Rapid Spectrophotometric Analysis of the Chemical Composition of Tobacco

Part 1: Total Reducing Sugars*

by

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INTRODUCTION

Knowledge of the sugar levels in tobacco is important to all segments of the tobacco industry. Quite often measurement of sugars can reveal a great deal concerning the vitality of living plants. Nutritional deficiencies, such as low boron levels, can be detected by measuring sugar variations (1). Fusarium wilt results in a significant decrease in sugars (2). Baron and Guthrie (3) measured abnormally low levels of sugar in plants infested with aphids.

Excluding chlorophyll degradation, the major changes in flue-curing are the loss of starch and an increase in reducing sugars (4). Levels of reducing sugars can reach as high as 30 percent by weight. Air-curing extends the period of senescence several days during which time the leaf uses the sugars for energy in an effort to sustain life. Consequently, Burley tobaccos and cigar tobaccos reach the end of the air-curing process with extremely low levels of sugars (often less than 1.0 percent) (5). Quantification of the levels of sugars at the end of curing is often used as a judgement criteria of proper curing practices, especially when other parameters are subtle. Ultimately sugars play an important role in determining the mildness and aromaticity of smoke. Budener (6) developed the ratio

> sugar + starch cell membrane substances

in an effort to objectively express mildness (the higher value means milder tobacco). *Pyriki* (7) and *Shmuk* (8) developed similar criteria, but *Shmuk* used a simple soluble carbohydrates to proteins ratio.

The slowness of current analytical techniques for measuring the sugar content of tobacco can be attributed to a dependence upon tedious chemical extraction procedures. While many of these methods are quite accurate, results obtained are usually not timely enough to provide a basis for making rational judgements concerning various processes. The need for a simple, rapid technique for estimating tobacco leaf reducing sugars has existed for a long time. Harvey and Palmer (9) stated that measurements within 20 percent of the true value would be adequate for field tests.

Several chemical techniques have been developed which have increased rate of analysis (samples/hour) of tobacco. Oakley (10) modified the procedures set forth by Van Handel (11), automated certain steps and developed methods for reducing sugars which gave a throughput of 10 samples per hour. Harvey et al. (12) published a

^{*} Received for publication: 19th October, 1976.

Paper No. 5095 of the Journal Series of North Carolina Agricultural Experiment Station, Raleigh, North Carolina. This project was, in part, supported by a grant from the North Carolina Tobacco Foundation, Inc.

procedure which describes an automated method for measuring reducing sugars. This method has been used in the Tobacco Chemistry Laboratory of North Carolina State University for more than 4 years. Not including sample grinding time, sample rates have been as high as 25 samples per hour.

Recent developments in spectrophotometric technology coupled with concomitant computer hardware developments have enabled researchers to more intensively investigate spectroscopic techniques involving the near infrared region of the spectra. Where spectrophotometry was once restricted to liquids which adhered to Beer's Law, it is now capable of making valid measurements on turbid and solid samples. Norris et al. (13) have shown that moisture content of grains can be determined by direct spectrophotometry. Ben-Gera and Norris (14) developed a non-destructive method for measuring the fat and moisture content of ground meat. Norris et al. (15) have applied the infrared reflectance technique to prediction of forage quality and were able to measure acid detergent fiber, neutral detergent fiber, lignin, and protein content as well as predict animal digestibility. This paper describes a technique for measuring total reducing sugars in cured tobacco which, with the exception of initial calibration, eliminates dependence on chemical extraction procedures. The method requires little sample preparation and gives a rapid reading. Furthermore, the analytical technique lends itself to instrumentation which can be automated for routine laboratory measurements at rates in excess of 200 samples per hour.

EXPERIMENTAL

Instrumentation

A computerized high-density spectrophotometric facility made possible an investigation of the near-infrared reflectance spectra of tobacco samples. A block diagram of this research instrument is shown in Figure 1. The system operates in the single beam mode using a Cary 14 monochromator^{*} with a wavelength range of 0.185 to 2.85 micrometers (μ m). For the measurements reported in this paper, large area lead sulfide cells were used to sense the energy diffusely reflected from the sample. In this mode the effective wavelength range is 0.8 to 2.6 μ m.

