Enzymatic Determination of Starch in Fresh Green, Lyophilized Green, and Cured Tobacco

Elisabeth T. Oakley

A procedure for the determination of starch in cured, fresh green, and lyophilized green tobaccos is described. The naturally present sugars are extracted with an aqueous ethanol distillate, and then the starch is solubilized in boiling buffer solution. The starch is converted to glucose enzymatically and the glucose is determined colorimetrically by a manual glucose oxidase method. Quantitative data are presented for starch in cured bright tobacco and in fresh and freeze-dried green bright tobaccos. Comparison is made of starch values obtained by a colorimetric method based on the starch–iodine complex. Accuracy of the method was established by repetitive extraction of residual tobacco after the completion of the procedure. Relative standard deviations at the 2σ level are ±12% on cured bright tobacco with 3.2% starch, ±14% on fresh, green tobacco with 15.2% starch, and ±3% on lyophilized green tobacco with 15.4% starch.

The starch content of tobacco is an important indicator of the maturity of the green leaf and is generally at its peak prior to the onset of the ripening stage (Bacon et al., 1952). The extent of the conversion of the starch to sugar during maturation and later during the curing process is known to influence leaf quality (Tso, 1972).

Methods found in the literature for the determination of starch in tobacco include boiling water extraction, with automated colorimetric measurment of a starch–iodine complex (Gaines, 1970), and preextraction with aqueous ethanol or methanol followed by dissolution in dilute perchloric acid and measurement of the starch–iodine colored complex (Rosa, 1971; Sensabaugh and Rush, 1972). Larsson et al. (1979) published an enzymatic method using amyloglucosidase, to hydrolyze starch from potatoes to glucose, and glucose oxidase for quantitation of the glucose produced. An adaptation of this procedure was made by Salomonsson et al. (1980) for analysis of barleys. A similar enzymatic method for starch in raw and cooked cereal has been included as an Official First Action by the AOAC (1980).

Difficulties experienced in our laboratories with accuracy and reproducibility of starch determinations, especially on lyophilized green tobacco, led to an investigation of the enzymatic method for our use. In this way, dependence on a standard of potato starch, or of starch extracted from the various types of tobaccos, is eliminated, and pure glucose is used as a standard. The starch value, then, can be considered absolute rather than relative, since it is not dependent on a non-tobacco starch or a tobacco starch standard with different composition (i.e., amylose:amylopectin ratio and/or solubility) (Kakie and Sugizaki, 1971; Gaines and Cutler, 1974) from that of the sample to be analyzed.

The tobaccos used for this work were uncased flue-cured bright tobacco of a bodied grade from the upper mid-stalk position and green (unripe) leaves from the middle stalk position of field-grown Coker 319 harvested at the "early button" stage of growth. Greenhouse-grown Coker 411 harvested at the early flowering stage was also analyzed.

EXPERIMENTAL PROCEDURES

Ethanol Extraction. A schematic representation of the experimental procedure is shown in Figure 1. An accurately weighed 300–500-mg sample of ground cured or ground lyophilized green tobacco is put into a 10 × 50 mm glass extraction thimble and extracted with 80% aqueous ethanol (v/v) in a micro Soxhlet extraction apparatus (Labglass, Inc.) for 18 h. For fresh green tobacco a larger Soxhlet apparatus or Goldfisch fat extraction equipment with 25 × 85 mm glass thimbles is needed to accommodate the greater bulk of the 3.5 g of cut pieces of turgid green leaf required to give 300–500 mg dry weight of tobacco.

The extracted residue is dried (100 °C, 1 h) to remove the ethanol, and then it is carefully removed from the glass thimble and transferred quantitatively to a glass-stoppered Erlenmeyer flask. The thimble and any glass wool used to hold the sample in place during the extraction are also put into the flask.

Enzymatic Conversion of Starch to Glucose. A volume of 0.1 M sodium acetate–acetic acid buffer (pH 5) sufficient to cover the residue and thimble is added to the Erlenmeyer flask. A few drops of Dow-Corning Antifoam "A" are also added to control frothing. The sample is boiled under reflux for 1 h to "solubilize" the starch.

The solution is cooled to <60 °C, and a thermophilic enzyme preparation, amyloglucosidase from Aspergillis niger suspended in 3.2 M ammonium sulfate (Boehringer Mannheim Biochemicals), is added to the flask at a rate of 1 μL/mg of tobacco sample. The flask is stoppered and incubated at 60 ± 1 °C for 16 h to effect conversion of the "solubilized" starch to glucose.

