EXTRACTION AND STRUCTURAL STUDY OF HEMICELLULOSE B FROM YORKSHIRE FOG (*HOLCUS LANATUS*)

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SUMMARY

(1) Hemicellulose B has been extracted from Yorkshire Fog (*Holcus lanatus*).

(2) The homogeneity of Branched Hemicellulose B was determined by Fractional Precipitation.

(3) Gas-liquid chromatographic study showed that the polysaccharide contains xylose, arabinose and hexose.

(4) The uronic acid content was quantitatively determined by specific colour reactions.

(5) Quantitative analysis of the monosaccharides from the acid hydrolysis product by partition chromatography on cellulose column.

(6) Methylation by methods of Haworth, Kuhn and Purdie followed by hydrolysis yielded the following methyl ethers

2,3-di-O-methyl-D-xylose,

2-O-methyl-D-xylose,

2,3,4-tri-O-methyl-D-xylose,

2,3,5-tri-O-methyl-L-arabinose.

(7) The methyl ether monomers were quantitatively determined by gas-liquid chromatography.

(8) From the results a simplified structure can be suggested for the branched hemicellulose B, consisting of 16 β-D-xylo-pyranose residues linked together by 1–4 glycosidic bonds, and with a terminal side chain D-glucuronic acid residue linked through C-2 to the xylose unit. L-arabinose unit probably occurs as a side chain and is linked through position 3 to every fourth D-xylose unit.

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Introduction

1.1 Hemicellulose

The structural carbohydrates are mixtures of polysaccharides which together with lignin, constitute the cell wall. Usually they are divided into three fractions: pectic substances, hemicellulose and cellulose.[1]

Pectins, widely distributed in land plants are characterised by a main chain of 1-4 linked galacturonic acid units. They may also contain rhamnose, galactose, xylose, arabinose and fucose in varying amounts. These sugars form part of the main chain in some pectins and branch chains in others.[2]

Cellulose is generally present in the plant cell as aggregates of fibrils or partly crystalline bundles. They consist of parallel chains of β 1-4 linked glucose residues, where the chains are in perfect alignment, the cellulose has a crystalline structure. The cellulose fibrils are generally embedded in other polysaccharide material, e.g. the hemicelluloses in the higher plants.[3]

The name hemicellulose was first proposed in 1891 by Schulze, who was examining products extracted from leguminous seeds, brans and green tissues. He isolated a group of carbohydrates similar to cellulose which were characterised by their behaviour with dilute mineral acid, being more easily hydrolysed than cellulose. This group of polysaccharides are chemically and structurally related to cellulose, for which reason, Schulze designated the group hemicellulose.

The use of the term hemicellulose has changed frequently. Some investigators wish to confine the term to the non-cellulosic carbohydrates and to define hemicellulose as being composed of (1) pentosans, chiefly xylan and araban, yielding on hydrolysis the pentoses, xylose and arabinose; (2) hexosans, chiefly mannan and galactan, capable of being hydrolysed to the hexoses, mannose and galactose; and (3) polyuronides, mostly in the form of polyuronic acids. The wood and cellulose chemists have been accustomed to define the term hemicellulose as that portion of a plant material which is soluble in cold alkali solution.

In general, the name is restricted to land plants and is applied to the group of carbohydrates found in the cell walls of plants in association with lignin as an amorphous phase enveloping the cellulose strands and which can be extracted from plant material with dilute alkali, either hot or cold.[4] A wide range of molecular sizes and molecular shapes exist among hemicelluloses. Because of these differences and the differences in their acidic properties, the polysaccharides which constitute the hemicelluloses vary among themselves in solubility. As a plant tissue ages, the solubilities of the polysaccharides decrease to some extent. Decreased solubility is most pronounced when the tissue material is dried. Insolubilization is not inherent in the drying process itself because it is possible to dry plant material in such a way as to prevent loss of solubility or chemical reactivity.

The isolation of the cell wall carbohydrates is based upon their differences in solubility. The extent to which a soluble

polysaccharide is extracted from a plant tissue or holocellulose depends upon the ease with which the solvent penetrates the insoluble phase and the facility with which the soluble component can diffuse from the matrix to the surrounding solution.

	Holocellulose	
	Hemicellulose	Cellulose
Lignin containing the non-carbohydrate material of the cell wall	shorter chains containing: xylose units mannose units uronic acid units methyoxyl groups acetyl groups (possibly galactose and arabinose units)	longer chain length including: α -cellulose "True" cellulose β -d-glucopyranose units

Figure 1. Relation of Hemicellulose to other cell wall components[3]

1.2 Molecular Structure of Hemicellulose

The group of polysaccharides called hemicelluloses are usually mixtures which include some or all of the xylans, the glucomannans and the arabogalactans. Sometimes other plant polysaccharides such as the β-glucans of barley and oats, some mannans and some galactomannans are regarded as hemicelluloses.

