Determination of Uronic Acids in Tobacco by Decarboxylation

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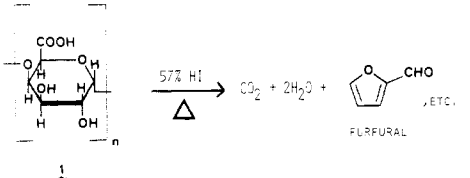
A simple, two-step procedure has been developed for measurement of the uronic acid content of tobacco. Following extraction with aqueous ethanol, a ground tobacco sample is decarboxylated with hydriodic acid. The liberated carbon dioxide is adsorbed in a sodium hydroxide solution, causing a decrease in conductivity, which is measured. The results are not dependent on whether the uronate polysaccharides are present as salts, esters, or acids. For samples of bright lamina, depending on the mean for a set of measurements, the relative standard deviation varied from 3.6% to 9.6%. The value for the pectin content of bright lamina estimated by using this uronic acid decarboxylation procedure varied by ≤9.3% from comparable values obtained by an involved gravimetric procedure and a pectinase–carbazole method.

Uronic acid constituents have been determined in suspension-cultured tobacco cells and their extracellular polysaccharides (Katō and Noguchi, 1976; Yamaoka and Sato, 1977; Mori and Katō, 1981; Akiyama and Katō, 1982) and in the laminas and stems (or midribs) of fresh green and flue-cured bright tobaccos (Eda et al., 1977, 1982; Eda and Katō, 1980; Bourne et al., 1967; Bokelman et al., 1983). It was found that the total content of the polymeric form of D-galacturonic acid in pectic fractions was much higher than the corresponding contents of 4-*O*-methyl-D-glucuronic acid in hemicellulosic fractions and D-glucuronic acid in the plant gum arabinoglucuronomannan (Mori and Katō, 1981; Eda et al., 1977; Eda and Katō, 1980).

Uronic acid constituents cannot be determined in the same manner as the neutral sugar components of polysaccharides since they are degraded to a significant extent when subjected to hydrolytic conditions. These constituents have most commonly been determined by colorimetric procedures such as the carbazole method (Bitter and Muir, 1962) and the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). However, all colorimetric procedures have low accuracy for the determination of uronic acids in complex polysaccharides due to the interfering color contributions from neutral sugars and other components.

In order to avoid the problem of spectrophotometric interferences, this study was undertaken to develop a method for the determination of uronic acids in tobacco based upon decarboxylation. This method is a modification of earlier procedures used for the determination of uronic acids in wood by Bylund and Donetzhuber (1968) and in dietary fiber by Theander and Åman (1979). As shown for α-1,4-linked anhydro-D-galacturonic acid (1) in



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pectin, a sample is decarboxylated with hydriodic acid. The liberated carbon dioxide is adsorbed in a sodium hydroxide solution, causing a decrease in conductivity, which is measured.

EXPERIMENTAL SECTION

Reagents and Solutions. All chemicals used were of analytical reagent grade. Deionized, charcoal-filtered water was used for the preparation of 30% potassium iodide, 30% cadmium sulfate, and 0.02 M sodium hydroxide. The sodium hydroxide solution was prepared from an analytical concentrate (J. T. Baker Chemical Co.). Distilled, unstabilized 57% hydriodic acid (Aldrich Chemical Co., Inc.) was stirred over amorphous red phosphorus (Fisher Scientific Co.) for 2 h or longer and then filtered through a sintered glass funnel of medium porosity just prior to use. The filtered solution had a pale amber color and was believed to contain a small amount of dissolved phosphorus.

