Rapid Spectrophotometric Analysis of the Chemical Composition of Tobacco

Part 2: Total Alkaloids*+

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INTRODUCTION

The percent total alkaloids of flue-cured tobacco is an important quality factor. Accurate methods have been reported for estimating the total alkaloids in tobacco samples (1, 3, 4, 5, 8, 8a). However, these methods are destructive, slow in most cases, and involve tedious chemical extraction procedures. Development of a simple, rapid, reliable and non-destructive technique for measuring total alkaloids will greatly benefit the tobacco industry.

Several researchers have demonstrated the potential of computerized spectrophotometry for rapidly measuring the chemical composition and quality of agricultural products. *Birth* (2) determined the chlorophyll content of peaches, detected alternaria in oranges, and established a quality index for pork. *Norris* and *Barnes* (10) developed prediction equations for crude protein, neutral detergent fiber, acid detergent fiber, and lignin in grains and forages. *Shenk* et al. (12) measured the composition of forage diets and predicted the animal response to the diets. *McClure* et al. (9) developed prediction equations for total reducing sugars in tobacco. *Finney* and *Norris* (6) established the feasibility of measuring moisture in corn kernels.

The absorption spectra of a product contain much information about the chemical constituents and their concentrations in a multicomponent system. Traditionally, if one has an n-component mixture, he can measure n absorption peaks and solve a system of n simultaneous equations. In fact, the accuracy can be increased by overdetermination, i.e. analyzing more than n data points. Because of the computational complexity, the classical "overdetermined" problem was limited to a few extra points. Advancement of computer technology in recent years has made it possible to have dedicated computers for spectral data retrieval and analysis. An on-line computer can greatly facilitate the analysis of a multicomponent system. The computer can process the digitized spectra and find the wavelengths at which the spectral data will best correlate with a constituent of a multicomponent system. The power of a computerized spectrophotometer lies in the fact that thousands of data points can be acquired and analyzed in a short period of time.

The theory of compositional analysis of biomaterials by computerized spectrophotometry has been discussed by *Hamid* (7). Briefly stated, the concentration C of a given chemical constituent can be related to a measured optical parameter P as follows:

$$C = K_0 + \sum_{i=1}^{\ell} K_i P_{\lambda_i} , \qquad [1]$$

where K_0 is the constant term, K_i is the experimentally determined coefficient of the ith term, P_{λ_i} is the magnitude of the optical parameter at the wavelength λ_i , and ℓ is the number of terms in the prediction equation.

P may be $\log (1/R)$, $dR/R d\lambda$, $d^2 (\log (1/R))/d\lambda^2$ or any other optical parameter which is related to the chemical constituent under study. Reflectance R is defined as the ratio of the detector signal of the sample to the detector signal of the ceramic standard in the reflectance mode (7, 9). A general stepwise multiple linear regression (called GSMLR) has been developed (7) to determine the functional relationship between concentration and one of the above optical parameters. This program considers all the wavelengths at which the spectra were encoded and GSMLR selects the wavelengths at which P best correlates with C. At every step of regression the P entering the regression will be the one which has the highest partial correlation with C. The program will continue adding terms until the desired accuracy is achieved.

^{*} Received for publication: 9th February, 1978.

Paper No. 5543 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh, N.C.

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.

⁺ Part 1: Total reducing sugars (9).

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MATERIALS AND METHODS

A total of 135 independent samples of cured tobacco were selected to provide a range of total alkaloids from approximately $0.5 \, 0/0$ to $6.5 \, 0/0$. All samples were pulverized in a Wiley mill with a U.S. 40 screen. Total alkaloid analyses (8) were made by the Tobacco Chemistry Laboratory (North Carolina State University, Raleigh, North Carolina). The samples were split into two groups according to their odd or even sample numbers. The 68 odd-numbered samples were used to develop the calibration equation whereas the 67 even-numbered samples were used to test the calibration equation.