(A) D-Xylans

Xylan is the commonest hemicellulose because it is removed by hemicellulose extraction procedures and is often the principal component of hemicelluloses. Purified hemicellulose is, in many instances, identical to xylan.[5] It occurs in practically all land plants and also in some marine algae. It is most abundant in annual crops, particularly in agricultural residues such as corn cobs, corn stalks, green hulls and stems where it occurs in amounts ranging from 15 to 30%. Hard woods contain 7 to 12% (see Tables I, II and III). Low strength vegetable fibres of commerce such as jute, sisal, manila hemp and coir may contain 5 to 20% xylan, whereas high strength fibres such as ramie, flax and cotton are almost devoid of xylan.[6] The xylan of esparto grass is of particular interest because it has been shown[7] to consist mainly of xylose residues. After complete methylation and hydrolysis the principal products obtained were 2,3,di-O-methyl-D-xylose, 2,3,4,tri-O-methyl-D-xylose, 2-O-methyl-D-xylose and a trace of 2,3,5,tri-O-methyl-L-arabinose. Acetolysis of the methylated D-xylan gave the disaccharide (I), therefore

TABLE I Chemical composition[a] of six North-American species of wood[4]

Species	Cellulose	Lignin	O-Acetyl	Hexuronic acid[b]	Residues[c] of				
					Galactose	Glucose	Mannose	Arabinose	Xylose
Acer rubrum Red maple	44.1	24.0	3.8	3.5	0.6	46.6	3.5	0.5	17.3
Betula papyrifera White birch	41.0	18.9	4.4	4.6	0.6	44.7	1.5	0.5	24.6
Fagus grandifolia American beech	42.1	22.1	3.9	4.8	1.2	47.5	2.1	0.5	17.5
Abies balsamea Balsam fir	44.8	29.4	1.5	3.4	1.0	46.8	12.4	0.5	4.8
Picea glauca White spruce	44.8	27.1	1.3	3.6	1.2	46.5	11.6	1.6	6.8
Pinus banksiana Jack pine	41.6	28.6	1.2	3.9	1.4	45.6	10.6	1.4	7.1

[a] All values in per cent of extractive-free wood

[b] As $C_6H_{10}O_7$ minus H_2O

[c] As $C_6H_{12}O_6$ minus H_2O

TABLE II Carbohydrate composition* of sixteen species of European Hardwood

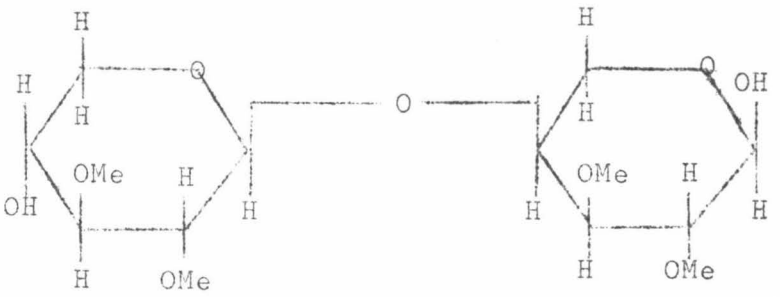
Species	Residues of:				
	Galactose	Glucose	Mannose	Arabinose	Xylose
Acer platanoides Norway maple	2.0	60.5	4.0	1.0	32.5
Alnus glutinosa Black alder	2.5	73.5	3.5	1.0	19.5
Alnus rugosa Speckled alder	3.5	67.0	1.5	1.0	27.0
Betula pubescens Birch	1.0	55.0	2.5	2.5	39.0
Betula verrucosa Silver birch	1.5	58.5	0.5	0.5	39.0
Corylus avellana European hazel	2.0	69.5	2.0	2.0	24.5
Fagus sylvatica European beech	4.0	65.0	1.5	1.5	28.0
Fraxinus excelsior European ash	3.0	60.0	2.5	2.5	32.0
Populus balsamifera Balsam poplar	3.5	68.0	2.5	2.5	23.5
Populus tremula European trembling aspen	1.5	64.5	3.0	1.0	30.0
Prunus padus European cherry	2.5	65.5	2.5	1.0	28.5
Quercus robur English oak	2.5	68.5	2.0	1.0	26.0
Salix alba White willow	3.0	74.0	2.5	1.0	19.5
Sorbus aucuparia European mountain ash	1.5	66.5	2.5	2.0	27.5
Tilia cordata Linden	1.5	58.5	3.5	2.0	34.5
Ulmus glabra Scots elm	2.5	68.5	2.0	1.0	26.0

* All values in per cent of total neutral carbohydrates.

