Characterization of Hemicelluloses in Tobacco Stems

Harry Jacin and R. J. Moshy

A method for the isolation and partial characterization of hemicelluloses in tobacco stems has been developed. The carbohydrate composition of a hemicellulose fraction extracted with 5% KOH consists of a xylan, to which other carbohydrates are attached. Some of the lignin in tobacco stems is probably chemically bound to the hemicellulose. Gel-permeation chromatography has shown that some of the hemicellulose in tobacco consists of mixed molecular weight materials, which can be fractionated on a Sephadex G-150 column into a relatively large molecular weight substance (70,000 to 100,000) and one of smaller molecular weight (6000 to 15,000).

The importance of reconstituted tobacco leaf in cigar and cigarette manufacturing practice has resulted in an increased utilization of tobacco stems. The trend toward a reconstituted tobacco sheet free of non-tobacco additives requires a thorough knowledge of the carbohydrate constituents in the tobacco, since some of them serve as structural components of the sheet while others act as the necessary adhesives. There are few reports in the literature on the hemicellulose in tobacco (Mizuno and Kimpyo, 1957; Nagasura, 1954, 1956), and nothing on the hemicelluloses in tobacco stems.

The objectives of this study were to isolate the hemicelluloses from tobacco, to resolve them partially into chemical types, and finally to characterize the hemicelluloses with respect to their carbohydrate composition and molecular weight.

EXPERIMENTAL

Fractionation. The fractionation procedure used to isolate the hemicelluloses from tobacco is presented in Figure 1.

The procedure up to the delignification step has been described (Jacin *et al.*, 1967).

Two delignification methods were used in parallel: the sodium chlorite–acetic acid method of Wise *et al.* (1946) and the Angell and Norris (1936) procedure where the material was refluxed with 50% ethanol containing 1% NaOH.

In the sodium chlorite procedure the pectin-free tobacco representing 8.635 grams of original stems was suspended in 160 ml. of water. To the mixture were added 1.5 grams of sodium chlorite and 10 drops of glacial acetic acid, while nitrogen was being bubbled through the mixture. The operation was carried out in a hood. The passage of nitrogen was continued and after an hour the same quantities of reagents were added to the mixture. After another hour the addition of reagents was repeated. The mixture was left overnight in the hood. A well-bleached tobacco sample was dialyzed against running water for 24 hours. The material in the dialysis bag was filtered on a Büchner funnel. The filter cake was suspended in 200 ml. of 5% KOH solution and stirred magnetically at room temperature for one hour. The mixture was filtered through a Büchner funnel into a flask containing sufficient acetic acid to neutralize the available KOH and to make the filtrate acidic. The filtrate obtained was mixed with 2 volumes of absolute ethanol, stirred, and left in the refrigerator overnight. The filter cake was suspended in a 10% KOH solution and treated in the manner described above for the 5% suspension. The filter cake treatment was subsequently repeated with 20% KOH. The alcohol solution was removed from the precipitates by decantation. The precipitates were transferred into dialysis tubing and dialyzed against running water for 24 to 48 hours. The contents from the dialysis tubing were concentrated under vacuum and freeze-dried.

In the alternate delignification procedure the pectin-free tobacco was suspended in 200 ml. of 50% ethanol solution containing 1% NaOH. The mixture was refluxed for 12 to 16 hours and filtered on a Büchner funnel, and the filter cake was washed several times with 50% ethanol solution. The washed filter cake was treated with 5, 10, and 20% KOH, in a manner described above. The effect of the delignification procedure on the yield of hemicellulose was noted only in the case of the hemicellulose fraction extracted with 5% KOH. The 10 and 20% KOH extracted materials were unaffected. The 5% KOH extracted hemicellulose yield was higher by about 0.5% when the sodium chlorite–acetic acid delignification procedure was used. The hemicellulose yields from a number of tobacco stem types are shown in Table I. The sodium chlorite–acetic acid delignification procedure was used on these stems.