Two grams of each sample were loaded into a Technicon solid sample container with a quartz window. The reflectance spectrum, from 1.0 to 2.6 μ m, of each sample was recorded on a computerized spectrophotometric system (7). A specially prepared flat white ceramic disc, which has a flat reflectance curve over the visible nearinfrared region, was used as a reflectance standard.

The bandpass of the monochromator was fixed at 7.0 nm. The spectra were scanned at 10 nm/s. Reflectance readings were recorded at wavelength intervals of 1.0 nm providing 1600 digitized data points which were stored on cassette tapes for further analysis. Each data point was the average of 100 readings. This technique was employed to improve the signal-to-noise ratio of the spectrophotometric system output. Each reflectance spectrum, consisting of 1600 digitized data points, was stored on cassette tapes for further analyses.

DATA PROCESSING

Three additional optical parameters were calculated from the reflectance spectra. These were: [1] log (1/R), [2] d R / R d λ and [3] d² (log (1/R)) / d λ^2 . Calculation of these parameters was as follows (7):

- 1. A twenty-five points least-squares polynomial smoothing was followed by the calculation of log (1/R).
- 2. A twenty-five points least-squares polynomial smoothing was followed by the calculation of the 25-point quadratic first derivative, and dividing the result by the smoothed reflectance.
- 3. A twenty-one points moving point average smoothing was followed by calculation of 21-point second derivative by linear interpolation.

In each of the above three cases the spectra were compressed to approximately 550 data points. The exact number of data points depended on the type of data processing used in the transformation. The transformed spectra were stored on separate cassettes for further analyses.

The stepwise multiple linear regression program, GSMLR (7), was run for each of the three optical parameters, i.e. $\log (1/R)$, $dR/R d\lambda$, and $d^2 (\log (1/R)) / d\lambda^2$. A maximum of fifteen steps were allowed by the regression procedure. The F value for entry and deletion of a variable was 3.0. Each step of regression would either add a variable to the regression equation or remove the one from the set of variables already included. Samples which produced residuals greater than twice the standard error of regression could be eliminated from the data set.

RESULTS AND DISCUSSION

Log (1/R) and d R / R d λ spectra of a typical tobacco sample with 3.1% total alkaloids are plotted in Fig. 1. The log (1/R) spectrum looks much like an absorption spectrum [log (1/T)] in that the peaks signify the presence of an absorber. For example, the most prominent absorber in the tobacco of Fig. 1 is water which exhibits absorption peaks at 1.94, 1.45, and 1.19 µm. The slight shift of the 1.45 water band toward longer wavelengths is probably due to overlapping absorbers. Absorption bands also occur in the log (1/R) curve at 1.725, 2.145, 2.31, 2.345, and 2.5 µm. These points can be easily identified from the first derivative curve by simply noting the points where d R / R d λ changes from negative to positive.

There are overlapping bands in the log (1/R) curve, and these overlapping bands are better resolved in the derivative spectrum. As can be seen in Fig. 1, in the vicinity of an absorption band the first derivative spectrum has two peaks, one corresponding to the maximum positive slope of the absorption band and the other corresponding to the maximum negative slope. Positive peaks in the d R / R d λ spectrum occur at the maximum negative slope of the log (1/R) spectrum and negative peaks occur at the maximum positive slopes. The first derivative is zero at absorption maxima and minima.

Fig. 2 shows the log (1/R) and d² (log (1/R)) / d λ^2 spectra of the same tobacco as in Fig. 1. Note that a negative peak occurs in the second derivative spectrum at the absorption maxima and a positive peak at absorption minima. The second derivative is zero at the points of inflection in the log (1/R) curve. Minima in the second derivative curve (Fig. 2) clearly indicate the same ab-



Figure 1. Log (1/R) and d R / R d λ spectra for a 3.1 % total alkaloid tobacco sample.

