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ISOLATION AND *A/B* CLASSIFICATION OF HEMICELLULOSES

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ABSTRACT

A novel method has been developed for isolation of hemicellulose from alkaline extracts of plants. The method combines dialysis and ion exchange and yields hemicellulose *A*, *B*, and *C* fractions. The conventional separation of hemicelluloses into these fractions by acidification of the alkaline extracts has been shown to be subject to unpredictable variability, which can be avoided by inclusion of a heat-treatment of the solution during the fractionation procedure.

A method of sub-fractionation of hemicellulose *A* has been proposed which involves dissolution in alkali and subsequent dilution with water.

INTRODUCTION

Hemicelluloses can be isolated from plant materials by extraction with alkaline solutions[1], and their recovery by neutralisation and precipitation with ethanol has led to a widely used conventional procedure originally established by O'Dwyer[2]. In this convention, hemicellulose *A* is the water-insoluble fraction which is precipitated on neutralisation of an alkaline extract to pH 4.5–5.0 with acetic acid; hemicellulose *B* is isolated by precipitation with 3 volumes of ethanol, and the remaining portion, usually discarded, is designated hemicellulose *C*.

Whistler and Gaillard[3] have drawn attention to the variety of procedures used to prepare and isolate hemicelluloses, and have expressed a need for uniformity in approach to enable comparative evaluation of hemicelluloses from different plant sources. Our attempts to correlate findings on tropical herbage with temperate species have been similarly hindered by lack of an established method of analysis among workers in this field. It is appreciated, of course, that the approach adopted by many workers will be dictated by the economics of man-hours and facilities available. Thus, schemes involving assessment by methods using acid-digestion[4] will provide faster and less costly surveys than those utilising solvent extraction[5]. The latter, however, has the advantage than it enables quantitative isolation of groups of polysaccharides which may be assessed for their individual characteristics.

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Gaillard's discovery that linear polysaccharides can be separated from branched polymers by precipitation as iodine complexes[6] has extended the possibility of comparisons between hemicelluloses from plants belonging to different plant families[7]. These studies have led to the proposal that pasture plant hemicelluloses consist of a mixture of the following major polysaccharide types: (*i*) Linear *A* (hemicellulose *A*), a water-insoluble hetero-xylan containing uronic acid and a small proportion of arabinose. (*ii*) Linear *B*, a more-soluble hetero-xylan containing much more arabinose and less uronic acid than linear *A*. (*iii*) Branched *B*, a water-soluble, highly branched polymer which, in addition to pentoses, is rich in galactose and uronic acids.

Both grasses and legumes contain representatives of these three fractions, and compositional differences which may reflect differences between monocotyledons and dicotyledons have been noted[7].

Measurement of the relative amounts and structural characteristics of these polysaccharides could provide a valuable means of assessing pasture quality, and work in our laboratory is developing along these lines. As a stumbling block to this approach, Gaillard[7] has noted that the separation of hemicelluloses into *A* and *B* fractions is not sharp and we have confirmed this observation. In fact, the separation by conventional methods has proved to be remarkably irreproducible, and this paper describes attempts to improve the experimental procedures.

EXPERIMENTAL AND RESULTS

Materials. — Hemicelluloses were prepared from guinea grass (*Panicum maximum*), spear grass (*Heteropogon contortus*), and sugar-cane hybrid varieties Q.57 and Q.63. A sugar-cane bagacillo from the latter was also separated into two fractions (predominantly pith and predominantly fibre) by differential flotation, and these were investigated separately.

Methods. — Prior to treatment with alkali, the plant materials were subjected to extraction with ethanol, cold and hot water, and ammonium oxalate, and delignified with chlorite[8]. Hemicelluloses were removed from the holocellulose (15 g) by rolling in polypropylene (use of glass vessels at this stage results in extraction of silica from the container and its ultimate isolation in the *C* fraction) centrifuge bottles in 10% aqueous sodium hydroxide (300 ml) saturated with nitrogen, for 3–12 h. After centrifugation and filtration through cloth, the hemicelluloses were recovered in two ways.

(*a*) *Conventional neutralization.* This involved acidification of the cold (5–10°) alkaline solution to pH 5 by the addition of 50% acetic acid. After standing for a minimum of 3 h, the solution was centrifuged at 2,500 r.p.m. for 20–30 min, and 3 volumes of ethanol were added to the stirred supernatant solution to precipitate hemicellulose *B*.

