Certain Fluorescence Characteristics of Intact Bright-Leaf Tobacco*

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Introduction

The evaluation of many biological products depends primarily on their physical attributes. Tobacco, a classical example, has long been evaluated by such sensory characteristics as color, body, and texture. In recent years, however, tobacco authorities have seen the inadequacies of grading tobacco by a set of techniques based on fallible human opinion.

That instrumentation is needed in this area is further manifested by the fact that certain of the higher yielding, newer varieties appear acceptable when purchased but are later found to be deficient. Jones, *et al.* (1960), attribute this to the complexity of the germ plasm in the newer varieties being developed by tobacco breeding programs. It is apparent, therefore, that subjective methods are no longer capable of defining trade preferences.

Some concentrated efforts have been extended to the study of chemical aspects of the cured leaf with the intent to determine in a relative way the quality of tobacco. The technique involves the quantitative determination of certain chemical constituents in the leaf such as nicotine, nornicotine, reducing sugars, nitrogen, etc. Today this method is used extensively throughout the tobacco industry. However, it has the disadvantage of slowness, is very tedious, needs trained personnel to make the evaluation, requires elaborate laboratory facilities, and is necessarily a destructive process; that is, the leaf must first be ground into a fine aggregate and then dissolved in proper chemicals in order to determine the levels of the chemical components of interest.

The development of simple, rapid, reliable, and nondestructive methods for assaying leaf quality would greatly benefit the tobacco industry. For example, such methods would offer the possibility of measuring the intrinsic quality of tobacco leaves harvested in the field at the so-called mature state. Furthermore, with careful instrumentation nondestructive analyses of leaf characteristics related to quality could conceivably be determined at any stage of growth or of the curing operation.

Fluorescence analysis has shown great promise in investigations of biological interest (Duggan *et al.*, 1957). With improvements in instrumentation and an increased knowledge of errors in measurements (Hercules, 1957; Parker and Barnes, 1957; Sprince and Rowley, 1957), fluorescence analysis has become even more adaptable as an analytical tool. Spectrofluorometric analysis has long been used to determine the fluorescence spectra of phosphors (Plymale, 1947; Studer, 1948; Klick *et al.*, 1951). Many of the compounds in tobacco leaves are each characterized by a particular fluorescence spectrum when exposed to ultraviolet radiation (Duggan *et al.*, 1957). Earlier investigations (Johanson, 1953; Kiser, 1957; and McClure, 1958) have shown that tobacco leaves do have definitive fluorescent properties. They found that the bottom side of a leaf fluoresces more than the top, and that fluorescence is proportional to extreme levels of fertilization.

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Figure 1. The spectrofluorometer used to measure the fluorescence spectra of intact tobacco samples.

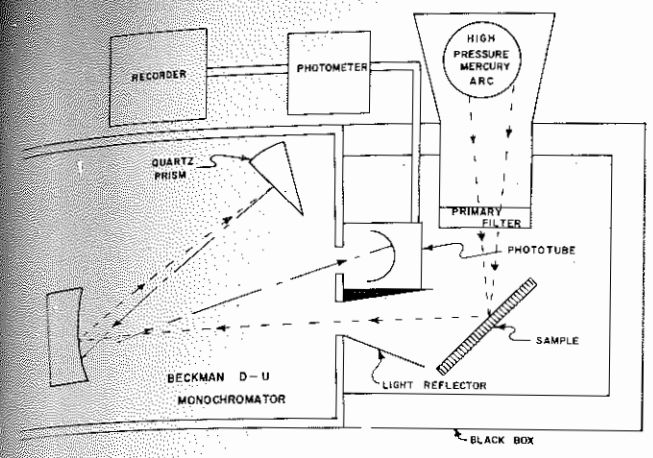

Figure 2. Schematic of the spectrofluorometer.