D-Galacturonic acid monohydrate, D-glucuronic acid lactone, pectic acid (polygalacturonic acid, grade III, catalog no. P-3889), and methyl pectate [poly(galacturonic acid)methyl ester from citrus fruits, grade I, catalog no. P-9135] were obtained from the Sigma Chemical Co. The commercial sample of D-galacturonic acid monohydrate was determined by potentiometric titration to be of greater than 99% purity. The commercial sample of D-glucuronic acid lactone was recrystallized twice in absolute ethanol to yield a product with a mp of 176–177 °C [literature mp 177 °C (Weast, 1968)] and a specific rotation of $[\alpha]^{21}_D$ +18.8° (water, *c* 5.19) [literature value of $[\alpha]^{22}_D$ +19.4° (water) (Weast, 1968)].

The pectic acid sample was purified as follows. A 5.00-g sample of the commercial material was dissolved in 0.1 M NaOH, from which it was precipitated with 3 volumes of ethanol. The precipitate was dissolved in water, dialyzed against distilled water, passed through a Rexyn 101 column in H^+ form to remove cations, centrifuged, and freeze-dried. The final lyophilized sample had a mass of 3.13 g.

A sample of calcium pectate was prepared from the purified pectic acid. Pectic acid (0.1001 g) was dissolved in 22 mL of 0.1 M NaOH, from which it was precipitated with 90 mL of absolute ethanol. This precipitate was collected on a sintered glass funnel and then dissolved in 45 mL of deionized water. The pectinaceous material was reprecipitated by the addition of 20 mL of 1.0 M $CaCl_2$. This final precipitate was collected on a sintered glass funnel and washed with 90 mL of deionized water and 40 mL of acetone. The product was dried for 16 h at 40 °C in a vacuum oven to yield a white, flaky material (0.1005 g).

Tobacco Samples. One sample each of the following aged, uncased, cured tobaccos were used in this study: bright lamina, bright stems, burley lamina, and burley stems. The bright lamina came from a single grade of heavy, or bodied, flue-cured bright tobacco harvested at the upper midstalk position. The burley lamina was a blend that was representative of the burley used in current commercial cigarette products. All the tobacco samples were ground to pass a 20-mesh screen.

Instrumentation. The instrumentation for the uronic acid decarboxylation procedure is illustrated in Figure 1. The glass apparatus was obtained from Billerud AB, Säffle, Sweden. It consisted of a reaction flask, a condenser with

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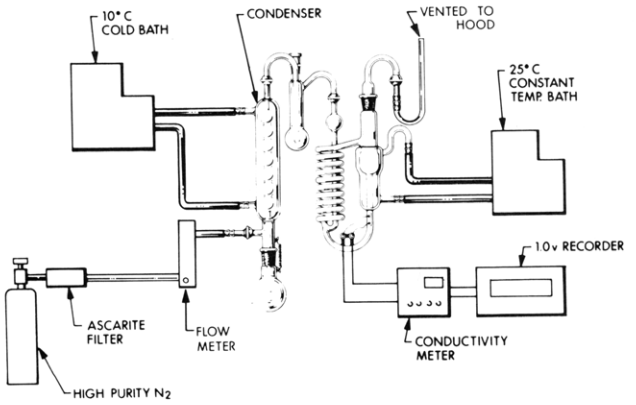


Figure 1. Instrumentation for the uronic acid decarboxylation procedure.

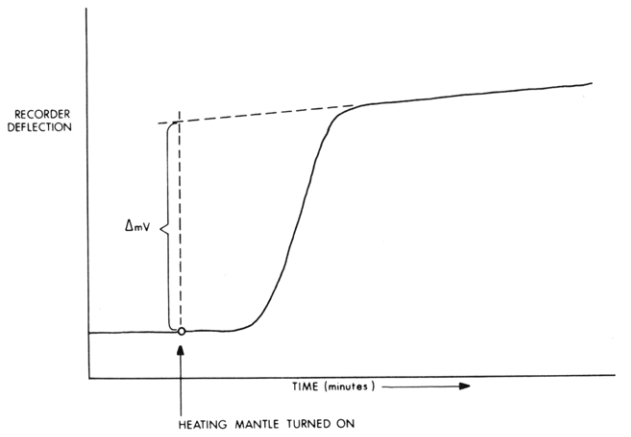


Figure 2. Illustration of the technique for measuring the uronic acid decarboxylation of an aqueous ethanol extracted tobacco sample.