(*b*) *Resin neutralisation.* The combined alkaline extract and washings were transferred to alkali-washed dialysis tubing (Visking Co., Chicago) and neutralised by a procedure involving dialysis of the alkali from the extracted polymers and sorption of the sodium ions by continuous circulation through Amberlite IRC-50(H^+) resin. The features of the scheme are illustrated in Fig. 1.

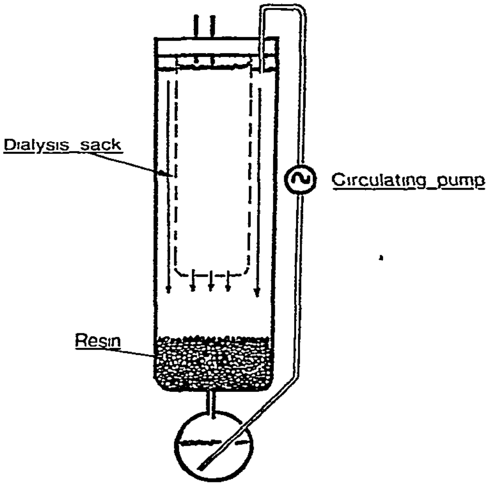

Fig. 1. Apparatus used for the recovery of hemicelluloses from alkaline extracts of plant cell-walls.

A suitable column was filled with water and resin layered on the bottom by sedimentation. In practice, a volume of resin (ml) numerically equal to the number of milli-equivalents of sodium ion used in the extraction proved sufficient for rapid and complete neutralisation. A dialysis tube was suspended from the top of the column of adequate capacity to allow for the considerable increase in solution volume during dialysis.

Circulation was effected by a pump at flow rates from 10–40 ml/min. This produced final pH values which varied from 5–7 in 6–8 h. It was found that, in the absence of stirring, a neutral pH front developed in the dialysis tube, moving from top to bottom and coincident with the partial precipitation of polysaccharide.

The dialysis tube was removed, drained, and washed, and the hemicellulose *A* was separated from the contents by centrifugation and dried to constant weight after solvent exchange with methanol. The centrifugate was concentrated to 300–500 ml, and hemicellulose *B* was recovered by drop-wise addition to 3 volumes of ethanol and centrifugation of the precipitate. This procedure was adopted when it was found that precipitation was frequently incomplete when ethanol was added to the concentrate.

The circulating solution from the column was combined with washings from the resin and produced insignificant amounts of hemicellulose *B* by precipitation with ethanol. Hemicellulose *C* was recovered from the resultant centrifugate by concentration and lyophilisation.

α-Cellulose was washed free from alkali with water and then acetone and dried to constant weight.

A comparison of the alkali extractives from spear grass and Q.63 sugar-cane pith and fibre neutralised by the two procedures is shown in Table I. Recoveries of α-cellulose in three analyses of dormant spear grass stem holocellulose were 45.3, 45.9, and 45.6% of the holocellulose dry-matter; 3% of the holocellulose dry-matter (recovered as hemicellulose *C*) was non-dialyzable.

TABLE I

HEMICELLULOSE EXTRACTION OF (*a*) SPEAR GRASS AND (*b*) Q.63 SUGAR-CANE PITH AND FIBRE EXPRESSED AS PERCENTAGES OF THE DRY WEIGHT OF HOLOCELLULOSE

Growth stage	*Plant section*	*Hemicelluloses*					
		Resin method			*Acetic acid neutralisation*		
		A	B	C	A′	B′	C′[a]
(*a*) Vegetative	leaf	0.6	30.4	17.2	1.3	30 8	17.6
(*a*) Early Seed	leaf	0	31.4	22.7	3.4	36.0	16 2
	stem	18.1	15.9	15.7	2.2	40.0	13.1
(*a*) Dormant	leaf	1.7	31.4	19.6	1.2	37.4	19.4
	stem	16.1[b] 17.6[b]	20.5[b] 20 3[b]	17.2[b] 16.5[b]	2.7	38.5	13.5
(*b*)	fibre	17.1	12.7	25.1	15 7	16.5	23.7
	pith	18.3	16.6	23.5			

[a] Hemicellulose *C* calculated by difference. [b] Duplicate experiments.

Dissolution and reprecipitation. — (*a*) *Hemicellulose* A. A sample of hemicellulose *A*, isolated by the conventional procedure from Q.57 sugar cane, was redissolved in 0.5M sodium hydroxide, and the solution was acidified with 50% acetic acid to pH 5.0; 32.6% was recovered as hemicellulose *A*, and 60.6% precipitated in 3 volumes of ethanol. The compositions of the fractions determined by gas–liquid chromatography[9] (g.l.c.) are shown in Table II.