TABLE III Percentage composition of polysaccharides[10]

Source	D Glucuronic acid	D Galactose	L-Arabinose	Other	Reference
Acacia cyanophylla	24	49	7	L-Rhamnose 20	20
Acacia Karroo (Hayne)	12	50	36	L-Rhamnose	21
Acacia mollissima (Black wattle)	9	42	42	L-Rhamnose 7	22
Acacia pycnantha	5	65	27	L-Rhamnose 1-2	43
Acacia verek (arabic)	16	52	19	L-Rhamnose 14	24
Amygdalus (Almond tree)	10	30	40	D-Xylose 20	25
Anogeissus latifolia wall (Ghatti)	12	27	41	D-Mannose 8	26
Asparagus adscendens	10			D-Glucose 40, D-Mannose 40, D-Xylose 10	
Asparagus filicinus	5			D-Mannose 45, D-Glucose 40, D-fructose 10	
Boswellia carterii (Olibanum or frankincense)		58	8	4-O-Methyl-D-glucuronic acid 33, trace of L-fucose and L-rhamnose L-Rhamnose 25	28
Brasenia schreberi Gmel (watershield)	22	47	9	D-Mannose 16	
Cetraria islandica	3	8		D-Glucose 89, D-Mannose 3	29
Citrus limonia (Lemon)	22	55	22	4-O-methyl-D-glucuronic acid	30
Citrus maxima (Grapefruit)	31	53	16	4-O-methyl-D-glucuronic acid	
Combretum verticillatum		15	38		31
Commiphora myrrha (Myrrh)		30	12	4-O-methyl-D-glucuronic acid 38	32
Curculigo orchioides	42			D-Mannose 23, D-glucose 35	
Dilsea edulis	10	80		D-xylose 7	33
Fagara xanthoyloides		61	21	4-O-methyl-D-glucuronic acid 17	34
Ferula species (Asafetida)	11	55	33	L-Rhamnose	35
Hakea acicularis	8	58		D-xylose 8, D-mannose 7	36
Hevea brasiliensis	6	4	20	D-Glucose 60, L-rhamnose 2, D-xylose 8	37
Mimosa pudica	21			D-xylose 79, trace of D-glucose	
Moringa pterygosperma (Drum stick)	10	37	53	Trace of L-rhamnose	38
Prosopis juliflora (Mesquite gum)		31	51	4-O-methyl-D-glucuronic acid 18	39
Prunus armeniaca (Apricot)	16	44	41		
Prunus cerasus (Cherry)	12	21	55	D-mannose 10, trace of L-rhamnose and D-xylose	40
Prunus domestica (Egg plum)	15	40	34	D-xylose 11	33
Prunus insits (Damson)	16	30	38	D-mannose 15, trace of D-xylose	41
Prunus persica (Peach tree)	7	36	43	D-xylose 14, trace of L-rhamnose	42
Prunus serrulata	6-8			D-xylose 92-94	
Prunus species (Purple plum)	12	38	38	D-xylose 12	25
Prunus virginiana (Chokecherry)	9	26	29	D-mannose 13, L-rhamnose 23	43
Puya chilensis (Chagual)	15	36	7	D-xylose 43	36
Ulva lactuca (Green marine algae)	19			D-xylose 9, L-rhamnose 31, D-Glucose 8	44
Zea mays (Cornhull)	7-12	7	35	D-xylose 48	45

the glycosic linkage is (1 —— 4) and the D-xylose residues are in the pyranose form.[17]



(I)

From this evidence the main features have been formulated as

β -D-xylp-(1— 4)-(β -D-xylp)$_x$—(1— 4)-(β -D-xylp)-(1— 4)
3
|
1
β -D-xylp-(1— 4)-(β -D-xylp)$_z$-(1— 4)-β -D-xylp

xylp = xylopyranose x+y+z = 75 ± 5

The presence of chains of (1— 4) β linked xylopyranose residues appears to be a general structural feature throughout most of the xylan group of polysaccharide (see Table IV).

(B) Arabino-Xylans

Perlin[8] has isolated from wheat flour a xylan fraction which contained almost as much arabinose as xylose. A study of this arabinoxylan by the methylation procedure and by other methods has shown that single L-arabofuranose residues are attached to a main chain of xylose residue, in many instances substituting the hydroxyl at both C-2 and C-3 of the same xylose residue. These arabofuranose residues

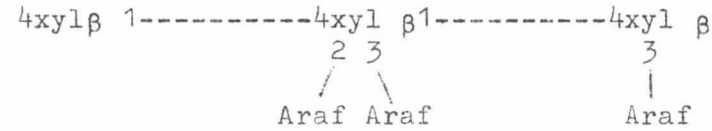
TABLE IV Xylans from the gramineae[6]