Figure 2. Log (1/R) and d² (log (1/R)) / d λ^2 spectra of a 3.1 % total alkaloid tobacco sample.



sorbers as were found in the first derivative spectrum. There are two notable exceptions. First, the band at 1.45 μ m does show the presence of overlapping absorbers. Second, although some of the maxima or minima are "hidden" in log (1/R) curve, they are indicated in the second derivative curve. Furthermore, since the second derivative process is more "noisy" than the first derivative, some of the minima, particularly in the region between 2.4 and 2.6 μ m, may be the result of noise due to low detector sensitivity in this region.

The constants for the calibration equations for the three optical parameters $[\log (1/R), dR / R d\lambda, and d^2 (\log (1/R)) / d\lambda^2]$ as developed by the stepwise multiple linear regression program, GSMLR, are given in Table 1. The data in Table 1 should be interpreted as follows:

% TA = K₀ +
$$\sum_{i=1}^{\ell} K_i P_{\lambda_i}$$
, [2]

where 0/0 TA is the percentage total alkaloids, K_0 is the intercept, K_i is the coefficient of the ith term in the equation, P_{λ_i} is the magnitude of the optical parameter selected for the ith term and corresponding to wavelength λ_i , and ℓ is the number of terms in the regression equation.

The equations for log (1/R) and d R / R d λ contained nine terms while the equation for d² (log (1/R)) / d λ^2 contained 13 terms. d R / R d λ gave slightly better results [s_c* = ± 0.2596] than log (1/R) [s_c = ± 0.2801]. The d² (log (1/R)) / d λ^2 [s_c = ± 0.1695] was indicated a better parameter than either log (1/R) or d R / R d λ .

Figures 3, 4 and 5 show, respectively, the log (1/R), d R / R d λ , and d² (log (1/R)) / d λ^2 spectra of two samples of tobacco, one containing 0.78 % and the other containing 6.07 % total alkaloids. While the two curves in each of the figures exhibit similarities and dissimilarities, it is difficult by the visual inspection to associate the differences with alkaloid levels at various wavelengths. The wavelengths selected by GSMLR are marked on the curves; the numbers on the markings indicate the order in which they were selected. Figure 3. Log (1/R) spectra of two tobacco samples of different levels of total alkaloids.



Figure 4. d R / R d λ spectra of two samples of tobacco of different levels of total alkaloids.



Figure 5. d² (log (1/R)) / d λ^{3} spectra of two tobacco samples of different levels of total alkaloids.



By superposition the spectrum of a tobacco sample is made up of the spectra of the individual constituents contained in the sample (9). Therefore, one would expect the stepwise multiple linear regression procedure to pick wavelengths, at least the initial one or two wavelengths, which would have a direct relationship to the particular constituent under study. As can be seen in Table 1, GSMLR picked approximately 1.66 μ m as