TABLE II

THE COMPOSITION OF HEMICELLULOSE FRACTIONS PREPARED BY DISSOLUTION AND REPRECIPITATION OF HEMICELLULOSE *A* FROM Q.57 SUGAR-CANE

Sample	*Components (absolute % glycan)*				
	Ara	*Xyl*	*Gal*	*Glc*	*4-O-Me-GlcA*
Hemicellulose *A* (original preparation)	4.5	70.0	0.7	8.2	6.2
Hemicellulose *A* (reprecipitated with acid)	3.5	55.9	1.4	18.9	3 9
Hemicellulose precipitated with ethanol	5.0	83.2	0 4	1.1	3.9

When an attempt was made to redissolve in 0.5M sodium hydroxide a sample of hemicellulose *A* isolated by the conventional procedure from Q.63 sugar-cane, a portion did not dissolve and was recovered by centrifugation at 19,250 *g* at 5° for 30 min (fraction *1A*). A second precipitate was obtained by centrifugation after dilution of the solution with 5 volumes of water (fraction *2A*). A third fraction (*3A*) was recovered by acidification to pH 5.0 with 5M acetic acid, and fraction *4A* was isolated after precipitation with 3 volumes of ethanol. The weight recoveries, optical rotation in M sodium hydroxide at 30°, and composition (g.l.c.) are shown in Table III.

TABLE III

PROPERTIES OF HEMICELLULOSE *A* FROM Q.63 CANE AFTER DISSOLUTION AND REPRECIPITATION

	Orignal	*Fraction*			
		1A	2A	3A	4A
Recovery (%)		3.1	44.0	6.7	42.2
$[\alpha]_D^{30}$ (degrees)	−95.3	[a]	−105	−80.1	−100
Arabinose (%)	5.2	5.0	4.9	5.2	5.4
Xylose (%)	89.7	88.7	89.4	75.4	80.9
Galactose (%)	0.3	0.2	0	0.3	0
Glucose (%)	1.5	1.7	0.4	0.8	9.3
4-*O*-methylglucuronic acid (%)	4.7	3.7	3.6	4.0	4.9

[a] Not determined.

Hemicellulose *A* and fractions *2A* and *4A* were fractionally precipitated from 0.5M sodium hydroxide by the addition of ethanol. The amounts of the individual, precipitated fractions were determined by optical rotation after redissolving the centrifuged precipitate in M sodium hydroxide. The results are shown in Fig. 2. Fractions *2Aa* and *2Ab* were subsequently recovered by dialysis of the alkaline solutions and lyophilization; surprisingly, neither fraction was precipitated from solution during the dialysis. When hydrolyzed, they showed molar ratios for arabinose/xylose of 9.4/100 and 8.1/100 for *2Aa* and *2Ab*, respectively. 4-*O*-Methyl-D-glucuronic acid contents were 1.7 and 1.5%, respectively.

(*b*) *Hemicellulose* B. Hemicellulose *B*, isolated from Q.63 sugar-cane by the conventional procedure and dried by solvent exchange to methanol, was dissolved in water as follows. Gradual addition of a minimal amount of ethanol and continuous agitation with a glass rod ensured wetting of the solid, and this was followed by the slow drop-wise addition of water with efficient mixing. In one instance, the sample, after dissolution by the above procedure, was heated on a boiling-water bath for 10 min The pH remained at 6–7 throughout the heating period which produced a clear solution. In another analysis, dissolution was effected with 0.1M sodium hydroxide. The effectiveness of these procedures is shown in Table IV.

Free-boundary electrophoresis in borate and phosphate buffers indicated at least three components in the crude hemicellulose *B* preparation. Mobilities of 1.530×10^{-4}, 4.516×10^{-5}, and 2.968×10^{-5} cm^{3}sec^{-1}volt^{-1} were measured in borate buffer.