Thin-Layer Chromatography of Sugars. Samples of the isolated hemicellulose (0.1 to 0.3 gram) were hydrolyzed in 0.2*N* HCl at 100° C. for 16 hours. Aliquots of the hydrolyzates were spotted on silica gel G plates (Jacin and Mishkin, 1965). The development was carried out in 1-butanol–acetic acid–water (5:4:1). The sugars were made visible by spraying the plates with a 1-naphthol sulfuric acid reagent followed by heating. Figure 2 shows a photograph of a plate containing unhydrolyzed and

American Machine & Foundry Co., 689 Hope St., Stamford, Conn. 06907

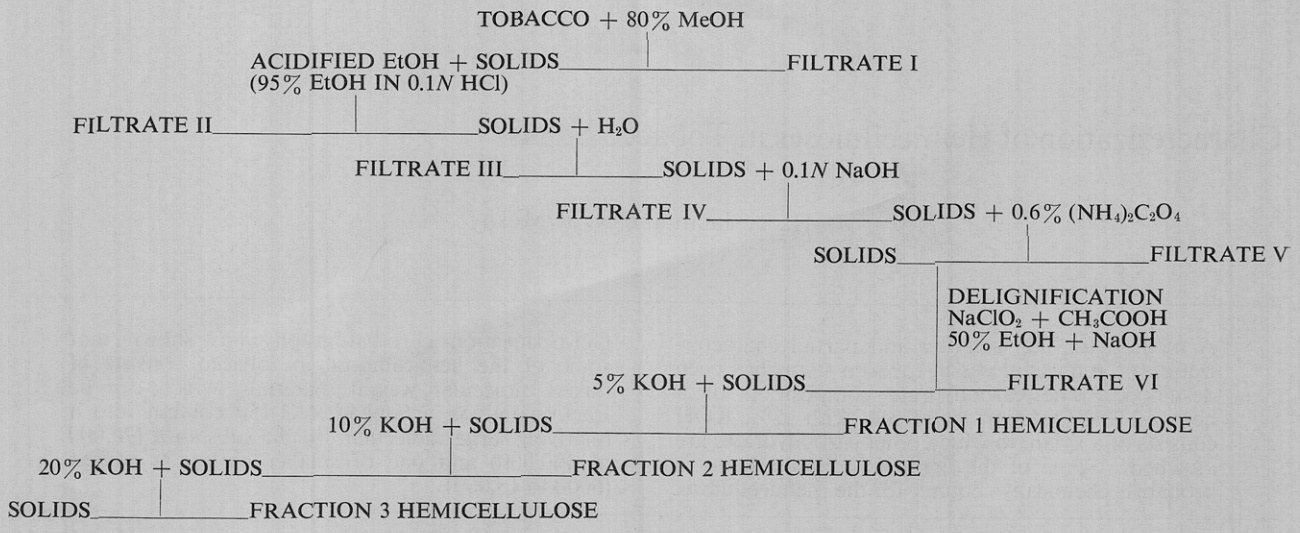

Figure 1. Isolation of hemicelluloses from tobacco

Table I. Hemicellulose Yield

Tobacco	% of KOH Solvent Used to Extract Hemicellulose	% of Hemicellulose
Bright stem	5	5.3
	10	5.2
	20	2.0
Havana seed leaf	5	4.1
	10	3.2
	20	1.6
Manila stem	5	6.4
	10	3.4
	20	1.8
Sweated Manila stem	5	6.0
	10	6.1
	20	3.0

hydrolyzed samples of hemicellulose isolated from Maryland stems. The delignification was carried out by the sodium chlorite–acetic acid method.

The ethanol sodium hydroxide procedure gave a hemicellulose, fraction 1, which contained xylose only, whereas the fraction 1 hemicellulose obtained by the sodium chlorite–acetic acid delignification method contained glucose and xylose, and in some tobaccos, also arabinose. Both delignification procedures gave hemicelluloses of similar carbohydrate composition for the two other hemicellulose fractions. A photograph of a thin-layer chromatography plate containing the acid hydrolyzate of a fraction 1 hemicellulose is shown in Figure 3. Gas chromatographic tests confirmed the TLC findings.

Gas Chromatography of Sugars. The determination was carried out on a Perkin-Elmer 800 gas chromatograph using a flame ionization detector (Sweely *et al.*, 1965). The conditions were as follows: The column was stainless steel 20 feet long by 1/8-inch o.d. The coating was Carbowax 20 M terminated with terphthalic acid on a Chromosorb-P (60- to 80-mesh) support. The column temperature was 180° C. The injection block temperature was 340° C. The nitrogen flow was 60 ml. per minute. The determinations were made isothermally. A Honeywell recorder of 1-mv. sensitivity and 2 seconds' full scale deflection was used. The chart speed was 1/2 inch per minute. The peak areas were determined with a planimeter.