Source	Mode of linkage of L-arabinofuranose end-groups[a]	Mode of linkage of D-glucuronic acid end-groups[b]	Other structural features	References
Esparto grass	none present	none present		46
Esparto grass	(1—3)-D-xylp	none present	β-D-xylp-(1—2)-L-Ara	47
Wheat straw	(1—3)-D-xylp	(1—3)-D-xylp (Me)	branched	48
			xylan chain	48
Wheat straw	(1—3)-D-xylp	(1—2)-D-xylp	.....4-D-Gp-1.....	49
Wheat straw	(1—3)-D-xylp	none present	3	
Wheat straw	none present	(1—2)-D-xylp		
Wheat straw	(1—3)-D-xylp	(1—2)-D-xylp (Me)	some branched	50
			xylan chains	51
Oat straw	(1—3)-D-xylp	(1—2)-D-xylp (Me)		52
Cocksfoot grass	(1—3)-D-xylp	(1—2)-D-xylp (Me)		
Wheat leaf	(1—3)-D-xylp	(?)(1—3)-D-xylp		53
Barley husks	(1—3)-D-xylp	(—2)-D-xylp (Me)	β-D-xylp-(1—2)-L-Araf	
			branched	
			xylan chain	
Corn (maize) cobs	(1—3)-D-xylp	not known	.....2-L-Araf-1.....	7
Corn (maize) cobs	(1—3)-D-xylp	(1—2)D-xylp (Me)	β-D-xylp-(1—2)-L-Araf	7
		(1—4)-D-xylp		55
Corn (maize) cobs	(1—3)-D-xylp	none present	.....4-D-Gp-1.....	49
			3	
Maize fibre	(1—3)-D-xylp	not known	α-D-xylp(1—3)-L-Ara	56
			L-Galp-(1—4)-D-xylp-(1—2)-L-Ara	
Maize hulls	not known	(1—2)-D-xylp	α-D-xylp(1—3)-L-Ara	57
			β-D-Galp-(1—4)-D-xylp	58
			β-D-Galp-(1—5)-L-Araf	
Wheat bran	(1—3)-D-xylp	(1—2)-D-xylp	.....3-L-Araf-1.....+	59
	(1—3)-D-xylp-(2—1)			8
Wheat flour	(1—3)-D-xylp	none present		
	(1—3)-D-xylp-(2—1)			
Barley flour	(1—3)-D-xylp	none present		60
	(1—2)-D-xylp			
	(1—3)-D-xylp(2—1)			61
Rye flour	(1—3)-D-xylp	none present		

[a] D-xylp = D-xylopyranose, L-Araf = L-arabinofuranose, D-Gp = D-glucopyranose and Galp = galacto-pyranose residues

[b] (Me) indicates that D-glucuronic acid residues are present, wholly or in part, as the 4-methylether

were readily removed by acid hydrolysis, leaving a xylan similar to the arabinose free material from esparto. The general molecular structure of wheat flour xylan is



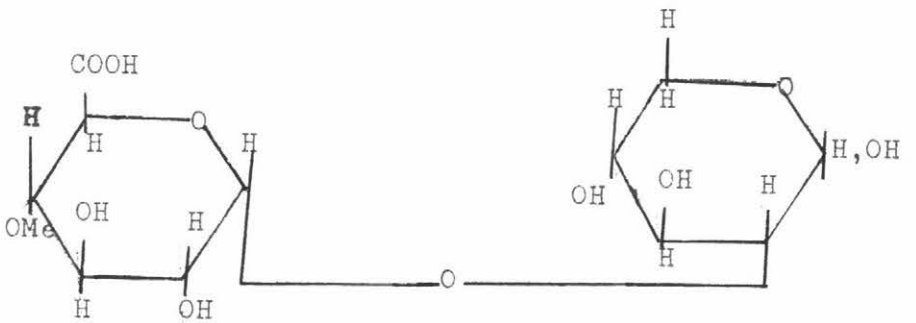
However, some xylans, such as the hemicelluloses from corn (maize) cobs, maize fibre and maize hulls and barley husks also contain non-terminal L-arabinofuranose. Such L-arabinofuranosyl linkages are easily released under mild conditions of hydrolysis, therefore oligosaccharides produced from them have L-arabinose residues at the reducing end.[18]

Bishop and Whitaker[9] have isolated an enzyme from myrothecium verrucaria which hydrolyses linear chains of β -(1— 4) linked D-xylose units. Application of the enzyme to wheat straw hemicellulose yields among other products, L-arabinofuranosyl-(1— 3) β -D-xylopyranosyl-(1— 4)-D-xylose.

(C) Glucurono-xylans

Many polysaccharides of the xylan group contain residues of D-glucouronic acid or 4-O-methyl-D-glucuronic acid.[10] As glycosiduronic acids are very resistant to mineral acid hydrolysis, aldobiouronic acids may be isolated from the graded acid hydrolysis of xylan containing hexuronic acid residues and the mode of linkage of D-glucuronic acid to D-xylose may be determined by the

identification of the hydrolysis products of the derived methylated aldobiouronic acid. Jones and Wise[11] have shown that O-(4-O-methyl-α -D-glucosyluronic acid)(1— 2)-D-xylose can be isolated from aspen wood hemicellulose.



D-glucuronic acid or its 4-methyl ether are most commonly linked to position 2 of D-xylose, although similar aldobiouronic acids containing (1— 3) (wheat straw) and (1— 4) linkages (corn cob) have also been observed.

In the structural study of hemicellulose of Lucerne, Aspinall and McGrath[12] they have found that the polysaccharide contains an essentially linear chain of 1— 4 linked β -D-xylopyranose residues with side chains of 4-O-methyl-α -D-glucopyranosyluronic acid residues attached to C-2 of approximately every ninth residue. The main chain also contains occasional rhamnose residues.