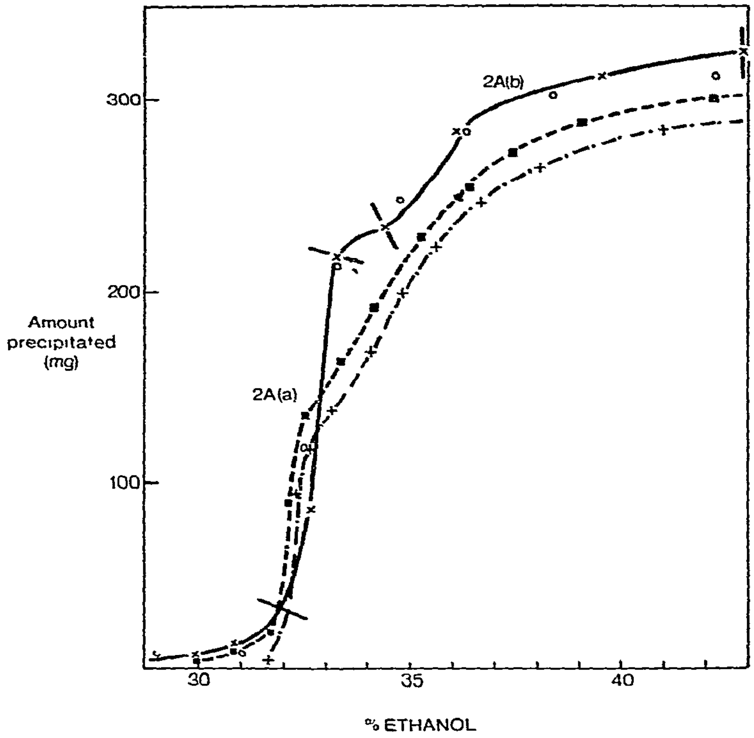

Fig. 2. Heterogeneity of hemicellulose *A* from sugar cane as revealed by fractional precipitation with ethanol. ■, Original hemicellulose *A*; +, fraction *4A*, ox, fraction *2A* (duplicate experiments)

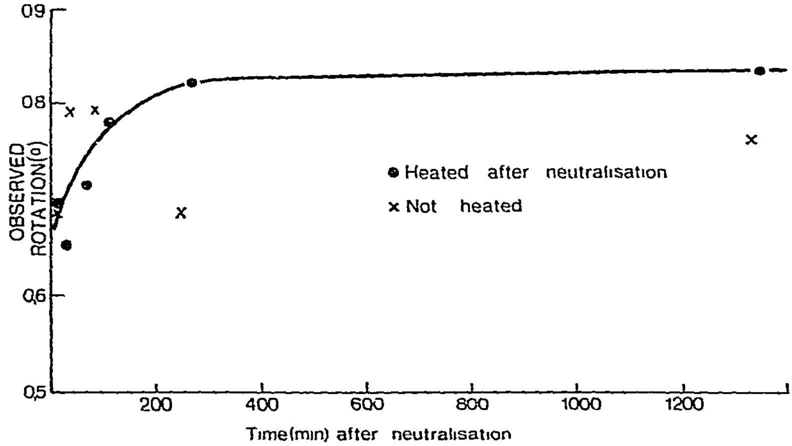

Fig. 3. Recoverable hemicellulose *A* from sugar cane by centrifugation of the neutralised extract.

Factors influencing the A/B *separation.* — A 10% sodium hydroxide extract (10 ml) from Q.63 sugar-cane holocellulose was cooled in each of four centrifuge tubes in an ice bath before drop-wise addition of 50% acetic acid (6 ml) to produce a

TABLE IV

SOLUBILITY OF HEMICELLULOSE *B* FROM Q.63 CANE, USING DIFFERENT METHODS OF DISSOLUTION

Solubility	*Treatment*			
	Cold water[a]		*Cold water and heat*	*0.1*M *Sodium hydroxide*
Insoluble (%)	63.7	61.7	31.4	51.8
Soluble (%)	34.1	37.0	64.8	42.3
Recovery (%)	97.8	98.7	96.2	94.1

[a] Duplicate experiments.

final pH of 5.0. The tubes were placed in a bath at 30° for 3.5 h before being spun at 17,300 *g* for 10 min at 20°. The separated precipitate was dissolved in M sodium hydroxide (15 ml) for determination of its optical rotation. Values of −0.834, −0.686, −0.715, and −0.825° were found.

The influence of the centrifugal field was investigated by using the preparative procedure outlined above, all centrifuge runs being for 10 min. The results are shown in Table V.