At the end of the incubation period, the sample is filtered by gravity on Whatman No. 541 paper into an appropriate-sized volumetric flask. The flask, residue, and filter paper are washed thoroughly with the pH 5 buffer, the washings are added to the volumetric flask, and the flask is made to volume with buffer.

Enzymatic Glucose Determination. The glucose-containing filtrate is diluted appropriately, if necessary, with buffer to contain 20–80 μg/mL glucose. Glucose is determined enzymatically by a modification of the method described in "Sigma Chemical Company Technical Bulletin No. 510" (1979) with their glucose oxidase kit, No. 510-A. One-milliliter aliquots of the diluted sample filtrates and a series of standard glucose solutions at 0, 20, 40, 60, and 80 μg/mL prepared in 0.2% benzoic acid are pipetted into separate Bausch & Lomb Spectronic 20 cuvettes. At carefully timed intervals of 30 s, 4 mL of enzyme color reagent (PGO enzymes and o-dianisidine hydrochloride) prepared in buffer at double strength (i.e., two capsules of PGO enzymes per 100 mL of buffer, rather than one capsule) from that recommended by the vendor are added

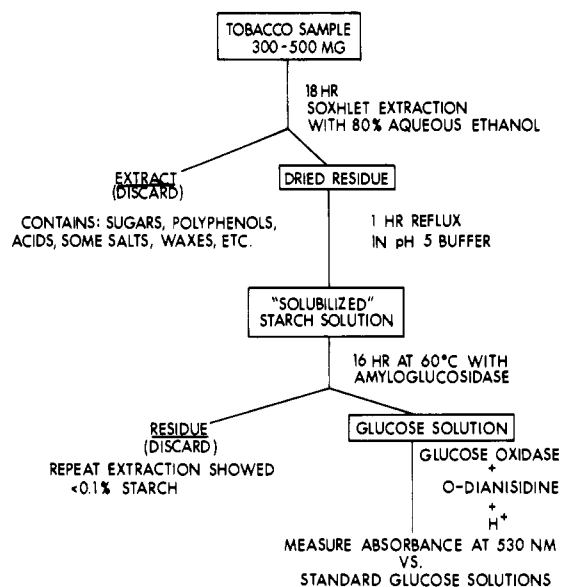Research Center, Philip Morris U.S.A., Richmond, Virginia 23234.

Figure 1. Schematic representation of the starch procedure.

to each cuvette. Immediately after the addition of reagent to *each* cuvette, it is mixed for 10–15 s on a Vortex Genie and then placed in a block heater at 37 °C. After *exactly* 20-min incubation the cuvettes are removed at 30-s intervals and 3 mL of 25% aqueous sulfuric acid (v/v) is added to each immediately. The contents are mixed well and the absorbance at 530 nm is measured within 30 min on a Bausch & Lomb Spectronic 20 spectrophotometer with the "0"-μg standard used as the reference. A standard curve is constructed of concentration in μg/mL glucose vs. absorbance, and sample concentration is determined from the standard curve. The percentage of glucose in the original weighed sample is calculated and then multiplied by 0.9 as shown below to obtain percent starch. The starch values are corrected to a dry weight of tobacco basis.

$$\% \text{ starch} = \\ \% \text{ glucose} \times \frac{162.14(M_r \text{ of anhydroglucose unit})}{180.16(M_r \text{ of glucose})}$$

$$\therefore \% \text{ starch} = \% \text{ glucose} \times 0.9$$

RESULTS AND DISCUSSION

Ethanol Extraction. A series of experiments established an 18-h Soxhlet extraction in 80% aqueous ethanol as the optimum conditions for complete extraction of naturally present sugars from flue-cured and green tobaccos with no loss of starch. Analysis of the residue by the reducing sugar procedure of Davis (1976) verified that all of the sugars had been removed. Analysis of the sample for starch by Gaines' method (1970) before and after the ethanol extraction showed little or no loss after correction for weight loss of the sample. Approximately 30–50% of the sample weight is lost.

In order to develop a viable routine analytical procedure, the ethanol extraction is performed on an accurately weighed sample and the sample is handled quantitatively throughout. This obviates the need to determine accurately the weight loss during the extraction for later correction to the original tobacco weight. The micro Soxhlet apparatus selected is ideal for the extraction of individual samples. The 300–500 mg of cured or lyophilized ground tobacco fits nicely into a 10 × 50 mm glass thimble and is held in place by a small wad of glass wool. The thimble is easily fitted into a 125-mL Erlenmeyer flask with a 19/22 inner standard taper joint. The flask in turn is compatible

with the condenser unit of the micro Soxhlet apparatus for reflux.

