Determination of the Cellulose Index of Tobacco

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A procedure is described for the determination of cellulose index on ground tobacco lamina, stems, and processed tobaccos. Soluble components are removed with hot dilute alkali, and the residue is treated with concentrated acid to hydrolyze the cellulose. The glucose generated is determined by an automated colorimetric method using *p*-hydroxybenzoic acid hydrazide (PAHBAH), and after correction for the uptake of water during hydrolysis, the results are reported as cellulose index. The method is empirical and not a true value for cellulose since the one-step extraction does not remove all noncellulosic sources of glucose and the colorimetric procedure used for the sugar determination is not specific for glucose. The relative standard deviation at the 2σ (95% confidence) level is ±5.0% for a bright lamina with a cellulose index of 7.1. Comparison of the cellulose index with cellulose values determined by the fractionation procedure of Bokelman et al. (1983) shows the ratio to be approximately 1.25 to 1 or the cellulose index to be about 25% higher than the true cellulose value.

Cellulose is the most widely distributed structural polysaccharide, constituting about half of the cell wall of wood and other plants (Fieser and Fieser, 1957). Tso (1972) observed that it is the principal constituent of cell wall and vascular tissue of tobacco. Recent increased interest in suspension cell culture and genetic manipulation of tobacco has renewed efforts to provide a rapid method for the determination of cellulose in tobacco materials. Gravimetric procedures involving time-consuming multistep extraction of noncellulosic components (Christy and Samfield, 1960; ASTM Standard Test Methods) were deemed impractical for screening large numbers of samples. Van Soest and Wine (1968) used permanganate to remove lignin from acid-detergent fiber (essentially lignin, cellulose, and insoluble minerals), then ashed the residue, and calculated the loss in weight as cellulose. Hellendoorn et al. (1975) determined the indigestible fiber content of foods gravimetrically after the enzymatic digestion of protein and starch. Updegraff (1969) published a procedure for cellulose in biological materials that required an acetic acid/nitric acid extraction of noncellulosic polysaccharides followed by dissolution of cellulose in 67% sulfuric acid and colorimetric determination with anthrone.

Recent reports by Bokelman et al. (1983) and by Ryan (1984) describe an improved method for cellulose in which the tobacco is extracted with aqueous ethanol, treated with a thermophilic enzyme to remove starch, and then sequentially extracted with dilute potassium hydroxide and an acid-detergent solution. The residue is hydrolyzed with sulfuric acid to convert the cellulose to glucose, which is determined as the alditol acetate by capillary gas chromatography. The value obtained is felt to be an accurate measurement of the cellulose content of the tobacco. The method described here is a more rapid, abbreviated adaptation of portions of the above procedures using a single extraction, hydrolysis of the cellulose in the residue, and determination of the sugar produced by an automated colorimetric procedure (Davis, 1976). This is not a true value for the cellulose content since the one-step extraction does not completely remove all noncellulosic sources of reducing sugars and the automated colorimetric method used for the sugar determination is not specific for glucose. The glucose generated is corrected for the uptake of water during hydrolysis, and the results are reported as cellulose index.

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The method is applicable to the analysis of cured lamina, stem, and reconstituted materials. It is not appropriate for freeze-dried green samples because of difficulty with the filtration after the alkaline extraction.

EXPERIMENTAL SECTION

Extraction and Filtration. A 1.000-g sample of tobacco material that has been ground to pass a 20-mesh screen is weighed into a 250-mL Erlenmeyer flask equipped with a 24/40 joint. Seventy-five milliliters of 0.5 N potassium hydroxide and a few drops of 1-octanol are added. Then the sample is boiled under reflux for 1 h. Immediately upon removal from the hot plate, 0.5 g of Celite analytical filter aid (Johns-Manville) is added, and the sample is swirled briefly and allowed to stand 2–3 min. It is then filtered rapidly while hot on a 45 mm diameter glass fiber filter pad (catalog no. 701, Virginia Lab Supply Corp., Richmond, VA) fitted into a Coors size 02 porcelain Büchner funnel, with normal laboratory vacuum. The flask and pad are washed thoroughly with hot distilled water until the pH of the washings is 7–8. The residue and pad are transferred carefully to a small disposable aluminum dish and dried in an oven at 100 °C for 2–3 h. The filtrate and washings are discarded.