An aliquot of the hemicellulose hydrolyzate (5 ml.) was evaporated to dryness under nitrogen. To the dry material was added 1 ml. of trimethylsilyl reagent (TRISIL, Pierce Chemical Co., Rockford, Ill.). After 15 minutes at room temperature aliquots of the mixture were injected into the gas chromatograph. The ratio of the sugars was calcu-

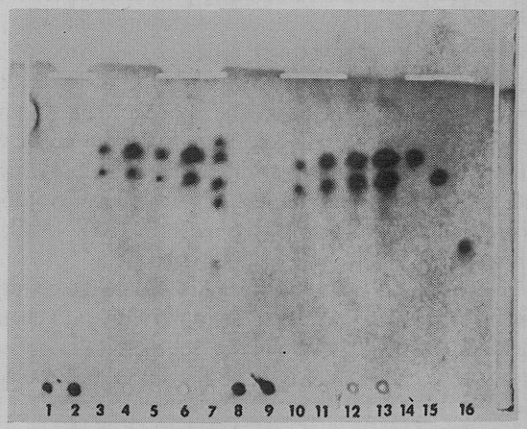

Figure 2. Tobacco hemicelluloses and their hydrolyzate products

1,2. Maryland stem hemicellulose fraction 1
3,4,5,6. Hydrolyzate of above hemicellulose showing glucose and xylose
7. Mixture of known sugars in ascending order, galacturonic acid, galactose, glucose, xylose, rhamnose
8,9. Maryland stem hemicellulose fraction 2
10,11, 12,13. Hydrolyzate product of above material showing glucose and xylose
14. Xylose
15. Glucose
16. Galacturonic acid

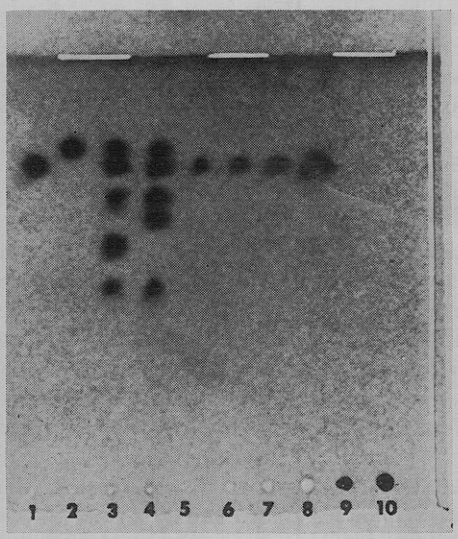

Figure 3. Carbohydrate composition of a fraction 1 hemicellulose where delignification was carried out by alcoholic sodium hydroxide

1. Xylose
2. Rhamnose
3. In ascending order galacturonic acid, sucrose, fructose, glucose, xylose, rhamnose
4. Galacturonic acid, galactose, glucose, xylose, rhamnose

5,6,7,8. Hemicellulose hydrolyzate, shows xylose only

9,10. Unhydrolyzed hemicellulose fraction

lated from the obtained peak areas. The results are shown in Table II.

Gel-Permeation Chromatography. The work was carried out on columns (2.5 × 50 cm.) of Sephadex G-150 (Pharmacia, Piscataway, N. J.) using 0.1*M* sodium chloride as eluent. The gel preparation and column packing were carried out according to instructions supplied by Pharmacia. Eluent was allowed to flow for 48 hours before the columns were calibrated with dextrans of known molecular

Table II. Ratio of Monosaccharides in Hemicellulose Isolated from Tobacco, in Per Cent

Tobacco	% of KOH Used to Extract Hemicellulose	Arabinose	Xylose	Glucose	Unidentified
Bright stem	5	2.5	74.0	23.5	...
	10	2.2	86.6	9.2	2.7
Havana seed leaf	5	3.7	49.5	43.4	0.4
	10	...	73.0	20.6	6.6
	20	...	90.0	7.0	3.8
Manila stem	5	1.8	54.0	43.5	0.4
	10	...	79.0	21.0	...
	20	6.2	70.0	20.0	3.8

weight (Anderson and Stoddart, 1966). Polysaccharide (about 20 mg.) dissolved in 0.5*M* sodium chloride (1 ml.) was applied to the column. Fractions collected from a 2-ml. siphon by an automatic collector were screened by the phenol–sulfuric acid method (Du Bois *et al.*, 1956). Elution volumes were estimated to the nearest milliliter from peak maxima. The calibration results are shown in Table III.