^{*} $s_c = standard error of calibration.$

								Con	stants					a.	- 	-2	6
Parameter		0	-	2	3	4	5	9	7	8	6	9	=	12	13	<u>-</u>	ň
	Wavelengths (μm)		1.666	1.012	1.654	2.263	1.684	1.699	1.723	1.459	1.474						1000 0
(H/I) 60	Coefficients (K _i)	6.931	2158.77	-29.05	-1508.91	-49.14	-1033.05	897.45	-428.91	-460.36	453.73					6006.U	1.082.0
Яр	Wavelengths `(µm)		1.657	1.714	2.125	1.537	1.870	1.750	1.072	2.383	2.353			-			
Rdλ	Coefficients (K _i)	5.379	-8995.17	8261.49	625.08	7968.11	-2204.38	-4094.33	-2492.30	-1511.31	1279.05					0.9664	0.2596
3 ² (log (1/R))	Wavelengths (um)		1.668	1.203	1.635	1.695	1.377	1.797	1.980	2.241	2.448	2.517	1.902	1.572	2.577		
d	Coefficients (K _i)	6.305	-897.33	-514.08	-809.67	-208.05	-312.39	113.61	130.94	-115.19	-79.90	-38.33	-118.47	-456.09	8.71	0.9867	0.1695
				Table	a 2. Effe	sct of diffe	rent numb	er of wav	elenaths i	n callbratic	on equatio	SU SU					
		Num	ber of		log (1/R)			P	R/Rdλ			1 ² (log (1	۲ <u>۲</u> ((R/)				
		wave	elengths	r ²	°S	S.	_	r ²	š	Sp	L2		Sc	Sp			
			- - -	0.4727	0.9627	1.016	2 0.7(679 (0.6388	0.6816	0.7736	0.6	309	0.6196			
			N	0.7770	0.6309	0.651	2 0.8	132 (0.5775	0.5281	0.8605	0.4	991 (.4475			
			n	0.8705	0.4846	0.546	5 0.8-	441 (0.5318	0.5262	0.9264	0.3	355 (.5092			
			4	0.9289	0.3620	0.479	2 0.8	697 (0.4901	0.5247	0.9422	0.3	262 (.4889			
			5	0.9349	0.3491	0.464	4 0.9	486	0.3102	0.4452	0.9508	0.3	336 (.4959			
			9	0.9389	0.3411	0.473	6 0.9	543 (0.2950	0.4636	0.9585	0.2	312 (.4657			
			7.	0.9530	0.3017	0.497	3 0.9	588 (0.2826	0.4761	0.9629	0.2	379 0	.4684			
			8	0.9556	0.2957	0.494	0.0	633	0.2690	0.4887	0.9672	0.2	544	.4668			
			6	0.9609	0.2801	0.521	1 0.9(664	0.2596	0.5068	0.9716	0.2	384 0	.4511			
		-	0								0.9752	0.2	252 C	.4376			
		-	=								0.9783	0.2	22	.4724			
		-	2								0.9846	0.18	303 0	.5414			
		-	13								0.9867	0.16	3 9 5 0	.5463			
	r ² = Coeff	iciant of de	termination														

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the initial wavelength for all three parameters. The spectrum of nicotine in carbontetrachloride has a complex absorption band between 1.6 and 1.8 μ m (13). Thus, it appears that the initial wavelength is directly associated with the major alkaloid absorption. While it is intuitively assumed that the choice of other wavelengths were made to correct for the presence of other absorbers, these choices cannot be verified at this time.

The real test of a calibration equation is its ability to predict a chemical constituent in an independent set of samples. A prediction program, PRED (7), was used to predict total alkaloids in even samples with calibration equations containing different wavelengths. The coefficient of determination (r^2) and standard error of calibration (s_c) given by GSMLR as well as standard error of prediction (s_p) given by PRED are listed in Table 2 along with the wavelengths in the calibration equation. The following observations can be made from Table 2:

- 1. The d² (log (1/R)) / d λ^2 gave the best results while d R / R d λ gave better results than log (1/R) equations.
- 2. The prediction standard error was generally larger than the calibration standard error.
- 3. The prediction error did not always decrease with additional wavelengths. For both log (1/R) and d R / R d λ , the prediction error decreased up to 5 wavelengths and then increased consistently with additional wavelengths. For d² (log (1/R)) / d λ^2 , it did not follow the same pattern. However, the minimum error of 0.438 was obtained for 10 wavelengths after which it increased consistently.

These results indicate that calibration equations should be evaluated carefully. A calibration equation developed from a small set of samples may not be equally good for an independent set of data, and a calibration equation containing too many wavelengths may not necessarily be a better prediction equation. Similar results were reported by *Shenk* et al. (12) for the compositional analysis of forages.

It is not obvious why the standard error jumps up or down up to 9 wavelengths for $d^2 (\log (1/R)) / d\lambda^2$. Two possible explanations can be given for this phenomenon. First the second derivative is very sensitive to noise. Since the log (1/R) spectrum contained significant amount of noise at the lower and upper extremes, due to the low sensitivity of the detector, some noise was carried over to the second derivative spectrum even after smoothing the data. Secondly, the second derivative has very sharp peaks and effects of compressing the curves will be more severe in this case which means that the wavelengths selected by the GSMLR might have been ± 1 nm off the optimum wavelengths.