The monochromator slits were fixed at 2.5 mm which gives an effective bandpass of 10 nm. The instrument operates in a single-beam mode with a reference stored in the computer for system-response correction. The reference sample was freshly packed barium sulfate powder, so the sample spectra represent reflectance values relative to that of barium sulfate. Figure 1. Block diagram of computerized spectrophotometer for reflectance measurements.



Materials and Methods

A total of 152 tobacco samples were selected from material held for analyses by the Tobacco Chemistry Laboratory, North Carolina State University. Samples were chosen without regard to variety or previous history to provide a range of total reducing sugars from approximately 0 to 30 percent as determined by the method proposed by *Harvey* et al. (12). Of the 152 samples, 129 samples were Virginia type, 16 samples were Burley, and 7 samples were Turkish.

The samples were dried to a moisture level of 2-3 percent and pulverized in a Wiley mill with a 40 mesh seive.

Reflectance (R) measurements were made on a smoothed surface of the comminuted tobacco prepared by pouring a subsample into a stainless steel cup with inside dimensions of 4.445 cm (1.75 in.) diameter and 9.525 mm (0.375 in.) depth and smoothing the surface with a flat spatula. All samples were prepared by the same person in order to assure as much uniformity as possible. The \log_{10} (1/R) spectrum of each sample was recorded for the range 1.4 to 2.4 µm. Each spectrum recorded consisted of 2000 data points; each point was stored in memory and written on cassette tape for later statistical analyses. For data analysis the 2000-point curves were smoothed by a computer program which averaged adjacent points (9 on either side of the selected point) and compressed the curves to 500 points. Compressed curves for all samples were recorded on a new cassette along with the compositional data for each sample.

A step-wise multiple-linear-regression program was used to analyze the data and determine the optimum wavelengths for predicting the sugar content. The reflectance data were treated in two different ways for the multiple-regression analyses. We tested both $\log (1/R)$ values and the second derivative of $\log (1/R)$ values for predicting sugar content. The second derivative technique gave slightly better results so they are reported in more detail. The multiple-regression calculation deter-

^{*} The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

mines the coefficients K_0 to K_9 and the wavelengths λ_1 to λ_9 to give the best fit to the following equation for total reducing sugars (TRS):

$$TRS = K_0 + K_1 \frac{d^2 \log (1/R_{\lambda_1})}{d \lambda_1^2} + \dots + K_9 \frac{d^2 \log (1/R_{\lambda_9})}{d \lambda_2^2},$$

where R_{λ_i} is the reflectance at wavelength λ_i and the values of λ_i are allowed to vary from 1.402 to 2.380 μ m (step width: 0.002 μ m) with the restriction $\lambda_1 \neq \lambda_2 \neq \ldots \neq \lambda_9$.

RESULTS AND DISCUSSION

Plotting the reflectance data as log (1/R) vs. wavelength gives a spectrum similar in appearance to an absorption spectrum with peaks occurring at the position corresponding to an absorbing compound. Figure 2 shows the spectra of two samples of comminuted tobacco with different levels of TRS. There exist similarities and dissimilarities between the two spectra. Water has an absorption band at 1.45 and 1.94 μ m as well as at shorter and longer wavelengths. These two absorption bands show up in Figure 2 with both bands distorted to longer wavelengths by the presence of other absorbers. Absorption bands also appear at 1.73, 2.11, and 2.32 μ m in both spectra. These absorption bands existed in all the spectra recorded.

Dissimilar features exist near 1.55, 2.11, 2.22, and 2.32 μ m. The spectra around 1.55 and 1.65 differ primarily in slope, although a slight difference in optical density does exist. A definite increase in absorption exists for the 19.0%-TRS sample at 2.11 μ m, perhaps due to the higher sugar levels. The two-spectra switch

Figure 2. Reflectance spectra log (1/R) for two samples of comminuted tobacco with different levels of total reducing sugars.



Figure 3. Reflectance log (1/R) and second derivative spectra for a high-sugar tobacco sample. Vertical lines indicate wavelengths chosen for predicting sugar content using second-derivative data.



position at 2.32 μ m with the 0.2%-TRS sample having the higher value.

The second derivative and log (1/R) spectra of the 19.0%-TRS sample are shown in Figure 3. Secondderivative spectra show positive peaks at the position of absorption bands, but they show greater definition of overlapping absorption bands.