A plot of peak elution volume *vs.* molecular weight (on semilog paper) was linear in the molecular weight range of about 2000 to 110,000. The elution volume of "blue dextran" (mol. wt. 2×10^6) was taken as the void volume.

The elution patterns of a pair of fraction 1 hemicelluloses from a Sephadex G-150 column are shown in Figure 4. Each hemicellulose showed two peaks, indicating the presence of a relatively large molecular weight component and a smaller molecular weight material. The molecular weights of each component were determined from the plot of peak elution volume *vs.* molecular weight, and are listed in Table IV.

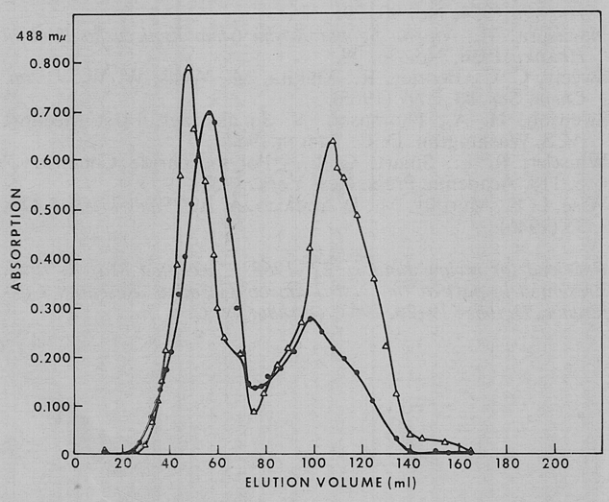

Figure 4. Elution pattern of hemicelluloses from a Sephadex G-150 column

● Sweated Manila stems
▲ Bright stems

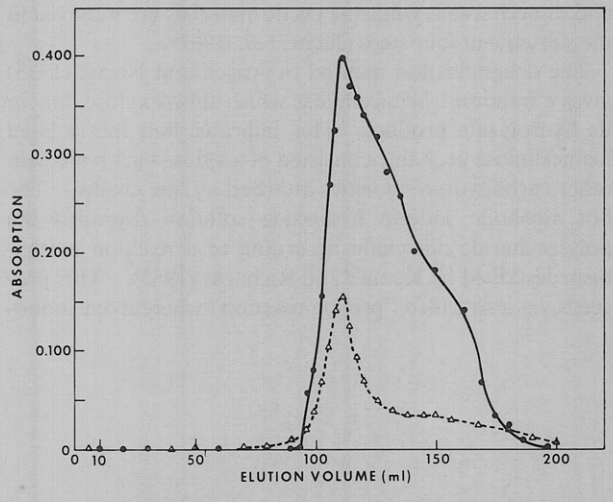

Figure 5. Elution pattern of a tobacco hemicellulose from a Sephadex G-75 column

● Test for sugars 448 mμ
▲ Test for lignin 280 mμ

Table III. Calibration of Sephadex G-150 Column with Dextrans of Known Molecular Weight

Substance	Molecular Weight	Elution Volume Peak, Ml.
Blue dextran	2×10^6	46.0
Dextran-150	1.5×10^5	46.0
Dextran-110	1.1×10^5	48.0
Dextran-80	8×10^4	53.0
Dextran-40	4×10^4	73.0
Dextran-20	2×10^4	88.0
Dextran-10	1.1×10^4	105.0
Sucrose	345	152.0

Table IV. Molecular Weights of Tobacco Hemicelluloses as Determined by Gel-Permeation Chromatography

Tobacco	Component 1	Component 2
Maryland stems	95,000	8,000
Havana seed leaf	110,000	5,500
Bright stems	75,000	11,000
Manila stems	95,000	8,000
Sweated Manila stems	110,000	9,000

Purity of Isolated Hemicellulose. The delignification procedure of Wise *et al.* (1946) has been shown to be nondestructive of carbohydrates (Whistler, 1953). However, the isolated polysaccharide retains 2 to 4% lignin. An examination of an isolated hemicellulose fraction on a Sephadex G-75 column revealed that the material contained a small amount of lignin which was determined spectrophotometrically (Kringstad and Ellefsen, 1964). The elution pattern is shown in Figure 5.