Based on the discussion in the previous paragraph, the optimum prediction equations for the three quantitative parameters are given in Table 3. Again the prediction equations should be interpreted according to equation 2.

Figure 6. Predicted values of total alkaloids plotted vs. chemistry values.



Results of prediction on 67 even samples using the best prediction equation [the one given for $d^2 (\log (1/R)) / d\lambda^2$] are shown in Fig. 6.

Deviations between the chemistry values and predicted values can be attributed to three sources: anomalies of chemical methods (measuring irregularities), instrument noise and sample preparation. To evaluate the magnitude of errors due to instrument noise, a tobacco sample with 3.1% total alkaloids was packed in the sample holder and 20 spectra were run on the same sample. The standard deviation between the predicted values of total alkaloids was 0.21 %. The experiment was repeated by packing and unpacking the same sample to evaluate the errors associated with sample preparation and instrument noise. Surprisingly, the standard deviation of predicted values was 0.17%. Normally, one would expect this number to be larger than 0.21%. This can be explained by the negligible errors associated with sample preparation and a large error associated with the variance estimate. McClure et al. (9) reported 50% errors due to sample preparation for sugars. In their case, the samples were prepared by pouring a subsample into a stainless steel cup and smoothing the surface with a flat stapula which could explain the errors associated with sample preparation.

Thus $0.19 \, 0/0$ (average of 0.17 and 0.21) error can be attributed to the instrument noise which is approximately 43 0/0 of the total prediction error. The other 57 0/0of the deviations may be attributed to variations of the chemical methods. Optimum prediction equations for measuring concentration of total alkaloids in tobacco samples. Table 3.

							Constants								
Parameter		0	1	2	3	4	5	9	7	8	6	10	r²	Sc	Sp
log (1/R)	Wavelengths (um)		1.666	1.012	1.654	2.263	1.684								
	Coefficients (K _i)	7.012	1723.93	-40.87	-1306.28	-65.36	-302.71						0.9349	0.3491	0.4644
ЧR	Wavelengths (μm)		1.657	1.714	2.125	1.537	1.870			×					
Rdλ	Coefficients (K _i)	5.749	-8704.89	8867.96	1870.60	-8445.72	-1502.18						0.9486	0.3102	0.4452
d ² (log (1/R))	Wavelengths (μm)		1.668	1.203	1.635	1.695	1.377	1.827	1.797	1.980	2.241	2.448			
d λ²	Coefficients (K;)	5.277	869.65	-427.78	-604.08	-274.41	-191.67	-106.42	157.01	153.83	-83.91	-47.02	20/6.0	7977.0	0.43/6
	r² = Coeffi	cient of deter	rmination.			B _c = Standa	rd error of ca	alibration.			s _p = Stan	dard error of	prediction.		

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CONCLUSIONS

This paper discusses the potential of a computerized near-infrared spectrophotometer for compositional analysis of total alkaloids in cured tobacco samples.

Reflectance spectra were recorded for 68 samples with a total alkaloids range of 0.78 to 6.1 %. Three quantitative parameters, $\log (1/R)$, $dR / R d\lambda$ and $d^2 (\log (1/R)) / d\lambda^2$ were investigated. Spectral data were correlated with chemical analyses of total alkaloids using the method proposed by *Harvey* et al. (8). It was found that $dR / R d\lambda$ was a better quantitative parameter than $\log (1/R)$ and $d^2 (\log (1/R)) / d\lambda^2$ was found better than either the $\log (1/R)$ or $dR / R d\lambda$.

While the standard error of calibration decreased with additional wavelengths in the calibration equation, it was found that the standard error of prediction might actually increase with additional wavelengths after a certain number of wavelengths have been added in the calibration equation. The optimum prediction equations for both log (1/R) and d R / R d λ contained 5 wavelengths. The best prediction equation was found for d² (log (1/R)) / d λ^2 which contained 10 wavelengths and dropped the standard error of prediction to 0.438 %. Instrument noise was found to contribute about 43 % of variation; the remainder was attributed to the variations of the chemical methods.