Hydrolysis. The dried residue and pad are transferred quantitatively to a 500-mL Erlenmeyer flask equipped with a 24/40 joint. Five milliliters of 24 N sulfuric acid are added from a pipet, and the residue and pad are macerated thoroughly with a flattened glass stirring rod. The hydrolysis is allowed to proceed for 2 h at room temperature; then 163 mL of distilled water and a few small glass beads are added. The sample is boiled under reflux for 6 h, cooled, and filtered by gravity on Whatman No. 41 paper; the filtrate and washings are collected in a 250-mL volumetric flask. The filtrate is brought to pH 1.5–2 by the addition of 37 ± 1 mL of 3 N sodium hydroxide, and the flask is made to volume with distilled water.

Sugar Determination. The sugar produced by the hydrolysis is determined on a portion of the filtrate by the automated colorimetric method of Davis (1976) for reducing sugars. The percentage of glucose in the original sample is calculated, then multiplied by 0.9 [162.14 (M_r of anhydroglucose unit)/180.16 (M_r of glucose)] to give the empirical cellulose index.

RESULTS AND DISCUSSION

Extraction and Filtration. One-gram samples of ground, uncased bright lamina were used in the experimental work performed to find a rapid means of extraction

0021-8561/84/1432-1192$01.50/0

Table I. Precision of KOH Extraction and Comparison with Multiple Extraction Method

sample	% residue (DWB)[a] I, KOH extraction	II, multiple extraction	I/II ratio	RSD (2σ), %
bright lamina	16.6	12.2	1.36	±5.8 (n = 14)
bright stems	23.4	22.2	1.05	
burley lamina	23.8	20.0	1.19	
burley stems	30.6	28.6	1.07	
			$\bar{X}$: 1.17	

[a] DWB = dry weight basis—all residue, cellulose, and cellulose index values are reported on a dry weight of tobacco basis.

of interfering soluble components, such as sugars and pectins, and to find a rapid method of filtration. These samples provided a residual tobacco weight of 150–300 mg, suitable for hydrolysis and final determination of sugar content. The multiple extraction scheme of Bokelman et al. (1983) used boiling 0.1 N potassium hydroxide under reflux in the third step of the procedure. For the work described here 75 mL of a more concentrated base, 0.5 N potassium hydroxide, were used in a single-step extraction under reflux. A few drops of 1-octanol were added to the solution to reduce foaming. A number of different vacuum filtration techniques were investigated, and a 45 mm diameter glass fiber filter pad, fitted into a size 02 porcelain Büchner funnel, was selected as the best means for achieving rapid filtration. The addition of 0.5 g of Celite diatomaceous earth to the hot solution, followed by 2–3 min of standing before filtration on the pad, allowed the filtration to be completed in 5–10 min. Using only 0.25 g of Celite lengthened the filtration time to about 30 min. It is important that the filtration be done while the solution is hot in order to keep the starch and soluble pectins from precipitating. Therefore, 0.5 g of Celite was used in all subsequent work. Washing the residue with hot distilled water until the pH of the washings was 7–8 completed the filtration procedure.

The residue was transferred to a disposable aluminum dish and dried in a convection oven at 100 °C for 2–3 h to remove all the water so it would not dilute the acid used for the hydrolysis. While it is not necessary to determine the residue weight for the routine analysis, the dried residues were weighed in order to determine the precision of the extraction and to compare it with the multiple extraction of Bokelman and Ryan (1983) after the 0.1 N potassium hydroxide step. Correction was made for the tare weight of the pad and aluminum dish and also for the added Celite. Results for the bright lamina and stems and burley lamina and stems are shown in Table I. The precision of ±5.8% RSD at 2σ, shown for bright lamina, is acceptable for the procedure described here. It is apparent that more materials are extracted from lamina by the multiple treatments than by the single base treatment, but the values for stem are comparable by the two procedures. Additional tests revealed that reconstituted tobaccos made by two different processes contained residue percentages of 25.4 and 27.4. Values for expanded bright lamina and stems differ very little from their unexpanded counterparts (18.8 vs. 16.6 for lamina and 23.4 for both expanded and unexpanded stems).