an inlet for nitrogen flow, a washing flask, an absorption cell, and an Ingold electrode for conductivity measurement. An ultrapure-grade nitrogen cylinder, attached to an Ascarite gas purifier, was joined with polyethylene tubing to a Brooks flow meter (Emerson Electric Co.), which was attached to an inlet of the condenser that extended into the reaction flask. The condenser was connected to a water–glycerol circulating cold bath kept at ~10 °C. The absorption cell, which was vented to a hood, was connected to a water circulation bath maintained at 25 °C. The electrode was connected to a conductivity meter (Metrohm Model E518, distributed by Brinkmann), which in turn was attached to a Linear 1.0-V full-scale potentiometric recorder.

Analytical Procedure. Prior to performing uronic acid determinations on tobacco samples, it is recommended that they be extracted with aqueous ethanol to remove chlorogenic, oxalic, malic, and citric acids. In this laboratory tobacco samples are routinely extracted with 80% ethanol in a Soxhlet apparatus for 18 h as the first step in a general fractionation scheme (Bokelman et al., 1983). However, it is likely that shorter extraction times or other extraction conditions would also suffice to prepare tobacco samples for uronic acid determinations.

The cold bath and the 25 °C constant temperature bath are turned on in advance to attain equilibrium conditions. The conductivity meter is turned on ~30 min before starting a determination. The meter should be set on the 100 μS cm^{-1} range. A 27-mL solution consisting of 30% KI and 30% $CdSO_4$ in a 1:1 ratio is added to the washing flask, and 15.0 mL of 0.02 M NaOH is transferred to the adsorption cell. Next the EtOH-extracted tobacco sample (5–40 mg), 1 mL of deionized water, and 10 mL of 57% hydriodic acid (freshly filtered after stirring over red phosphorus) are added to the reaction flask. A heating mantle is attached to the reaction flask but not turned on at this time. Then the flow of nitrogen through the system is initiated and maintained at a rate of 60 mL/min. When a stable base line is obtained on the recorder, the heating mantle is turned on to start the decarboxylation reaction, which begins just before the mixture starts to reflux. The recorder draws a sigmoid curve (see Figure 2), taking about 40 min to complete a determination.

Recorder deflection was measured by the method of Bylund and Donetzhuber (1968). That is, during the determination the point on the curve is marked when the heating mantle is turned on. Later a vertical line is drawn through this point. Then a tangent is drawn from the end linear part of the sigmoid curve to intersect with the vertical line. The vertical distance between the point of intersection and the curve (ΔmV) is the quantity that is measured for the uronic acid determination.

RESULTS AND DISCUSSION

Hydriodic Acid Reagent. Controlling the composition of the hydriodic acid reagent was found to be critical to the reproducibility of this method. Hydriodic acid without a preservative soon darkens and generates a significant quantity of iodine, which can interfere with conductivity measurements. On the other hand, hypophosphorous acid, the preservative commercially used for hydriodic acid, decomposes upon heating to form phosphine, a gas that is poisonous, spontaneously flammable, and also capable of interfering with the conductivity measurements.

It was found that reproducible results could be obtained by treating commercial 57% hydriodic acid lacking a preservative with amorphous red phosphorus as described under Experimental Section. The filtered solution is thought to contain a small amount of dissolved phosphorus, since it actually turns to a lighter yellow color during the thermal decarboxylation process. Red phosphorus is relatively nontoxic unless it contains some of the white form as an impurity (Gosselin et al., 1976). White phosphorus may be detected by its characteristic garlic-like odor. In this laboratory, to minimize the possibility of exposure to toxic substances the red phosphorus used to treat the hydriodic acid is changed on a weekly basis and the adsorption cell is vented to a hood.