At the end of the 18-h extraction period the sample is left intact in the thimble and excess solvent is removed by vacuum. The remaining ethanol must be removed completely, generally by oven-drying at 100 °C, in order to prevent its inhibiting the enzyme activity during subsequent steps in the analysis.

The dried, extracted tobacco is carefully removed from the thimble and transferred to an Erlenmeyer flask. The thimble and glass wool are added to the flask also, since some of the tobacco adheres. Removal of the tobacco from the glass thimble permits better contact with buffer during reflux.
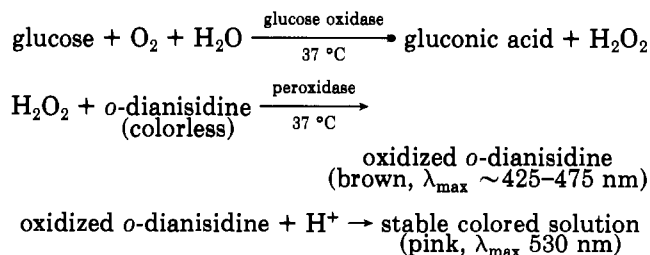
Fresh, green tobacco is treated in essentially the same way by using cut pieces of leaf about 10 × 10 mm in size, and, of course, larger Soxhlet apparatus and an Erlenmeyer flask large enough to hold a 25 × 85 mm thimble.

Enzymatic Conversion of Starch to Glucose. Reflux for 1 h in an adequate volume (generally 50 mL for the microapparatus and 150 mL for the larger apparatus) of 0.1 M sodium acetate-acetic acid buffer (pH 5) is sufficient to solubilize all of the available starch. Reflux for 2, 3, or 4 h produces essentially the same starch values as for 1 h. A second reflux in buffer of the residue from the 1-h reflux showed that 97–98% of the starch is removed in a single 1-h extraction. The addition of a few drops of Dow-Corning Antifoam "A" prevents foaming without affecting the final starch values.

The solution is cooled to <60 °C and the thermophilic enzyme preparation, amyloglucosidase, is used to effect hydrolysis of $\alpha(1$–$4)$- and $\alpha(1$–$6)$-glucan linkages to glucose ("Boehringer Mannheim Biochemicals Catalog", 1980–1981). Its use at 60 °C prohibits enzymatic activity of contaminating enzymes which might produce glucose as the end products from non-starch constituents. A ratio of 1 μL of amyloglucosidase to 1 mg of original tobacco is sufficient to completely hydrolyze the starch in samples containing up to at least 25% starch. A hydrolysis time of 16 h described by Salomonsson et al. (1980) is convenient and is efficient as shown by the lack of formation of a blue starch–iodine complex on addition of iodine solution to the hydrolyzed solution.

The sample solution is filtered on Whatman No. 541 paper to remove solid residue, washed, and made to an appropriate volume with buffer to contain 20–80 μg/mL glucose, which is the optimum range of the standard glucose curve. For high-starch samples, it is convenient to make two successive dilutions in order to avoid using large volumes of buffer.

Enzymatic Glucose Determination. The well-known reactions of glucose with glucose oxidase are shown here:

$$\text{glucose} + O_2 + H_2O \xrightarrow[37 \text{ °C}]{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \underset{\text{(colorless)}}{o\text{-dianisidine}} \xrightarrow[37 \text{ °C}]{\text{peroxidase}}$$

$$\underset{\text{(brown, } \lambda_{\text{max}} \sim 425\text{–}475 \text{ nm)}}{\text{oxidized } o\text{-dianisidine}}$$

$$\text{oxidized } o\text{-dianisidine} + H^+ \rightarrow \underset{\text{(pink, } \lambda_{\text{max}} 530 \text{ nm)}}{\text{stable colored solution}}$$

The glucose oxidase (PGO enzyme) is prepared in combination with the o-dianisidine chromogen in a single reagent designated enzyme–color reagent (ECR), by a modification of the instructions in "Sigma Chemical Company Technical Bulletin No. 510" (1979). Maximum sensitivity, linearity, and reproducibility of the standard curve are found by making the ECR at double strength

Table I. Comparison of Starch Values by Three Different Methods

	% starch (dry wt basis)		
sample	starch–iodine	PAHBAH	enzymatic
flue-cured bright	2.24	4.04	3.16
lyophilized green Coker 319 (field grown, 1974)	14.2	25.0	23.4
fresh green Coker 411 (greenhouse grown, 1981)		2.75	2.70
lyophilized green Coker 411 (greenhouse grown, 1981)		3.00	2.82
amylose from potato		93	92
commercial cornstarch		104	102