DISCUSSION

The fractionation procedure shown in Figure 1 gives a hemicellulose free of pectic material and other carbohydrates. The 80% methanol treatment removes mono- and disaccharides, while the pectic materials are removed in the subsequent four steps (Jacin *et al.*, 1967).

The delignification method of Angell and Norris (1936) gives a fraction 1 hemicellulose which shows xylose only in its hydrolyzate product. This indicates that the isolated hemicellulose probably consisted of a xylose backbone with other carbohydrate moieties attached as side chains. The hot alcoholic sodium hydroxide solution degraded the polysaccharide side chain according to a reaction mechanism described by Kenner and Richards (1953). This proceeds via a so-called "peeling reaction" wherein one monomer unit after another is peeled off from the reducing end of the side chain. In the case of glucose the monomers are converted into isosaccharinic acid. The monosaccharide ratio, as determined gas chromatographically, showed D-xylose to be the predominant monosaccharide in the hemicellulose isolated from tobacco.

Gel-permeation chromatography indicated that the isolated hemicellulose was of mixed molecular weight. One fraction had a degree of polymerization (DP) of about 500, while the other had a DP of about 30. In assessing the validity of these findings one must keep in mind that the column was calibrated with dextrans, which are linear polysaccharides, while the hemicelluloses investigated were most likely branched materials. The differences in shape of the hydrated molecules may affect the fractionation on a gel to a greater extent than the molecular weight (Kringstad and Ellefsen, 1964). The high DP value is, however, not entirely beyond the realm of possibility since DP values of 470 have been reported by Swenson (1962) for highly purified xylans.

The amount of lignin present in the hemicellulose is very small. This is evident from the absorbances in Figure 5. Although the amount of material used in the lignin determination was twice that used in the carbohydrate determination, the absorbancy remained low. The larger amount of material was used to obtain an absorbance in the range of at least 0.100. An attempt to remove the lignin completely is undesirable because of concomitant loss of carbohydrates under the more drastic conditions (Wise *et al.*, 1946).

LITERATURE CITED

Anderson, D. M. W., Stoddart, J. F., *Carbohydrate Res.* **2,** 104 (1966).
Angell, S., Norris, F. W., *J. Biochem.* **30,** 2155 (1936).
Du Bois, M., Gilles, K. A., Hamilton, J. R., Rebers, P. A., Smith, F., *Anal. Chem.* **28,** 350 (1956).
Jacin, H., Mishkin, A. R., *J. Chromatog.* **18,** 170 (1965).
Jacin, H., Moshy, R. J., Fiore, J. V., J. AGR. FOOD CHEM. **15,** 1057 (1967).
Kenner, J., Richards, G. N., *J. Chem. Soc.* **1953,** 2240.
Kringstad, K., Ellefsen, O., *Papier* **18,** 583 (1964).
Mizuno, T., Kimpyo, T., *Nippon Nogei Kagaku Kaishi* **31,** 297 (1957).
Nagasura, M., *Nippon Senbai Kosha Chuo Kenkyusho Kenkyu Hokoku* **1954,** No. 90, 79.
Nagasura, M., *Nippon Senbai Kosha Chuo Kenkyusho Kenkyu Hokoku* **1956,** No. 96, 48.
Sweely, C. C., Bentley, R., Makita, M., Wells, W. W., *J. Am Chem. Soc.* **85,** 2497 (1965).
Swenson, H. A., Thompson, N. S., abstract, 141st Meeting, ACS, Washington, D. C., March 1962.
Whistler, R. L., Smart, C. L., "Polysaccharide Chemistry," p. 118, Academic Press, New York, 1953.
Wise, L. E., Murphy, M., D'Addicco, A. A., *Paper Trade J.* **122,** 35 (1946).

Received for review January 22, 1968. Accepted May 1, 1968. Presented in part at the 21st Tobacco Chemists Research Conference, October 19–20, 1967, Durham, N. C.