Hydrolysis. The sulfuric acid hydrolysis procedure of Ryan (1984) required 1 mL of 24 N sulfuric acid/100 mg of sample. However, the greater bulk of the residue from the extraction described here, which included a glass fiber pad and 0.5 g of Celite as well as 150–300 mg of tobacco residue, made it necessary to use 5 mL of acid for the hydrolysis. Even with the larger volume of acid, magnetic stirring did not allow the acid to contact all of the sample, so a flattened stirring rod was used to macerate the residue and ensure that all of the tobacco residue was in contact with the acid. The addition of water to bring the normality to 0.72 and subsequent boiling for 6 h under reflux were done as described by Ryan (1984) to deesterify the hydrolyzed glucose.

Filtration to remove solid material, addition of base to adjust the filtrate to a pH of 1.5–2, and adjustment to a volume of 250 mL provided a solution that was then compatible with the automated equipment for total reducing sugars by the method of Davis (1976) using *p*-hydroxybenzoic acid hydrazide (PAHBAH).

Sugar Determination. The total reducing sugars were determined in the hydrolysate by using the specified glucose–fructose (1:1) standards made in 5% acetic acid and also a series of glucose standards made in the sulfuric acid–sodium hydroxide solution identical with the prepared hydrolysate. The results based on glucose standards in the H_2SO_4–NaOH solution were about 10% higher than those vs. the glucose–fructose standards in acetic acid. It was decided, in the interest of simplicity, to use the glucose–fructose standards for the analysis so that the routinely operated automated autoanalyzer reducing sugar channel could be used without modification. Also, the results using the sugar–acetic acid standards were nearer the more accurate cellulose values reported by Bokelman and Ryan (1983). However, the term cellulose index was chosen to differentiate the simplified procedure from the cellulose procedure of Bokelman et al. (1983).

Precision and Stability Studies. A precision study of the method was made by using ground, uncased bright lamina on four different days. Interspersed with the bright lamina, samples of various other tobaccos were analyzed to measure the consistency of the cellulose index estimates for different products (Table II).

The stability of the hydrolysate was investigated by using the series of samples from day 1 of the precision study. Portions of the solutions were analyzed on the automated reducing sugar channel on 5 different days. The samples were stored in a refrigerator at 5 °C between runs. Table III gives the sugar values in milligram per

Table II. Precision Study of Bright Lamina and Cellulose Index of Various Tobaccos

sample	n	cellulose index (DWB) range	mean	RSD (2σ), %
bright lamina	10	6.8–7.5	7.1	±5.08
bright stem	2	14.8, 14.8	14.8	
burley lamina	2	8.3, 8.3	8.3	
burley stem	2	18.6, 19.6	19.1	
reconstituted tobacco A	2	14.6, 15.3	15.0	
reconstituted tobacco B	2	10.8, 10.3	10.6	
expanded stems	2	14.4, 14.4	14.4	
expanded lamina	2	8.0, 7.8	7.9	

Table III. Stability Study of Hydrolysates for Sugar Analysis (Results Expressed as mg/mL Sugar)

sample	days of storage at 5 °C 1	2	3	6	8	mean
bright lamina	0.28	0.30	0.30	0.30	0.29	0.29
bright lamina	0.28	0.30	0.30	0.30	0.28	0.29
bright lamina	0.29	0.30	0.30	0.30	0.29	0.30
bright stems	0.60	0.62	0.63	0.60	0.61	0.61
burley lamina	0.34	0.35	0.36	0.34	0.34	0.34
burley stems	0.76	0.78	0.80	0.76	0.77	0.77

Table IV. Comparison of Cellulose Index with Cellulose Values

sample	I, % cellulose	II, cellulose index	I/II ratio	cellulose index × 0.8
bright lamina	5.9	7.1	0.83	5.7
bright stems	11.7	14.8	0.79	11.8
burley lamina	6.4	8.3	0.77	6.6
burley stems	15.4	19.1	0.81	15.3
			$\bar{X}$: 0.80	

milliliter for the various tobacco samples and demonstrates the stability of the solutions over a period of at least 8 days.

