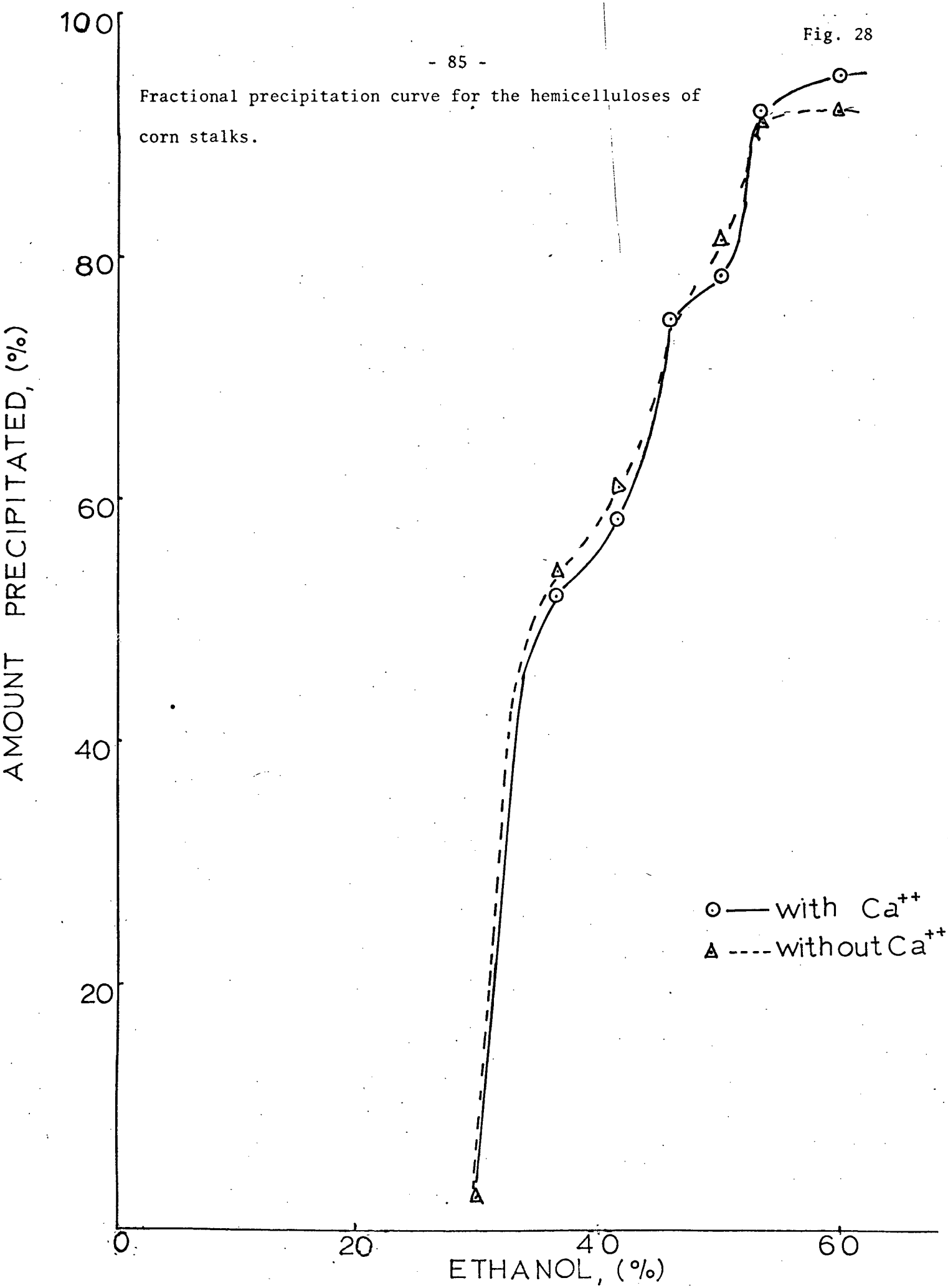
FRACTIONAL PRECIPITATION OF CORN STALK POLYSACCHARIDES IN ETHANOL-WATER MIXTURES

Fraction	Ethanol Added (%)	Amount of the Hemicellulose Precipitated %	
		With Ca^{++}	Without Ca^{++}
1	30	3	4
2	37	49	50
3	42	7	7
4	46	16	13
5	50	4	8
6	53	13	9
7	60	3	1

A typical precipitation curve shown in Fig. 28.

Fig. 28

Fractional precipitation curve for the hemicelluloses of corn stalks.



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