Calibration. A calibration curve (Figure 3) relating changes in conductivity in millivolts (ΔmV) as measured by the recorder to concentration of uronic acid in millimoles was prepared by using D-galacturonic acid monohydrate, pectic acid, and D-glucuronic acid lactone. It had been demonstrated previously that D-galacturonic acid and 4-*O*-methyl-D-glucuronic acid give similar molar conductivity changes (Theander and Åman, 1979). Potentiometric titration with 0.1 M NaOH was used to determine the exact content of carboxylic groups of the D-galacturonic acid monohydrate and pectic acid reference samples. D-Glucurnoic acid lactone could not be readily hydrolyzed under titration conditions. Therefore, for this latter sample the content of carboxylic groups was calculated based on its molecular weight, after ascertaining the purity of the recrystallized compound by measurement of melting point and specific rotation.

It is recommended that the calibration curve be checked on a regular basis, although no significant change may occur for a period of 6 months or longer. Whenever a calibration curve is being generated, it is advisable to first check the cell constant of the absorption cell with KCl standards (for example, as described in the instruction

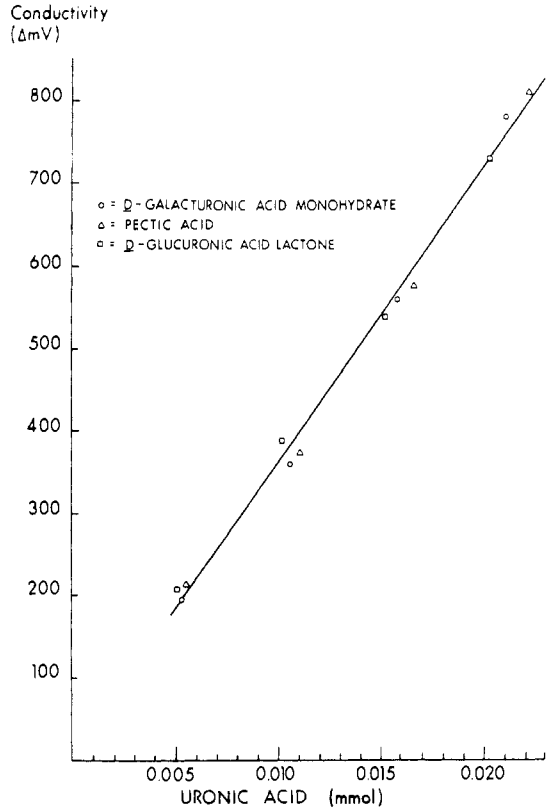


Figure 3. Typical calibration curve for the uronic acid decarboxylation procedure.

manual for the Metrohm Model E518 conductivity meter).

Precision and Detection Limit. Standard deviations were calculated on three sets of samples with ten determinations per set. Sample quantities for the three sets were selected to give a wide range of readout values. For 1.8-mg samples of D-galacturonic acid monohydrate from aqueous solutions, the mean value was 334.2 ΔmV with a relative standard deviation (RSD) of 9.6%. For individually weighed 3.0-mg samples of pectic acid, the mean value was 527.5 ΔmV with a RSD of 5.4%. A set of 15.0-mg samples of ethanol-extracted bright lamina gave a mean value of 728.3 ΔmV with a RSD of 3.6%.

On all of the above samples the standard deviation varied over only a small range from 26 to 32 ΔmV. Defining the detection limit as that quantity which causes a signal twice the size of standard deviation from the mean, the detection limit for hexuronic acid is 0.23 mg. This corresponds to 0.58% by weight of uronic acid in extracted tobacco based on a 40-mg sample. Since the extraction process removes non uronic acid constituents and a sample quantity greater than 40 mg could be used for determinations, the detection limit in unextracted tobacco is significantly less than 0.58% by weight of uronic acid.