TABLE V

VARIATION IN THE RECOVERY OF HEMICELLULOSE *A* FROM SUGAR CANE AS A FUNCTION OF CENTRIFUGAL FIELD

R.C.F. (× g)	*Optical rotation (degrees)*
1085	−0.431
3020	−0.646
3020	−0.658
5900	−0.540
9750	−0.589
17,300	−0.718
17,300	−0.694
27,000	−0.613
48,200	−0.714

The influence of equilibration time after neutralization, before centrifugal recovery of hemicellulose *A*, was studied in two ways. In the first, the conventional neutralization procedure to pH 5 was effected as described above, the samples being held for appropriate times at 30°. In the second approach, neutralization to pH 7 was effected by the addition of 50% acetic acid (3 ml), and the sample was then placed on a boiling-water bath for 10 min prior to being left at 30° for appropriate times. The results are shown in Fig. 3.

DISCUSSION

All of the hemicelluloses used in this work are derived from grasses, and they consist predominantly of mixtures of xylans containing small proportions of 4-*O*-

methyl-D-glucuronic acid and L-arabinose with trace amounts of D-galactose. In addition, they also contain small proportions of glucose. In some cases, this sugar may be derived from short-chain cellulose molecules, and in this case it is concentrated in the *A*-fraction (*cf.* Table II). In other cases, the glucose may either be a part of the xylan molecule or present as a glucan, such as starch, since it is reprecipitated only on addition of alcohol (Table III).

The procedure which we have devised for simultaneous dialysis and deionisation of alkaline extracts containing hemicellulose is simple and is claimed to be more reproducible than the conventional neutralisation with acetic acid. In addition, it permits the facile examination of the hemicellulose *C* fraction which is commonly discarded because of the difficulty of removing large amounts of sodium acetate in the conventional procedure. In all of the samples which we have examined, however, the *C*-fraction has a low carbohydrate content and high ash content (e.g., 2% and 40%, respectively[8]) and has been mostly of low molecular weight (and therefore dialysable). We conclude that this fraction consists predominantly of degradation products (derived mainly from lignin but also possibly to some extent from oxidative and alkaline degradation of polysaccharides) together with sodium silicate (derived from silica in the grasses).

The results in Table I indicate that our resin method of *A*/*B* fractionation gives reproducible results. They also show that the relative yields of the *A* and *B* fractions may differ widely between the conventional and the resin methods of isolation. These differences are very marked in the samples of spear-grass stem which gave very little *A* fraction by the conventional method, but a considerable *A* fraction (and correspondingly less *B* fraction) by the resin method. Such differences did not occur with sugar-cane hemicelluloses, and we are at present unable to explain them in the spear-grass samples. In view of these differences, we were concerned to investigate the factors influencing the precipitation of the *A*-fraction from solutions. When a conventional hemicellulose *A*-fraction was redissolved in alkali and reneutralised with acetic acid, only one-third of the original sample was re-precipitated as hemicellulose *A*, suggesting that in the original isolation of this fraction a factor such as ionic strength was operative in causing excessive precipitation. The observation, however, suggested a possible procedure for sub-fractionation of the *A* fraction, which has always been difficult to purify because of its limited solubility. The *A* fraction from a sugar cane was therefore redissolved in 0 5M sodium hydroxide, and the solution was diluted with water to produce a precipitate. Further subfractions were obtained by neutralisation and by addition of ethanol, and their composition is shown in Table III. The fraction produced on dilution of the alkaline solution with water has been shown[10] to be relatively homogeneous by ultra-centrifugation, and it is probable that the above procedure will prove generally useful for purification of hemicellulose *A*-fractions. Fractions 3 and 4, which were precipitated on acidification and addition of alcohol, respectively, showed an increase in the ratio of arabinose to xylose, and in this respect they tend to resemble the hemicellulose *B*-fraction. The glucose content also increases in fraction 4, but this is probably due to a contribution from a chemically distinct glucan. Thus, the major factor so far detected as being responsible for the limited solubility of the *A* fraction is a rather lower content of arabinose residues.

The relative yields of *A* and *B* fractions in the conventional procedure are generally irreproducible (*cf.* Ref. 7) and, for example, could not possibly form the basis of a reliable system of chemical taxonomy. Thus, we have found, in carefully duplicated experiments, variations of up to 10% of the arithmetic mean yield of the *A* fraction. This variability is not due to variation in centrifugal field in separation of the fractions (Table V), but appears to be associated with variation in the extent of dispersion or solubilisation of the hemicellulose molecules, and most of this type of irreproducibility can be obviated by a simple heat-treatment (Fig. 3). We propose to consider the mechanism of this effect in more detail by study of the physical properties of the hemicellulose solutions.

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