Table II. Precision of Enzymatic Starch Determination

	no. of determinations	% starch	σ	RSD (2σ), %
flue-cured bright	12	3.16	0.19	±12.2
lyophilized green Coker 319 (field grown, 1974)	12	23.8	0.58	±4.8
fresh green Coker 319[a]	12	14.5	1.20	±16.6
lyophilized Coker 319[a]	6	13.4	0.34	±5.1
oven-dried Coker 319[a]	6	6.10[c]	0.11	±3.7
fresh green Coker 319[b]	12	15.2	1.04	±13.7
lyophilized Coker 319[b]	6	15.4	0.23	±3.0
oven-dried Coker 319[b]	6	15.0	0.83	±11.0

[a] Field grown; harvested June 19, 1981.　[b] Field grown; harvested July 14, 1981.　[c] Sample oven-dried after being held in the fresh, green state for 48 h at 5 °C.

from that recommended by the vendor and in buffer rather than in distilled water. This reagent is stored at 5 °C and is discarded after 5 days.

Full color development does not occur within 20 min at 37 °C but is 90% complete after 15 min ("Sigma Chemical Company Technical Bulletin No. 510", 1979). Therefore, exact control of the incubation time for standards and samples after addition of the ECR is critical for maximum reproducibility. The addition of sulfuric acid as described in "Fermco Laboratories Technical Bulletin No. 9" (1965) and by the AOAC (1980) stops the reaction and at the same time produces a shift from a brown, turbid solution with a broad absorbance band from 425 to 475 nm to a stable, pink, clear solution with an absorbance maximum at 530 nm.

Ratios of the sample solution to ECR to H_2SO_4 (1:4:3) are maintained for optimum color development. The total of 8 mL of volume is practical in that it can be contained in a Bausch & Lomb Spectronic 20 cuvette, and the cuvette can be used for the entire glucose oxidase determination.

Standard glucose solutions prepared in 0.2% benzoic acid are used to generate a standard curve which is linear over a range of 0–80 μg/mL glucose. The standards are stable over at least 2 months when stored at 5 °C. Although it is preferable to perform the glucose analysis on sample solutions on the same day that the enzymatic conversion of starch to glucose is accomplished, a brief storage study on six diluted samples showed no significant change in glucose values after 48-h storage at 5 °C.

Comparison of starch values obtained by an automated colorimetric method based on the formation of the blue starch–iodine complex and the enzymatic method described here is shown in Table I. Also shown are starch values obtained by analysis of a portion of the enzymatically generated glucose solution using an automated reducing sugar method published by Davis (1976) in which p-hydroxybenzoic acid hydrazide (PAHBAH) is the chromogen. In addition, values are given for the percentage of starch in pure amylose from potato and commercial cornstarch analyzed by the PAHBAH and enzymatic methods, omitting, of course, the initial ethanol extraction.

The starch–iodine method gives values which are about 29% lower for cured tobacco and 39% lower for lyophilized green tobacco than those obtained by the enzymatic method. Two factors may contribute to these low values: (1) poor extraction of starch with boiling water or, a more likely cause, (2) use of potato starch for construction of the standard curve which can give only relative values when compared to tobacco starch. The blue color of the starch–iodine complex is due principally to the amylose

moiety, while the amylopectin moiety produces a weak violet color (Kolthoff et al., 1969). The amylose:amylopectin ratio in potato starch is about 20:80 (Street and Close, 1956), while that of tobacco starch differs as a function of variety, maturity, and cultural and curing practices.

The PAHBAH readout for reducing sugars appears to give results that are 28% higher than that for the enzymatic method on cured bright tobacco, but the values for lyophilized green Coker 319 and fresh and lyophilized green Coker 411 agree and fall well within the combined relative standard deviations of the two methods. This leads to the assumption that compounds are formed during the curing process which have a positive interference on the reaction of reducing sugars with PAHBAH [see Davis (1976)] and which are not removed by ethanol extraction or by the dialysis which is performed on the automated PAHBAH system.

Excellent agreement is seen between the two glucose methods for pure amylose and commercial cornstarch, which indicates that enzymatic hydrolysis of starch to glucose is virtually quantitative. No values are reported for these materials by using the starch–iodine method since they could not be kept suspended uniformly in the sample cups on the automated system. Slightly low recovery for the amylose can be traced to the difficulty in preparing a uniform suspension and the presence of small, undispersed lumps of starch.