Comparison of Responses of Calcium Pectate, Methyl Pectate, and Pectic Acid. A comparison of the molecular weights for the anhydro monomeric subunits of pectic acid (176) and calcium pectate (195) indicates that for samples of equal mass calcium pectate contains 90.3% equiv of the uronic acid content of pectic acid. Since a 3.0-mg sample of pectic acid gave a value of 540 ΔmV (average of five determinations), a 3.0-mg sample of calcium pectate would be expected to give a value of 488 ΔmV. Five decarboxylation determinations on 3.0-mg samples of calcium pectate were averaged to yield a value of 492 ΔmV, which is in good agreement with the expected value.

The commercial sample of methyl pectate (see Experimental Section) had a galacturonic acid content of 88.9%

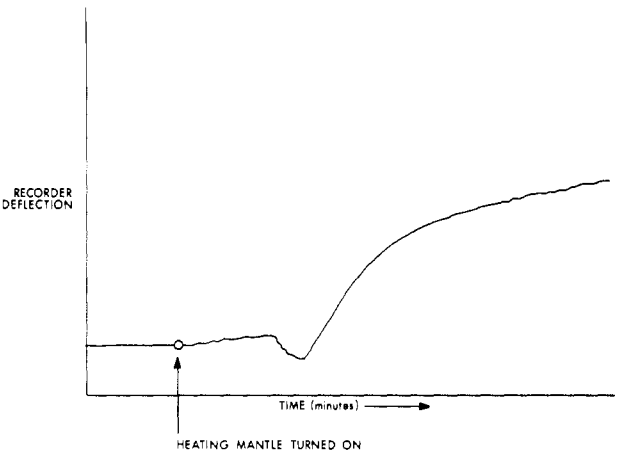


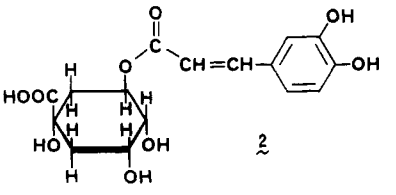
Figure 4. Typical response for citric, malic, or oxalic acid by the decarboxylation procedure.

and a methoxy content of 9.7%. Since a polymer consisting exclusively of methyl galacturonate residues would have a methoxy content of 16.3% (31/190), then 59.5% (0.097/0.163) of the galacturonic acid residues in this sample were present as methyl esters. Using this value and comparing the molecular weights for the anhydro monomeric subunits of pectic acid (176) and methyl pectate (190), it was calculated that for samples of equal mass the methyl pectate contained 95.5% equiv of the uronic acid content of pectic acid. Since a 2.0-mg sample of pectic acid gave a value of 350 ΔmV (average of five determinations), a 2.0-mg sample of methyl pectate would be expected to give a value of 334 ΔmV. Five decarboxylation determinations on 2.0-mg samples of methyl pectate were averaged to yield a value of 333 ΔmV.

On the basis of these experimental results, it was concluded that calcium pectate, methyl pectate, and pectic acid are all equally labile to decarboxylation under the conditions employed in this analytical procedure. Since the calibration curve (Figure 3) was prepared by plotting conductivity change vs. molar quantity of uronic acids, a molar value for uronic acid determinations is obtained that does not depend on whether the uronate polysaccharides are present as salts, esters, or acids.

Examination for Interference. It had been demonstrated by Theander and Åman (1979) that a series of neutral sugars, polysaccharides, proteins, and plant phenolic acids show only minor or no background degradation by this procedure. However, it was found that citric, malic, and oxalic acids do give a response by the decarboxylation procedure, as shown in Figure 4, although they do not produce a sigmoid curve. It is not known why a portion of the response curve produced by any of these acids has a negative deflection. A tobacco sample containing citric, malic, and oxalic acids may give a bimodal response curve, as shown in Figure 5, that still could be acceptable for the determination of uronic acids. However, these interfering compounds may be easily removed by extraction with aqueous ethanol as discussed previously.