Comparison of Methods. The cellulose index values obtained by the procedure described here were compared with the cellulose values reported by Bokelman and Ryan (1983) for the same four samples. The cellulose value can be closely approximated by taking 80% of the cellulose index. Data are given in Table IV.

CONCLUSIONS

A relatively rapid and simple procedure has been developed for the cellulose index of various cured tobacco materials. The RSD at 2σ is ±5.0% for a ground, uncased bright lamina with a cellulose index of 7.1. The cellulose index values are approximately 25% higher than the cellulose values for samples analyzed by the fractionation method of Bokelman and Ryan (1983).

ACKNOWLEDGMENT

I thank A. B. Smiley and Shirley Langley for the analysis of many samples for total reducing sugars and for conducting the stability study of the hydrolysates.

Registry No. Cellulose, 9004-34-6.

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Received for review December 8, 1983. Revised manuscript received March 23, 1984. Accepted April 19, 1984.

Persistence and Fate of Ethylenethiourea in Tomato Sauce and Paste

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Tomato sauce was fortified (at 5.00, 0.50, or 0.05 ppm) with [4,5-^{14}C]ethylenethiourea (ETU), and samples were analyzed weekly for 3 months. Recovered products were separated by thin-layer chromatography, located by autoradiography and quantified by liquid scintillation counting. The toxicant was rather stable in this matrix over the course of the investigation. The rate of decomposition depended upon the level of spiking and on batch to batch differences in the matrix. Five decomposition products identified by TLC; they were 2-imidazolinyl hydrosulfate, 2-imidazolidinesulfonate, 2-imidazolidone, 2-(methylthio)imidazolidine, and 1-(2-imidazolin-2-yl)-2-imidazolidinethione. As a consequence of the persistence of ETU in this matrix, a quarantine or holding time for canned tomato products prior to distribution or sale would not represent a suitable decontamination technique of this commodity.

Ethylenethiourea (ETU, 2-imidazolidinethione) is a toxicologically significant decomposition product of ethylenebis(dithiocarbamate) (EBDC) fungicides. EBDCs are widely used in fruit and vegetable production to control a variety of fungal pathogens. These fungicides are not systemic; however, surface residues on fresh produce can be converted to ETU during normal industrial processing involving heat treatment (Watts et al., 1974; Newsome and Laver 1973; Philips et al., 1977). The fate of ETU in the sterile environment of a processed food is controversial. It has been reported (Han, 1977) that ETU, during a 4-week storage (at 1.0 or 0.1 ppm), decreased to 1% of the initial amount in pickles, 1–5% in apple sauce, 0.1–0.2% in tomato sauce, and 9–12% in spinach. In contrast, Uno et al. (1978) have reported that ETU in tomato puree was stable for up to 200 days. Efficient decontamination procedures are available for the removal of EBDC surface residues from tomatoes and green beans (Marshall and Jarvis, 1979; Marshall, 1982) prior to processing. However, if a suitable holding time for a potentially contaminated food could be defined, these preprocessing washes would not be necessary. Most processed fruits and vegetables are canned during a short 2–3-week period of each year and sufficient storage facilities are available. Thus, a suitable holding time would represent an attractive decontamination procedure provided that the degradation products were innocuous.

Abiotic transformations of ETU have been described as being mainly oxidative (Rose et al., 1980). Ethyleneurea (2-imidazolidone, EU) results directly from the oxidation of ETU or from partial oxidation (to sulfinate or sulfonate) followed by hydrolysis (Marshall, 1979). In contrast, ETU itself is quite stable to hydrolysis. The interaction of ETU with substrates that function as sulfur acceptors has been less well studied. Thiourea reacts with a variety of halogenated heterocycles (Boarland and McOmbie, 1951; Polonovski and Schmitt, 1950; Scott and Watt, 1937; Ro-

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0021-8561/84/1432-1194$01.50/0