(D) Mannans

Polysaccharides based on D-mannose as the major structural unit occur in woods and in the seed of many plants. The mannans present in ivory nuts have been shown

to consist of chains of (1— 4) linked β -D-mannopyranose residues.[13] The glucomannans which comprise up to half the hemicellulose content of some coniferous woods contain units of both mannose and glucose in their molecular structure.[14] Many mannans and glucomannans contain a small proportion of D-galactose residues, which have been shown to be an integral part of the mannans or glucomannans.

(E) Arabino-galactans

L-arabino-D-galactans are the major wood glycans that can be extracted from wood with water. They are highly branched, water soluble and are found in many coniferous woods. Polysaccharides of this type have (1— 6) and (1— 3) linked D-galactopyranose residues and the L-arabinose residues appear as integral parts of arabinogalactans.

Jones and co-workers[15] have found that the addition of borate to the alkali permits the extraction of the glucomannan in reasonably pure form as the borate complexes. By controlled acid hydrolysis of Loblolly pine wood, Jones and Painter[16] have isolated 2-O-(4-O-methyl- α -D-glucuronosyl)-D-xylose and 4-O- β -D-glucopyranosyl-α -D-mannopyranose.

The greater part of cell wall material in wood, other than cellulose itself, are the xylans, (including arabinoxylans and glucuronoxylans) the galactoarabans and the glucomannans.

1.3 Isolation and Extraction of Hemicellulose from Yorkshire Fog (Holcus lanatus)

(A) Preliminary Extraction

The soluble carbohydrates are usually extracted from the dried and powdered material in two stages. Firstly, mono, di and oligo-saccharides are isolated by extraction with boiling ethanol benzene, and then the residue is extracted with warm water. Secondly, lipids and other extractives are removed. The removal of lipids and extractives before polysaccharide separation not only eliminates them as sources of impurities but opens the tissue to penetration by hydrophilic solvents.

(B) Water-soluble Polysaccharides

Fructosans and some organic acids can be extracted from the residue after benzene-ethanol extraction. The choice of 60°C for the temperature of water for extraction followed the practice of earlier work concerned with the extraction of fructosan from dried grass.[64] As the temperature increased, the amount of water soluble polysaccharide being extracted also increased. At 60°C, the extract came largely from the ionic fraction, and at the temperature higher than 60°C, an appreciable amount of extract came from non-ionic fraction. Therefore, the water at 60°C is the best condition for extracting water soluble polysaccharides.

(C) Pectin Extraction

The pectic substances consist of pectin together with

galactan and araban. They are found abundantly in the primary cell walls so that young plants may be rich in them. Mature plant material contains only small amounts and the pectic material is often associated with polymers of glucose and even of xylose. For the removal of pectic substance from the residue after extraction with ethanol-benzene and water, the method of Weihe and Phillips[65] using 0.5% ammonium oxalate at 80-85°C for twenty-four hours can be used, but has the disadvantage that, there might be some retention of ammonia by the grass fibre. Therefore, pectic substances such as polygalacturonic acids are better extracted by treating the residue with EDTA at 70°C for two hours.

(D) Extraction with Pepsin in Hydrochloric Acid

After those three previous extractions, the grass residue still contains a large amount of the original nitrogen and most of the protein. As an examination of the structural carbohydrates is a major object of this work, reagents to be employed in the removal of protein have to be mild enough not to bring into solution any appreciable quantity of the hemicellulose fraction. This precluded the use of alkali, therefore the proteolytic enzyme of pepsin was used. This has been shown to have no activity towards carbohydrates.

(E) Delignification

Lignin is a complex, three dimensional polymer of phenylpropane residues, formed by dehydrogenation polymerisation of a precursor which is probably of the coniferyl

alcohol type.[69] Lignin removal from the material to be used in structural investigation requires more than usual care because of the ease with which the water soluble hemicellulose fraction goes into solution once a substantial amount of the lignin is removed.[66] However, lignin must be removed as it often retards or prevents complete extraction of the hemicelluloses, either because of mechanical obstruction or by reason of attachement through covalent bonds. Bouveng and Lindberg[68] have observed that hemicelluloses containing lignin could not be separated into the pure components, but, after further delignification, they could be readily fractionated. Delignification may cause a loss in carbohydrate, therefore as mild treatment as possible is desirable. Delignification with chloramine in weakly acid solution was suggested by Gaillard[1] who reckoned that the treatment compares favourably with the usual acid chlorite treatment. For plant material with a moderate lignin content (6-11%) such as Yorkshire Fog, hay or fresh grass, delignification can be carried out directly after the extraction with EDTA or ammonium oxalate solution without causing any loss in carbohydrates. After the suspension was filtered and washed with ethanol, solution of ethanolamine was left in contact with the residue. The purpose of this is to remove secondary cell wall lignin without attacking that of the middle lamella.