Unlike the di- and tricarboxylic acids, chlorogenic acid (2) decarboxylates simultaneously with uronic acids so its



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presence would not be indicated by the shape of the

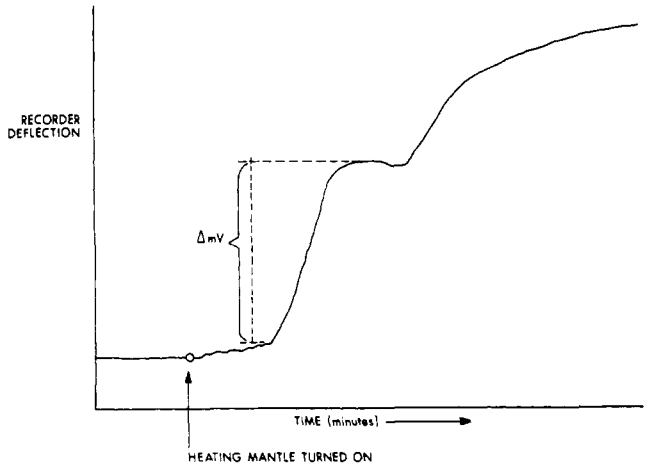

Figure 5. Bimodal response for an unextracted tobacco sample.

Table I. Interfering Compounds in Unextracted Tobacco Samples (All Values Expressed on a Percentage Dry Weight Basis)

compound	bright lamina	bright stems	burley lamina	burley stems
citric acid	0.7	0.5	5.3	0.9
malic acid	3.7	7.8	4.8	3.0
oxalic acid	1.3	1.4	2.9	1.9
chlorogenic acid	1.47	0.14	ND[a]	ND

[a] ND = none detected.

(sigmoid) response curve. Due to its high molecular weight the response of chlorogenic acid is only about half that of uronic acids on a weight basis. Fortunately, chlorogenic acid is also easily removed by extraction with aqueous ethanol.

The concentrations of interfering compounds found in the unextracted tobacco samples are listed in Table I. Citric, malic, and oxalic acids were determined by gas chromatography following conversion to their respective methyl esters (Harvey et al., 1970). Chlorogenic acid was determined by high-performance reversed-phase liquid chromatography using the procedure of Court (1977). No measurable quantity of chlorogenic acid was detected in the samples of burley tobacco.

Determination of Uronic Acids in Four Types of Tobacco. The tobacco samples were extracted with aqueous ethanol to remove interfering compounds. Decarboxylation values in millivolts were converted to millimoles of uronic acid by using the calibration curve (Figure 3). A residue weight for anhydrohexuronic acid of 0.176 g/mmol was utilized for conversion to weight of uronic acid. Finally, corrections were made for the weight loss caused by extraction and the moisture content of the starting material. The following values were obtained for percent of uronic acids in the unextracted tobacco samples on a dry weight basis: bright lamina, 9.6; bright stems, 11.1; burley lamina, 10.4; burley stems, 13.4.

Accuracy. The uronic acid content of a sample of bright lamina was determined to be 9.6% by this decarboxylation procedure. Values for the content of anhydro-D-galacturonic acid in pectin from bright tobacco have been reported that vary from 83.7% to more than 90% (Eda and Katō, 1980; Bourne et al., 1967). On the basis of this information and knowing that in tobacco D-galacturonic acid is by far the most prevalent uronic acid (Mori and Katō, 1981; Eda et al., 1977; Eda and Katō, 1980), it was estimated that the total pectin content of the bright lamina sample as measured by this procedure would be 10.7% [9.6%/0.9 ~ 10.7%]. For the sake of comparison with comparable tobacco samples, an involved gravimetric procedure (Phillips and Bacot, 1953) gave a value of 9.7% for "total pectic substances" and a pectinase–carbazole method (Norman, 1961; Dische, 1947) gave a value of 11.0% for pectin. The latter two values vary by ≤9.3% from the value for the pectin content of bright lamina estimated by using the uronic acid decarboxylation procedure.

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