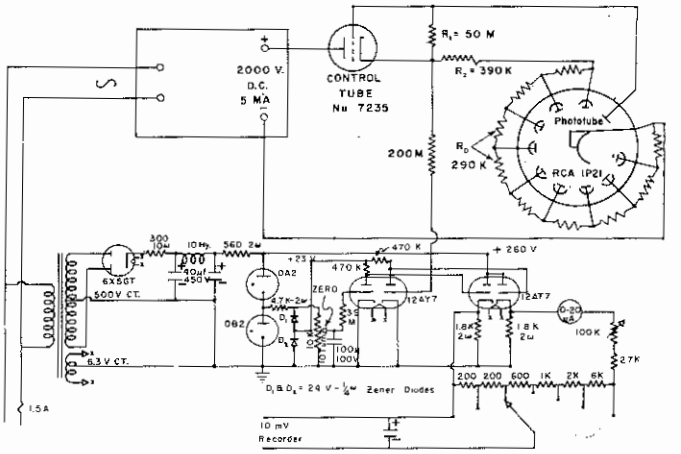

Figure 3. Electronic circuitry in the spectrofluorometer.

Studies of tobacco tissue up to this point, however, have been concerned with the visual observation of fluorescence or with the measurement of a representative portion of the fluorescent light emitted from tobacco leaves. The present study was undertaken to determine some comparative spectrofluorometric characteristics of intact tobacco.

Experimental

Instrumentation. The spectrofluorometer used to determine the fluorescence spectra of the tobacco leaf samples is shown in **Figure 1.** The primary components are numbered in the figure as follows: (1) an adjustable span recorder was used to record the signal from the photometer; (2) a specially designed photometer was used to measure the low intensity fluorescence; (3) a synchronous motor was adapted to drive the wavelength drum of the Beckman DU Monochromator (4); (5) a high pressure mercury arc lamp was employed as the exciting source; (6) a black box was designed to hold the samples as well as facilitate mounting of the photomultiplier detector.

The signal from the photometer was fed into a Minneapolis-Honeywell adjustable span recorder (0-1 mv to 0-50 mv with a 50 mv supply). A special gear train provided a chart speed of 12 in/min. An operational pen assembly was adapted to indicate wave length on the strip chart every 25 mu from 400 to 600 mu. It was actuated electrically by a microswitch and cam assembly in the wavelength drive of the monochromator.

A Beckman DU monochromator was used to scan the fluorescence spectra. A black box was designed and positioned over the entrance slit of the monochromator. The samples were placed inside the box at a 45° angle to the face of the entrance slit. Since the sample was positioned very near the entrance slit enough light entered the slit, without the aid of extra optics other than a silvered light reflector tube (see **Figure 2**).

Figure 2 shows the arrangement of the spectrofluorometer. The light source used to excite fluorescence in the tobacco samples was a GE S-4 high pressure mercury-arc lamp. This lamp was filtered with a Photovolt Hg-1 primary filter to give a spectrum of ultraviolet light peaking at 360 mu and having lower and upper cut-off points at 310 mu and 390 mu respectively. However, due to the characteristics of the 360 mu mercury line of the high pressure mercury arc, the radiation on each side of this line from 310 mu to 350 mu and from 370 mu and 390 mu was extremely small compared to the total over the whole range (less than 10%). Hence, the light striking the sample was essentially monochromatic at 360 mu.

By the nature of the fluorescence process, most of the fluorescent energy varied in intensity at wavelengths greater than 400 mu. This total spectrum of energy was transmitted through the monochromator where it was dispersed by a quartz prism. A synchronous motor was used to drive the wavelength drum of the monochromator in such a manner as to scan the spectrum from 400 to 600 mu.

The photometer was designed to measure light flux levels as low as 3.0×10^{-3} microlumen (McClure, 1957). An RCA 1P21 photomultiplier tube was employed as the detecting unit because of its high amplification factor and geometry. The circuit incorporating this photomultiplier tube is shown in **Figure 3.** The photomultiplier tube was operated at essentially constant anode current. As the fluorescence energy increased the anode current increased with a simultaneous increase in the IR drop across the anode resistor R_1. This increase in IR_1 drop caused the accelerating voltage across the photomultiplier tube to decrease by increasing the voltage drop across the triode. This triode control permitted maximum sensitivity at low light levels, and also prevented phototube damage at high light levels. The response of the system was approximately logarithmic over the entire range of accelerating voltages from 975 to 200 volts. However, since the accelerating voltage varied only from 975 to 900 volts for no light to maximum light conditions respectively, the change of voltage can be considered linear with respect to light intensity.