(F) Hemicellulose Extraction

The hemicelluloses are composed of a mixture of polysaccharides of different solubility, part being readily soluble in weak alkali and the remainder being extracted only by strong alkaline solution. Alkaline extraction of holocellulose is a useful mean for the isolation of a group of polymers which can later be separated. However, alkaline extraction of the holocellulose can bring many changes in the polysaccharides, even under oxygen free conditions, alkaline degradation might result. It is also possible that any naturally occurring ester groups are saponified.[3]

Solutions of potassium hydroxide, instead of sodium hydroxide are often used, because of the high solubility of potassium acetate in ethanol. The alkaline extraction is usually carried out at or below room temperature for limited periods of time and in an atmosphere of nitrogen. Under these conditions, any changes in the xylan brought about by the alkali are kept at a minimum.

Complete subdivision of the hemicelluloses into individual molecular species has not been accomplished. Separation difficulties arise not only from the presence of different kinds of molecules, but also from the occurrence of each molecular type in different degrees of polymerisation. Therefore, separation based on solubility would not bring about a complete segregation of species. A good general method for effecting initial subdivision of the hemicellulose group is the neutralisation of an alkaline hemicell-

ulose solution which causes precipitation of the high molecular weight polymers and leaves in solution the molecules of lower degree of polymerisation.

The more soluble fraction is often called polyuronide hemicellulose or hemicellulose B and the less soluble xylan cellulosan or hemicellulose A.[67] The difference in solubility of these polysaccharides depends mostly upon the chain length and upon the presence of an uronic acid unit in the chain. A short chain length of the molecule or the presence of an uronic acid unit may raise the solubility of the polysaccharide, but the type of sugar of which a polysaccharide is constituted has no effect on its solubility.

Hemicellulose A is the fraction of the total hemicellulose which is precipitated when an alkaline extract of the holocellulose is neutralised. When the supernatant is poured into ethanol, a white precipitate (Hemicellulose B) appeared. Usually, hemicellulose B has a higher uronic acid content than hemicellulose A.

(G) Fractionation of Hemicellulose B

Hemicellulose B is a mixture of several different polymers, both linear and branched. The separation of these individual polymers in a pure state is a tedious procedure. Generally it is achieved by repeated fractional precipitation from aqueous solution with ethanol or acetone. Gaillard[98] had observed that it is possible to separate the linear from the branched polymers in hemicellulose B

by dissolving the mixture in concentrated calcium chloride solution and precipitating the linear polymers with an iodine-potassium iodide solution. The branched polymers are then recovered from the filtrate.

TABLE V Composition of the linear A and B and the branched B polymers from some gramineae and leguminosae[98]

	T.pratense (red clover) (%)	M.sativa (lucerne) (%)	G.max (soya bean) (%)	L.perenne (grass) (%)	T.vulgare (wheat) (%)	Z.mais (maize) (%)
Linear A						
uronic acid	4.7	6.6	6.6	1.9	2.1	2.4
galactose	-	-	-	-	-	-
arabinose	-	-	-	12.9	5.7	5.3
xylose	95.3	93.4	93.4	85.2	92.2	92.3
glucose	-	-	-	-	-	-
Linear B						
uronic acid	1.0	1.5	4.3	0.4	0.3	0.1
galactose	-	-	-	-	-	-
arabinose	10.8	8.3	8.3	16.5	11.1	10.9
xylose	88.2	90.2	89.2	83.1	88.6	89.0
glucose	(11.6)	(11.4)	(4.0)	(11.0)	(12.0)	(21.5)
Branched B						
uronic acid	20.6	22.3	24.6	5.4	7.9	12.8
galactose	34.5	31.1	34.3	7.7	9.8	8.7
arabinose	27.6	34.2	24.0	23.8	26.5	24.4
xylose	17.3	3.1	3.4	63.1	55.8	54.1
glucose	-	9.3	6.9	-	-	-
rhamnose	-	-	6.8	-	-	-

1.4 Homogeneity of the Polysaccharide[70,71]

Most of the naturally occurring polysaccharides are complex mixtures and for the complete structural characterisation of a polysaccharide, it is necessary to ensure that the polymer is pure, has been isolated from a single species and is chemically homogeneous. By homogeneous it is meant that further fractionation of the polysaccharide yields products having the same specific rotation and the same ratio of mono saccharide building units. There is no standard method for the isolation of a polysaccharide from a mixture, a combination of several methods usually gives the best chance of success. Each biological material presents its own problems and the task of separation and purification is often difficult and tedious, but is an essential preliminary to structural determination.

Most extraction methods yield mixtures of polysaccharides contaminated with other substances such as protein. Furthermore, the extraction procedures may modify the actual structure of the molecule and also alter its molecular weight distribution. The ease of purification depends upon the nature of the contaminating substances. As it is very difficult to obtain an absolutely pure polysaccharide.

The methods used for the separation of polysaccharides in order to obtain homogeneity include graded extraction, fractional precipitation by addition of precipitant, or by cooling, precipitation by a specific complexing agent, ultracentrifugation, electrophoresis, chromatographic adsorption, gel filtration,

the use of enzymes and immunological tests.