The eight vertical lines indicate the wavelengths chosen by the multiple-regression program for predicting TRS content based on second-derivative values. The order in which they were selected was: 1.458, 1.914, 1.790, 2.028, 1.516, 1.592, 1.476, and 2.178 µm.

In a heterogeneous material like tobacco, the spectrum of a sample is made up of the various spectra of the individual constituents, mostly by superposition. Thus, it seems plausible that if one or more constituents vary from sample to sample, the multiple-linear-regression analysis would pick first the wavelength at which sugar absorbs and then those wavelengths which would compensate for the variation of other constituents. The computer selected 1.458 µm as the first wavelength so an attempt was made to determine if sugar has an absorption band at that wavelength. The $\log (1/R)$ spectrum of dry glucose crystals (Fig. 4), does not show a band at 1.458 µm but does show bands at longer wavelengths. The relatively sharp bands at 2.295, 2.33, and 2.37 µm are particularly significant because they do not appear on the tobacco samples even though we had samples of 29% TRS. This led us to conclude that the sugar in the tobacco sample must have different absorption characteristics than dry glucose crystals. By measuring a saturated solution of glucose in water mixed with barium sulfate against water-soaked barium sulfate we obtained a log (1/R) spectrum more like that of the tobacco. This spectrum (Fig. 4) has evidence of an absorber at 1.45 µm and it is suggested that this is the explanation for the choice of 1.458 µm for the first or best wavelength.

Figure 4. Reflectance log (1/R) spectra for dry glucose and glucose-water solution in barium sulfate.



The computer selected 1.914 μ m as the second wavelength. Water has an absorption band at 1.94 μ m. The water content of each sample did vary. Hence, it seems likely that the choice here was to correct for the variation in water content of the samples. The basis for selecting the other parameters is less obvious.

The relationship between the TRS determined by the *Harvey* Method and the infrared technique for 152 samples is shown in Figure 5. The correlation coefficient is 0.987 and the standard error is \pm 1.37 percent. Omitting six outlying points (Fig. 5) based on their deviation from regression being greater than two times the above standard error, gave a correlation coefficient of 0.990 and a standard error of \pm 1.15 percent.

Data in Table 1 show that the precision of the spectrophotometric technique compares favorably with that of the chemical method used in this study. Forty-five replications on a well-blended sample of tobacco gave a relative standard deviation at the 2-sigma level [RSD(2 σ)] of 16.31% for the chemical method. The spectrophotometric method gave a RSD(2 σ) of 10.09% for nine replications on the same sample.

Potential errors in the spectrophotometric procedure may

 Table 1.
 Comparison of the precision of the spectrophotometric technique and the Harvey et al. Method.

n	x	Variance	Average variation	Standard devlation (σ)	Coef- ficient of variation	RSD (2σ)
A. Sp	ectroph	otometric	: techniqu	10		
5a 5a	0.2 16.64	0.522 0.338	0.455	0.722 % 0.581 %	2.553 0.035	729 º/o 6.98 º/o
9b 9b	0.67 16.69	1.081 0.708	0.895	1.040 % 0.842 %	1.552 0.050	311 % 10.09 %
B. Ha	rvey et	al. Metho	d	*		
45c	13.75	1.258		1.121 %	0.086	16.31 %

a: Five replicates on the same subsample.

b: Measurements on nine independent subsamples from the same samples.

c: Assays of forty-five independent subsamples from the same sample.

Figure 5. Relationship between the chemically determined total reducing sugars and the values predicated by infrared reflectance data.



be classified as errors due to [1] sample preparation and [2] instrument noise. Replications in Table 1 marked with the superscript (a) were made on the same subsample to eliminate sample preparation as a source of error. The standard deviation (σ) for a concentration of 16.64 percent TRS was \pm 0.58 percent. This figure constitutes instrument noise. An analysis of variance (16) indicated the variance at 16.64 percent TRS was not significantly different (5% level) from the variance at 0.2 percent TRS. This means it is valid to average the two giving an average variance due to instrument noise of 0.455.

Making multiple measurements on independent subsamples from the same sample permits calculation of the variance due to sample preparation plus instrument noise. In Table 1 nine samples were measured for each of two concentrations of TRS. The variances were 0.708 and 1.081 for 0.67 and 16.69 percent TRS respectively. These variances are not significantly different at the 5 percent level according to the F test. Thus, the average total variance is 0.895.