The spectrofluorometer was allowed to warm up for 24 hours prior to all measurements to assure maximum stability of all circuit components. The monochromator slit width was .2 mm for the turgid material and .16 mm for the desiccated and cured samples. This adjustment was made to offset the increase in fluorescence in the desiccated and cured tissue.

Measurements were made as follows. With no sample in the black box and wavelength set at 395 mu, the recorder and photometer were zeroed. The recorder was then brought to zero after each sample was placed in the box for testing. After each twenty samples were run, a spectrum was plotted from a fluorescent glass standard to check instrument response. The instrument

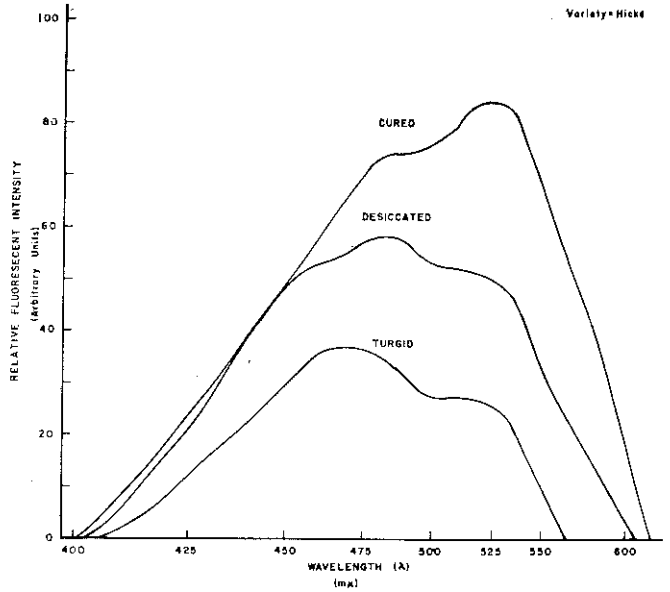

Figure 4. Relative fluorescence spectra of intact tobacco (Hicks: cured, desiccated, and turgid leaves).

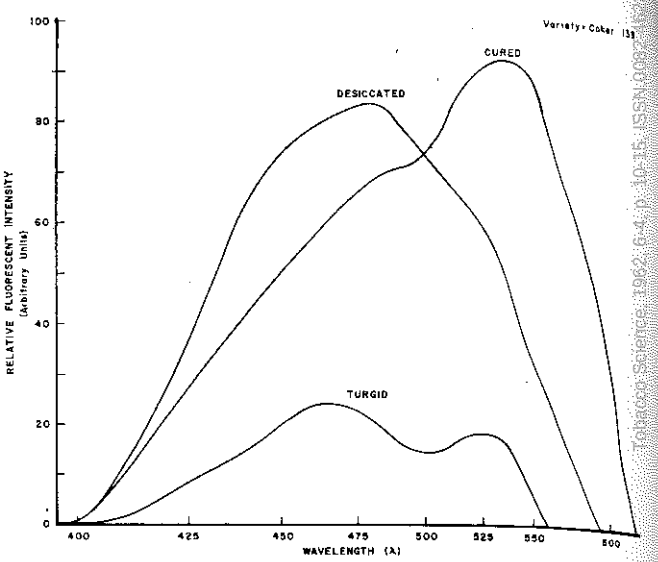

Figure 5. Relative fluorescence spectra of intact tobacco (Coker 139: cured, desiccated, and turgid leaves).

was adjusted against the standard to compensate for zero drift in the DC amplifier. Since adjustments were made on zero and span of the instrument, there was no need for adjustment of phototube sensitivity.