Fractionation of Polysaccharides

Methods of fractionation of polysaccharides are based upon many properties. Electrophoresis is one way of testing the chemical homogeneity of the material, but as with the ultracentrifuge, it can only be used to separate microquantities.

In most cases, a solution of the precipitant is added to an aqueous solution of the polymers. The separation may be poor due to the tendency towards coprecipitation and occlusion of other polysaccharides. However, the procedure is widely practised, as a useful method for purification.

Jones[72] found that cetyl trimethylammonium bromide could be used to precipitate the acidic components from a mixture of polysaccharides. Fehling solution is often employed to selectively precipitate polysaccharides, and solutions of cupriethylene diamine, copper chloride, copper sulphate and copper acetate have also been used.

1.4.1 Determination of the Homogeneity of Polysaccharide

The methods which can be used are

(a) Fractional precipitation with ethanol

(b) Precipitation using a specific complexing agent.

(a) Fractional Precipitation with Ethanol

This involves the precipitation of polysaccharide from solution by the gradual addition of ethanol to the aqueous solution of water soluble polysaccharide.

Fractionation is usually carried out at or near pH 7.0, where the polysaccharides are most stable, and

the carboxyl groups, present in the hemicelluloses containing uronic acids, are in the form of ionised salts. Under acidic conditions, significant hydrolysis of glycosidic bonds may occur and in basic solutions, alkaline degradation may take place.

(b) Precipitation using a Specific Complexing Agent

Copper salts have been widely used as precipitating agents to fractionate mixture of polysaccharides. Fehling's solution is often used possibly because it is a common reagent in the laboratory.

Usually an excess of precipitant is added, and the insoluble polysaccharide-copper complex is removed by either filtration or centrifugation. The jelly-like complex is then decomposed by an alcoholic solution of acid or a chelating agent and the precipitate is washed until free from inorganic ions.

Chanda and co-workers[46] in studying the constitution of xylan from esparto grass found that fractional precipitation as the copper complex progressively reduced the total arabinose content of xylan, suggesting that the so-called "araboxylan" or "xyloaraban" might be, not a homogeneous polysaccharide, but a mixture of a true xylan with an araban of the type present in pectic materials.

1.5 Quantitative and Qualitative Determination of Sugars by Gas-Liquid Chromatography[73,74,75,76]

Gas liquid chromatography is a valuable supplement to existing analytical methods, because of its greater selectivity, speed and simplicity. As a result it has been widely used for the separation and estimation of carbohydrates and related polyhydroxyl compounds. Since these substances are not themselves sufficiently volatile for gas chromatography, suitably volatile derivatives have to be made, and these are commonly prepared from the sugars by methylation or acetylation.

The work of Sweeley, Bentley, Makita and Wells[77,78] is one of the most significant advances in carbohydrate chemistry in the decade. Using a simple experimental procedure[79] they prepared the polytrimethysilyl ethers of sugars and chromatographed these derivatives on several different supports with spectacular qualitative results.

The sugars can be converted into their derivatives quantitatively, but problems arise when separating monosaccharides by gas liquid chromatography. The formation of as many as four glycosides per monosaccharide resulting from anomeric and ring isomerisation, and each of which produce a peak on the chromatogram. In a complex sugar mixture, containing a number of monosaccharides, the multiplicity of peaks produced would prevent complete separation of all the peaks from one another, as a result, accurate quantitative determination cannot be achieved. However, this difficulty can be overcome by converting the monosaccharides into their corresponding alditols, by reduction

with sodium borohydride and subsequent acetylation.[80] This procedure eliminates the problem of multiple peaks since the alditols cannot anomerise,[81] and the method is applicable to the quantitative analysis of the sugars in hemicellulose B.

1.6 Quantitative Determination of Uronic Acids[82,83]

The decomposition of polyuronides by hot hydrochloric acid with the formation of furfural and carbon dioxide is a common method for the determination of uronic acids. However it is not specific, as true sugars and amino sugars both give rise to the same products under the same condition, and also it usually requires a few mg of material for several determinations

Meyer, Bloch and Chaffee[84] have reported that it is possible to determine the uronic acids in certain polyuronides after isolation and methanolysis by the naphthoresorcinol reaction. However, in the presence of proteins and true sugars the results are unsatisfactory.

Since 1929, Dische's method has been widely used for the microanalysis of uronic acids. The sugars are treated with concentrated mineral acids (sulphuric acid, hydrochloric acid) to yield mixtures of products which react with various organic substances (indole, diphenylamine, carbazole and SH compounds) to give colours. Different groups of sugars such as pentoses, hexoses, desoxypentoses and even individual sugars of the same group (e.g. mannose and glucose) show marked differences in the speed of the development of the colour and in its absorption spectrum. The absorption due to the carbazole reaction of true

sugars can be deducted when the nature of the sugar and its approximate concentration are known.