Thus, it can be concluded that the computerized spectrophotometer has potential for compositional analysis of complex biomaterials. However, the technique should be used with caution. Equations developed on calibration samples should be evaluated carefully on an independent set of data. Furthermore, the technique is purely empirical and strictly correlative. The accuracy of its prediction cannot exceed the accuracy with which the calibrating samples are analyzed. Utility of equations developed will heavily depend on the calibrating samples and hence the calibrating samples should be selected carefully and should include evenly distributed samples over the entire range of composition. The prediction equations should not be used outside this range.

Equations developed by the computerized spectrophotometers can be used to build economical filter instruments for contactless monitoring of composition of biomaterials. Thus the computerized spectrophotometer has great potential in the field of quality and composition of biomaterials.

SUMMARY

This paper illustrates the potential of a computerized spectrophotometer for measuring total alkaloids in tobacco. Prediction equations were developed for three optical parameters. Of the three parameters investigated $d^2 (\log (1/R)) / d\lambda^2$ gave the best results and $dR / R d\lambda$ gave better results than $\log (1/R)$, where reflectance R is the ratio of the detector signal of the sample to the detector signal of the ceramic standard in the reflectance mode. The coefficient of determination r^2 for the prediction equation containing $d^2 (\log (1/R)) / d\lambda^2$ terms at 10 different wavelengths was 0.975. This equation predicted the total alkaloids in an independent set of samples with a standard error of 0.438 %. Instrument noise contributed 43 % of the variation, the remainder was attributed to anomalies of the chemical methods.

ZUSAMMENFASSUNG

Die vorliegende Arbeit veranschaulicht das Leistungsvermögen eines rechnergesteuerten Spektrophotometers bei der Messung der Gesamtalkaloide im Tabak. Für drei optische Parameter wurden Gleichungen zur Voraussage entwickelt. Die Untersuchung der Parameter zeigte, daß d R / R d l zu besseren Ergebnissen führte als log (1/R) und daß mit d[§] (log (1/R)) / d λ^{9} die besten Resultate erzielt wurden, wobei der Reflexionswert R das Verhältnis vom Detektorsignal der Probe zum Detektorsignal des Keramikstandards, gemessen als Reflexion, darstellt. Bei der Gleichung zur Voraussage mit Termen des Typs d² (log (1/R)) / d λ^2 belief sich der Bestimmungskoeffizient r2 bei zehn verschiedenen Wellenlängen auf 0.975. Diese Gleichung sagte den Gehalt an Gesamtalkaloiden einer unabhängigen Meßreihe mit einem mittleren Fehler von 0,438 % voraus. Das Geräterauschen trug mit 43 % zur Schwankung bei, der Rest wurde Ungleichmäßigkeiten der chemischen Verfahren zugeschrieben.

RÉSUMÉ

Cet exposé démontre les capacités d'un spectrophotomètre branché sur un micro-processeur dans la détermination des alcaloïdes totaux du tabac. Des équations de prédiction ont été établies pour trois paramètres optiques. L'examen de ces trois paramètres a révélé que les résultats de dR/Rdl sont supérieurs à ceux de log (1/R), et que les meilleurs résultats sont obtenus avec d² (log (1/R)) / d λ^2 . La valeur de réflexion R représente le rapport entre le signal détecteur de l'échantillon et le signal détecteur de l'étalon de céramique, mesurés en réflexion. Pour l'équation de prédiction avec des termes du type d² (log (1/R)) / d λ^2 , le coefficient de détermination r² était de 0,975 à dix différentes longueurs d'onde. Cette équation permettait de prédire la teneur en alcaloïdes totaux dans une série d'échantillons indépendants avec une erreur moyenne de 0,438%. Le 43% de la variation était imputable au bruit de fond de l'instrument, le reste a été attribué à la variation des méthodes chimiques.

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