Methods. Tobacco leaves tested were from the fourth and fifth generations of varieties as grown for curability stndies at the Oxford Experiment Station, Oxford, North Carolina (McClure and Weldon, 1959). Three varieties were tested: Hicks Broadleaf (referred to as Hicks hereafter), Coker 139 (C 139), and Dixie Bright 101 Cherry Red (101 CR).

In 1955 studies were begun on these and eleven advanced breeding lines to determine genetic influence on curability. At the end of the first growing season the individual plants (50 per variety) were reassembled for grading purposes. From these plants the five "best" and the five "poorest" plants were chosen on the basis of how they had cured. Choices were made by three qualified tobacco men on the basis of color, body, and texture within a particular variety. The five best plants were designated as A and the five poorest plants were B. In subsequent years the $A_1 \ldots A_4$, and $B_1 \ldots B_4$ selections were generated from the five best and five poorest plants of A and B respectively. It was apparent from the three indices, color, body, and texture, that this procedure had produced best (A) selections that were different from the poor (B) selections. The difference between these two selections was greater in 101 CR than in Hicks and C 139. For example, 101 CR-A_4 was void of almost all traces of cherry red color while 101 CR-B_4 was extremely red in appearance. For the other varieties the B selection generally possessed a greater amount of "toady" tobacco than the A selection. Furthermore, it was presumed that selection A was generally superior to selection B.

For this spectrofluorometric experiment on intact leaf samples, the three varieties were planted and grown by conventional cultural practices. They were grown in two replications of more than 50 plants per variety. At maturity fifty normal-appearing plants were tagged, twenty-five from each replication. Only ten of these plants were chosen at random for testing purposes when harvesting began.

Three different leaf conditions were tested; namely, turgid, desiccated, and cured conditions. All samples were discs two inches in diameter and were taken from between the fourth and fifth lateral veins. A turgid sample was taken from one side of a representative mature leaf at each of the five harvest stages. After measurements were made on the turgid samples they were quick-dried (desiccated) in an oven at 160° F for 24 hours. The samples were then removed and allowed to come to moisture equilibrium at 70° F and 70% R.H. (the ambient conditions for all measurements). The cured samples were taken from the same relative position but on the opposite side of the test leaves after being subjected to the conventional flue-curing process.

The turgid leaf samples were brought from the field in a high humidity chamber to preserve, as much as possible, the turgidity of the leaf material. The desiccated and cured samples were allowed to reach moisture equilibrium at ambient conditions (70° F and 70% R.H.) before their fluorescence spectra were recorded. However, preliminary investigations showed that ambient air changes of 10-20% R.H. had very little influence on the fluorescence of the leaf material.

In all, there were three varieties tested, an A and B selection for each variety, ten plants in each selection, and five primings. This gave a total of 300 samples of intact leaves to be subjected to spectrofluorometric analysis. All measurements were made on the bottom side of the leaf samples to reduce the effect foreign material deposits (deposits which were noted on the top of the leaves).

Experimental Results

Fluorescence Spectra of Bright-Leaf Tobacco. **Figures 4, 5** and **6** illustrate the spectral response of intact bright-leaf tobacco to ultraviolet light. The spectral distributions are representative of all the samples tested. It was found that all fluorescent curves were similar in shape but varied in height or intensity. The curves are relative; that is, the ordinate values are not absolute fluorometric units. However, since leaf differences were of primary consideration a relative indication was sufficient. Therefore, one curve may be compared to another and different points on the same curve may be compared.