Anthrone sulphuric acid and orcinol sulphuric reactions[85] are able to be used for identification of sugars, uronic acids and sugar amines, as they all give a colour reaction. The anthrone sulphuric reaction is better than orcinol sulphuric, as it has been reported that for equal quantities of glucuronic acid and galactose, the absorbance of glucuronic acid to galactose is 0.44 for the reaction with orcinol sulphuric acid, and 0.10 for the reaction with anthrone sulphuric acid. As it is impossible to obtain reproducible results with the same solution of sugars, because the absorbance varies, it is therefore necessary to introduce an internal standard into a series of determinations.

The simplicity, specificity of the carbazole and anthrone sulphuric acid reactions, and also the low degree of interferences from other constituents, encourage the application of these methods to quantitative determination of uronic acids in polysaccharides.

1.7 Paper Chromatography[19,38,86]

Since the introduction of paper partition chromatography in 1944, it has been successfully applied to the separation of sugars and also for their quantitative determination. The method gives a rapid means of separation as well as giving a strong indication of their identities. Though the final proof of their constitution depends upon their separation and ident-

ification by determination of their physical constants and the formation of characteristic derivatives.

1.8 Methylation Procedures: Which Include Methods of Methylation, Acid Hydrolysis Qualitative and Quantitative Identification of the Monomers[68,88]

Methylation analysis is a very important method for structural investigation. The procedure involves the preparation of an exhaustively methylated polysaccharide followed by hydrolysis and identification of the resulting partially-O-methylated derivatives. The purpose of methylation is to achieve an etherification of all the free hydroxyl groups in the polysaccharide. Complete methylation can convert all unsubstituted hydroxyl groups to methoxyl groups and each of free hydroxyl group present in the methylated monosaccharides that is liberated by hydrolysis corresponds to a substituted hydroxyl of the original product. Therefore, the glycosidic linkage and the ring size can be deduced.

The ideal methylating agent is one which can rapidly introduce the theoretical number of methyl groups and at the same time, cause no degradation of the sugar. However, it is very difficult to get these reagents with such properties, therefore, several methylations with different reagents are generally used in order to obtain full methylation.

The original, classical methods of Denham and Woodhouse[89] and Haworth[90] are still widely used. The polysaccharide is treated several times with 25-30% aqueous alkali and dimethyl

sulphate. It had been reported that the use of more concentrated sodium hydroxide solution[91] and an increase of the operating temperature and even the replacement of sodium hydroxide by potassium hydroxide[92] would give a more satisfactory result. In order to obtain a complete methylation, the product is usually methylated several times and different methods are applied.

In general, polysaccharides are insoluble in the organic solvents, therefore they are usually methylated first with methyl sulphate and aqueous alkali. This yields a partially methylated product which is soluble in methyl iodide required for the Purdie procedure. The method of Kuhn and his co-workers is also frequently used. The partially methylated product is treated with methyl iodide and silver oxide under reflux. The advantage of this method is the ease with which the methylated product may be recovered. Other methods such as dissolving the polysaccharides in N,N-dimethylformamide[194] and then treating the solutions with methyl iodide or treating the polysaccharide in methyl sulphoxide with sodium hydroxide and methyl iodide[93] have also been used extensively.

It is very difficult to estimate the completeness of methylation, and the usual method is the determination of the methoxyl content and a comparison of this with a theoretical determination of the suspected repeating unit which is defined as the simplest repeating structure which defines the polysaccharide molecule

The impurities, non-polysaccharide materials such as salts, condensation products and lignin and also part of the polysaccharide that is less completely methylated can be removed

by fractional precipitation from the methylated polysaccharide. Mixtures of chloroform and light petroleum or ether are usually used for this purification.

Since the methylated polysaccharides are usually insoluble in water, a non-aqueous or only partially aqueous medium is used for the initial hydrolysis. Methanolysis is usually carried out by refluxing the methylated product with methanolic hydrogen chloride. Other methods including formolysis[95] and prehydrolysis[96] in concentrated sulphuric acid can also be used.

The aim of hydrolysis is to depolymerise the methylated polysaccharide to the monomeric state under the conditions to give the least possible degradation of the resultant monomers. Because of the great resistance of glycosidic linkages to acid hydrolysis, uronic acid residues always appear as methylated aldobiouronic acids in the hydrolysate.

The mixtures of methylated sugars are separated by partition chromatography on a cellulose column, or by adsorption chromatography on carbon-celite columns. The components are tentatively identified on the basis of their mobilities on paper chromatography and electrophoresis. Since these procedures do not differentiate between D and L enantiomorphs, the components of the hydrolysate are then converted into appropriate derivatives, which can be identified by their melting points and optical rotations. Certain components of polysaccharides can be identified by specific colour reactions without previous direct hydrolysis and separation.[38]

Gas liquid chromatography is another important method in methylation analysis, both as an aid in identifying individual methylated sugars and for their quantitative measurement. It has been found that methyl ethers of the methyl glycosides of the heat labile monosaccharides were sufficiently stable and volatile to be analysed and the fully methylated methyl glycopyranosides were successfully separated by gas liquid partition chromatography.[97]