Precision data are given in Table II, reported as standard deviation and also as relative standard deviation at the 2σ or 95% confidence level. The precision expressed as RSD (2σ) appears considerably poorer for flue-cured bright than for the Coker 319, 1974 crop, lyophilized material. It can be noted, however, that σ is lower in the cured tobacco but calculated as RSD (2σ) gives a high value because of comparatively low starch content. The poorest precision is found for fresh, green samples. This may be because these samples were weighed and processed individually while the lyophilized and oven-dried samples were weighed from tobacco which had been dried first, then combined, ground, and mixed well before being weighed for starch analysis. The values for lyophilized tobacco appear to be lower than those for fresh samples harvested June 19, 1981, and slightly higher for samples harvested July 14, 1981, so that no clear-cut comparison can be made. It is obvious, however, that in both studies the precision for lyophilized samples is excellent.

One interesting observation is the rapid loss of starch from the fresh green sample harvested June 19, 1981, after it was stored for 48 h at 5 °C. This was noted when the first group of oven-dried samples were lost and a second

group was dried for analysis after 2 days of refrigerated storage in the green state. Almost 58% of the starch originally present was lost. This difference could not be accounted for by the oven-drying process since such a difference was not seen for samples harvested July 14, 1981, and dried immediately. This points up the need for rapid processing of green tobacco to obtain reliable results. The most practical approach appears to be *immediate* freeze-drying after sampling green tobacco to get it into a form which is stable and can be analyzed reproducibly.

It is also interesting to observe that the starch content of Coker 319 *field-grown* tobacco (Table II) harvested at the early "button" stage is about 5 times that of Coker 411 *greenhouse-grown* tobacco (Table I) harvested in the early flowering stage (~15% vs. ~3%). Similar low values were found for a Coker 319 *greenhouse-grown* tobacco that had a starch content of 3%. This appears to be another manifestation of the evidence that field-grown plants are more lush and full bodied than plants grown under the conditions maintained in our greenhouse. The magnitude of the difference is such that it cannot be due to varietal variation between the two Coker varieties.

No recovery studies were made of starch added to tobacco because it was felt that topically applied starch would wash off during the ethanol extraction.

CONCLUSIONS AND RECOMMENDATIONS

An enzymatic method for the determination of starch in tobacco in various forms has been developed. With slight modifications it has been applied to other starches. While it is a somewhat laborious method requiring solvent extraction, starch solubilization, and two enzymatic treatments before the manual colorimetric readout, it is felt to be an absolute representation of the starch content of materials rather than a relative value. It has been applied successfully to samples which could not be analyzed by an automated starch–iodine method.

Future improvements in speed and possibly in precision can be made by automation of the glucose oxidase readout using a Technicon AutoAnalyzer II system or a Yellow Springs Instrument Co. Model 27 industrial analyzer.

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N-Nitrosoproline, an Indicator for N-Nitrosation of Amines in Processed Tobacco

Klaus D. Brunnemann, John C. Scott, and Dietrich Hoffmann*

N-Nitrosoproline (NPRO) was isolated from processed tobacco; a method for its quantitative assessment was developed, based on the enrichment of NPRO by solvent distributions, derivatization to its methyl ester, and gas chromatography with a thermal energy analyzer (detection limit 0.5 ng/g of tobacco). Cigarette and cigar tobaccos contained 0.33–2.3 ppm of NPRO; chewing tobacco and snuff had levels of 0.45–21.8 ppm. On the basis of the analyses of 14 tobacco products, the formation of the noncarcinogenic NPRO is significantly correlated with the formation of N'-nitrosonornicotine and the sum of the carcinogenic tobacco-specific *N*-nitrosamines (TSNA). Fine-cut snuff is relatively rich in NPRO as well as in TSNA. The latter are the only known carcinogens in snuff tobaccos where they occur in relatively high concentrations. It is suggested that efforts be undertaken to inhibit *N*-nitrosamine formation, especially during the preparation of fine-cut snuff. NPRO was not detected in cigarette smoke (<1 ng/cigarette).

Tobacco and tobacco smoke contain three types of *N*-nitrosamines, namely, the volatile nitrosamines, *N*-nitrosodiethanolamine, formed from the residue of an

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Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595.

agricultural chemical, and the tobacco-specific *N*-nitrosamines (Hoffmann et al., 1981; Brunnemann et al., 1982). These agents are not present in freshly cut green leaves but are formed during tobacco processing (Hecht et al., 1978; Adams et al., 1983). All of the *N*-nitrosamines so far identified in tobacco products are known carcinogens (U.S. Surgeon General, 1982).