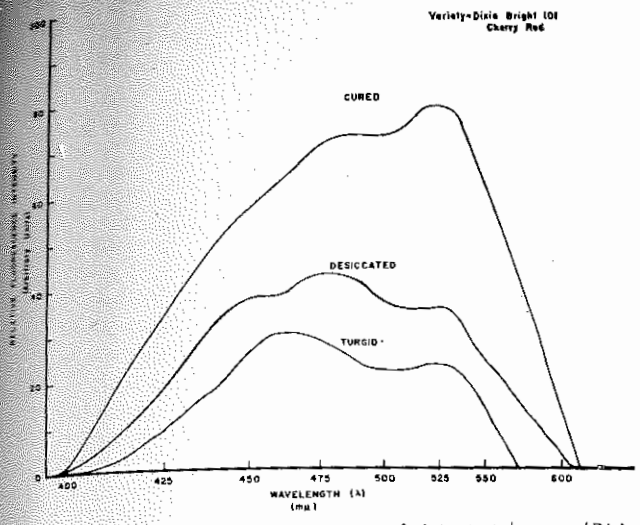

Figure 6. Relative fluorescence spectra of intact tobacco (Dixie Bright 101 CR: cured, desiccated, and turgid leaves).

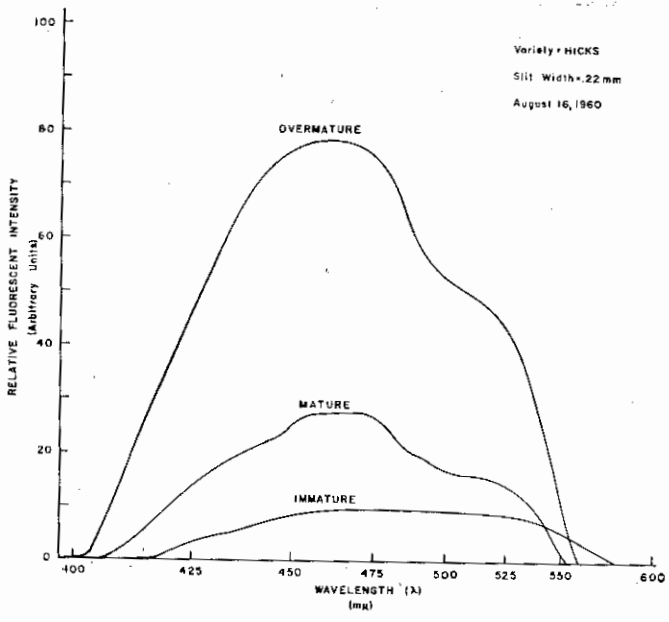

Figure 7. Fluorescence-maturity relationship of Hicks.

Figures 4, 5 and 6 indicate two prominent peaks in the fluorescence spectra of intact bright-leaf tobacco. The cured leaf had a major emission peak at 525 mu with a minor peak at 480 mu. This relationship held true for all cured leaves tested. The turgid leaf, on the other hand, had its major emission at 465 mu. The secondary peak occurred at 525 mu. For the desiccated material, the prominent band occurred at 480 mu and the minor band at 525 mu.

The experimental evidence shows two distinct phenomena. First, it is noted that the prominent peak of the desiccated leaf had shifted toward longer wavelengths as compared to that of the turgid leaf. This shift could be due to the change in the bond relationship between chloroplast pigments and other components of the chloroplasts (Thomas, *et al.*, 1956). Second, the fluorescence spectra show that during the curing cycle a chemical or physical change takes place which causes the major and minor peaks of the turgid leaves to switch. This is, the 465 mu peak becomes less prominent while the 525 mu peak increases to a maximum as the turgid leaf is cured. This change is marked due to the fact that the 525 mu peak increases much more rapidly than the 465 mu peak.

Maturity Determination of Turgid Leaves from Fluorescence spectra. Measurements were also made to determine whether fluorescence is a function of maturity. Tests were made on immature samples taken from leaves which were almost fully expanded but definitely immature, mature samples taken from leaves having an "optimum" amount of yellow-green color, and overmature samples taken from leaves having a very yellow but sound body.

Figure 7 shows the spectral distribution for the three maturity levels of Hicks (C139, and 101 CR follow the same pattern). The fluorescence peak which showed the greatest difference between maturity levels occurred at 465 mu for all varieties (the major fluorescence emission band of turgid leaves). This fluorescence-maturity relationship existed for all croppings in all varieties. Since these determinations were made on gross maturity differences, there remains a need for quantitative verification.