Based on the average variances over 50 percent of the variability of the spectrophotometric technique is due to instrument noise. The initial reaction is to assume the instrumental approach is poor. To the contrary, this is encouraging. Measurements made on this system utilized a double monochromator which considerably diminished the light reaching the sample. It should be recognized that instrument noise is related inversely to the amount of energy reaching the detector. If a filter monochromator were used instead of the double monochromator used in this study, energy output would increase more than 100fold, which in turn would reduce the error from instrument noise. Filter instruments are commercially available and can be adapted to make these measurements. Reduction of sampling errors could be accomplished by averaging the readings from several subsamples, by grinding to a more uniform particle size, more thorough mixing of sample, and high pressure packing of the subsample.

Figure 6. Predicting total reducing sugars from independent data. The line —— represents the calibration curve from the even-numbered samples. The data points are the predicted values for the odd-numbered samples.



Of utmost concern is the ability of the technique to predict TRS levels from a calibration equation developed from independent samples. In Figure 6 the solid line is the regression line based on the even-numbered samples in this experiment. The regression equation of this line was used to predict the sugar content of 72 oddnumbered samples. The data points shown in Figure 6 are the predicted values plotted about the calibration equation. Based on deviations from the calibration equation, the correlation coefficient is 0.986 and the standard error is \pm 1.36 percent.

An instrument design would be simpler with log (1/R) data than with the second derivative, but our results indicate less accuracy would be obtained. The standard error on the same samples was $1.41 \, 0/_0$ using log (1/R) data at eight wavelengths as follows: 2.100, 1.898, 2.216, 1.742, 1.788, 2.300, 1.722, and 1.600 μ m. Omitting the six samples having abnormally large errors reduces the standard error to $1.24 \, 0/_0$. The simplest practical instrument would be a two-filter instrument which according to our data would give a standard error of $2.4 \, 0/_0$. This is reduced to $2.0 \, 0/_0$ for four filters and $1.51 \, 0/_0$ for six filters.

SUMMARY

The log₁₀ (1/R) spectra, from 1.4 to 2.4 μ m, of 152 samples of comminuted samples of tobacco were recorded on a computerized spectrophotometer. The parameter d² (log (1/R)) / d λ^{2} was calculated for each spectrum and correlated with chemical analyses of total reducing sugars using the method proposed by *Harvey* et al. (12). A nine-term multiple-linear regression relating the above parameter at seven different wavelengths to total reducing sugar gave a correlation coefficient of 0.990 and a standard error of \pm 1.15 percent. Instrument noise was found to contribute over 50 percent of the total variance.

ZUSAMMENFASSUNG

Für 152 Proben pulverisierten Tabaks wurden die log₁₀ (1/R)-Spektren im Bereich 1,4 – 2,4 µm von einem rechnergesteuerten Spektrophotometer aufgezeichnet. Der Parameter d² (log (1/R)) / d λ^2 wurde für jedes Spektrum berechnet und zu chemischen Analysen der gesamten reduzierenden Zucker [Methode nach *Harvey* (12)] in Beziehung gesetzt. Eine lineare Regression mit neun Variablen, die den erwähnten Parameter bei sieben verschiedenen Wellenlängen den gesamten reduzierenden Zuckern zuordnete, ergab einen multiplen Korrelationskoeffizienten von 0,990 und einen mittleren Fehler von ± 1,15 %. Es zeigte sich, daß das Geräterauschen zu über 50 % zu der Gesamtvarianz beiträgt.

RÉSUMÉ

Les spectres $\log_{10} (1/R)$, de 1,4 à 2,4 µm, de 152 échantillons de tabac pulvérisé ont été enregistrés sur un spectrophotomètre couplé à un ordinateur. Le paramètre d² (log (1/R)) / d λ^2 a été calculé pour chaque spectre et correlé à des analyses chimiques des sucres réducteurs totaux faites par la méthode de *Harvey* (12). Un calcul de régression linéaire à neuf termes, rapportant le paramètre ci-dessus sur sept longueurs d'onde différentes, aux sucres réducteurs totaux a donné un coefficient de corrélation de 0,990 et une erreur standard de \pm 1,15%. Le bruit de fond de l'instrument contribue pour plus de 50% à la variance totale.

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