Determination of Leaf Differences. Each leaf condition (turgid, desiccated, and cured) was treated separately for determining leaf differences. Differences between varieties, selections, and croppings were of primary consideration.

A complete fluorescence spectrum, from 400 mu to 600 mu, was plotted automatically for each sample. Examination of the spectra indicated that the slope of the distribution in the blue-violet region was the best indication of leaf differences. The difference between two predetermined points on the relative fluorescence curves (Δf) was taken as the statistic for analyzing leaf differences because this number was proportional to the slope. For the turgid samples, the statistic was the difference between the intensity at 465 mu and at 420 mu. The Δf of the cured and desiccated tissue was the difference between the intensities at 450 mu and 400 mu.

Table 1 gives the mean Δf values for the three varieties tested. The differences between varieties were significant at the 0.01 confidence level for all three sample conditions. Note that Hicks and C 139 had Δf values which were increasingly larger for the turgid, desiccated, and cured leaves respectively. On the other hand, 101 CR switched for the cured leaf; that is, the cured leaf had a lower Δf value than the desiccated leaf for this variety. The average Δf value for 101 CR-A was 40.8 and for 101 CR-B was 26.6 giving the average 33.7 shown in **Table 1.** Hence,

Table 1. Δf Values[a] for Varieties

Variety	Turgid*	Desiccated*	Cured*
Hicks	24.2	36.7	47.0
Coker 139	28.0	55.8	77.9
D B 101	17.1	35.0	33.7

[a] *Each entry is mean of 100 observations.*
* *Differences significant at 0.01 confidence level.*

Table 2. Δf Values for Varieties and Selections[a] within a Variety

Variety	Turgid		Desiccated		Cured	
	A	B	A	B	A	B
Hicks	23.8	24.6	29.8	43.5	46.4	47.6
C 139	24.3	31.8	48.4	63.2	77.6	78.1
101 CR	15.4	18.8	30.1	40.0	40.8	26.6
Selection Average	21.1	25.1**	36.1	48.9*	54.9	50.8***

[a] *Each selection entry within a variety is the mean of 50 observations.*
* *Differences significant at 0.01 confidence level between selection averages.*
** *Differences significant at 0.05 confidence level between selection averages.*
*** *Differences significant at 0.10 confidence level between selection averages.*

Table 3. Δf Values[a] for Croppings

Cropping Number	Turgid*	Desiccated*	Cured*
1	27.9	40.6	46.2
2	19.1	26.8	50.9
3	21.1	58.2	62.7
4	28.7	43.9	50.7
5	18.7	43.0	53.8

[a] *Each entry is mean of 60 observations.*
* *Differences significant at 0.01 confidence level.*

the switch was primarily due to the predominance of cherry red color in 101 CR-B. It is noted also that C 139 had a Δf value higher than Hicks and 101 CR. Hicks had a Δf value higher than 101 CR, however, the average difference between these two (7.4) was not as great as the average difference between C 139 and Hicks (17.9).

Table 2 is a breakdown of the Δf values within each variety. Differences between selections were significant at the 0.05 level for the turgid and desiccated samples and at the 0.10 level for the cured samples. In general, it was noted that the A selection had a Δf value lower than the B selection. The one exception was 101 CR cured where the B selection had the lower Δf. The largest significant difference occurred in the desiccated material.

Table 3 gives the Δf values for all five croppings from lugs to the tips. The highest Δf values occurred in the third priming for the desiccated and cured samples but in the fourth for the turgid material. In the cured and desiccated leaf textures there is a drop in fluorescence from the first to the second priming while the cured samples demonstrate an increase. There is some support for the thought that this occurs due to the large amount of foreign material present on the leaf surface of the bottom leaves. If this foreign material fluoresced it could cause the high readings for the first priming of desiccated and turgid leaves. On the other hand, since the cured samples had been handled numerous times most of the foreign material dropped off.

Summary of Findings and Conclusions

The fluorescence spectra of bright-leaf tobacco have very definitive characteristics. The cured leaf had a prominent fluorescence peak at 525 mu. The turgid and desiccated leaves had major and minor fluorescence peaks at 465 mu and 480 mu respectively. The Δf values increased for the turgid, desiccated, and cured leaves in this sequence.

Measurements made on the immature, mature, and overmature leaves show definite differences in Δf values. The Δf values increased as the tobacco leaves became more mature.

The three varieties tested show definite differences. 101 CR, Hicks, and C 139 had higher Δf values respectively. The cured samples gave the best index of variety differences.

There were two very encouraging differences in this experiment. First, C 139, a discount variety, stood out from the other varieties according to the Δf values. This variety had the highest average Δf value of all three. Second, the B selection of all three varieties (with the one exception of 101 CR-B-cured) had a higher Δf value than did selection A. This seems to indicate that tobacco of low "quality" has a high Δf value; that is, a low Δf value is desirable in mature tobacco.

It must be pointed out, however, that the evidence presented here is not conclusive by any means. Further studies are being made of the fluorescence excitation spectra of the intact leaf. Intensive considerations are being given to the fluorescence spectra, and fluorescence excitation spectra of the major individual leaf compounds in their pure state. These studies will permit qualification of the optimum absorption band of the tobacco leaf. Other studies, such as spectrophotometric analysis in the ultraviolet, visible, and infrared, offer promising results in evaluating, non-destuctively, tobacco leaf properties related to quality. Hence, the final objective assay of leaf quality may depend on a combination of measurements, of which spectrofluorometric analysis could be one.

Literature Cited

Dement, J., Fluorochemistry. Chemical Publishing Co., Inc., New York (1945).

Duggan, D. E., R. C. Bowman, B. R. Brodie and S. Udenfriend, "A spectrophotofluorometric study of compounds of biological interest." *Arch. Biochem. Biophys.* 68:1-14 (1947).

Hercules, D. M., "Some factors affecting fluorescence maxima." *Science* 125, 1242 (1957).

Johansen, R., "Differential fluorescense in identification of tobacco trashy leaf." *Nature* 171, 753 (1953).

Jones, G. L., W. K. Collins and J. M. Kenyon, Measured Crop Performance: Tobacco. Research Report No. 23. Dept. of Field Crops, North Carolina State College, Raleigh (1960).

Kiser, F. W., A Study of Fluorescence Measurements of Tobacco Tissue. Unpublished report. Agricultural Engineering Dept., N. C. State College, Raleigh.

Klick, C. C., J. G. Shomacher and R. G. Stokes. "Absorption, Reflection, Luminescence Emission, and Excitation Measurements with the Beckman Model DU Quartz Spectrophotometer." Review of Scientific Instruments. 22: 776-82 (1951).

McClure, W. F., Comparative Fluorescence of Intact Tobacco. Unpublished report. Agricultural Engineering Department, N. C. State College, Raleigh (1958).

McClure, W. F., and N. W. Weldon

Curability Relationships Between Various Strains and Individual Plants Within Certain Strains of Tobacco. Unpublished report. Agricultural Engineering Department, N. C. State College, Raleigh (1959).

Parker, C. A. and W. J. Barnes. "Some experiments with spectrofluorimeters and filter fluorimeters." *Analyst* 82:606-18 (1957).

Penn, P. T. and J. A. Weybrew. "Some factors affecting the content of the principle polyphenols in tobacco leaves." *Tobacco* 146:20-24 (1958).

Prymale, Jr., W. S., "A sensitive photoelectric method for determining the chromaticity of phosphorescent and fluorescent materials." *J. Opt. Soc. Am.* 27:399-402 (1947).

Sprince, H. and G. R. Rowley, "Quinine calibration of the Aminco-Bowman spectrophotofluorimeter." *Science* 125:25 (1957).

Studer, F. J., "A method for automatically plotting spectral energy distributions of luminescent materials." *J. Opt. Soc. Am.* 38:467-470 (1948).