

1308

Report on the Determination of Total Alkaloids, Nicotine, and Nornicotine in Tobacco*

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The Analytical Methods Committee of the Tobacco Chemists Conference decided that a study of methods for the determination of total alkaloids (as nicotine) in tobacco should be undertaken. This decision was reached because of several factors:

1. The relatively new method of Cundiff and Markunas (1) had never been subjected to interlaboratory study.
2. The new apparatus and procedure of Griffith (2) had been adopted by several laboratories.
3. The only collaborative study since the establishment of the A.O.A.C. official procedure (3) in 1911 had been that of Willits,

et al. (4) in 1953, and this study was not completed to the point at which a complete procedure could be recommended.

Consequently it was decided that the following three methods should be tested: I—Cundiff-Markunas, II—Griffith; and III—a tentative procedure based on the results of Willits' study. In addition the collaborators were asked to use their own laboratory procedure, Method IV, if it differed from the three listed above. A copy of Method III was provided each collaborator and each was asked to supply the Committee with a copy of Method IV. Any deviations from the published procedures for Methods I and II or from the procedures provided for Methods III and IV were to be noted when the analytical results were submitted.

Six samples were prepared for collabora-

* The authors of this report comprise the Analytical Methods Committee of the Tobacco Chemists Conference, C. L. Ogg, Chairman.

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tive analysis. Five were tobacco samples of the following types: 1, burley; 2, flue-cured; 3, cigar filler; 4, Maryland; and 5, Turkish. The sixth sample was a synthetic mixture of nicotine picrate and nornicotine picrate on Filtercel. This mixture contained 1.21% nicotine, 0.38% nornicotine, and 1.63% total alkaloid as nicotine and was included to provide a test of the accuracy and precision of the Cundiff-Markunas method, the only method being tested which could differentiate between the two alkaloids normally found in tobacco in appreciable amounts. The tobacco samples were ground to pass a 1 mm sieve, equilibrated in air, mixed by rolling in a glass carboy, and placed in screw-capped glass jars. To minimize gain or loss of moisture between the time of bottling and analysis, the rims of the caps were sealed with refrigerator tape. Analytical results were requested on the "as received" basis and total alkaloids were to be reported as nicotine. The collaborators also were requested to make duplicate analyses of each sample by each method used and to report all data.

METHODS

Method I.—The procedure of Cundiff and Markunas (1) was to be followed. Any deviations from the recommended procedure were to be noted when the results were reported.

Method II.—Griffith's (2) apparatus and procedure were to be used as published.

Method III.—This was referred to as the tentative method and was based on the results of the study by Willits, *et. al.* (4). Details are as follows:

Apparatus

(a) *Distillation apparatus.*—A. O. A. C., Griffith-Jeffrey, or other suitable steam distillation apparatus may be used.

(b) *Volumetric flasks.*—1000 and 100 ml.

(c) *Pipets.*—Set ranging from 5 to 50 ml.

(d) *Absorption cells.*—1 cm, quartz.

(e) *Spectrophotometer.*—Beckman Model DU or other instrument capable of accurately measuring absorbance in 200–300 m μ range.

Reagents

(a) *Alkali-salt solution.*—Dissolve 100 g NaCl and 100 g NaOH in water and dilute to 1 L.

(b) *Silicotungstic acid solution.*—(For gravi-

metric determination only.) Dissolve 120 g SiO₂·12WO₃·26H₂O in water and dilute to 1 L. (Solution should be clear and free from green color.)

Procedure

Weigh accurately between 2 and 5 g of tobacco sample and transfer to distillation flask or apparatus. If final determination of nicotine is to be gravimetric, use sample weight which will give at least 0.1 g of nicotine; if spectrophotometric, use sample of at least 2 g.

Place 25 ml HCl (1+9) in suitable receiver (1 L volumetric flask is desirable) and place receiver so that condenser tube dips into HCl solution.

Add 50 ml of the alkali-salt solution to distillation flask so that sample is rinsed into bottom of flask. If a larger volume of liquid is required for proper function of still, add more alkali-salt solution; do not dilute.

Connect flask to apparatus immediately and steam distill with as rapid a current of steam as can be condensed efficiently. Effluent condensate should not be above room temperature.

Apply heat to the distillation flask to prevent steam condensate from diluting the alkali-salt solution. Use a burner, mantle, or other heat source to keep the volume in the flask approximately constant.

Collect approximately 900 ml of condensate (or distill additional 100 ml after condensate shows no nicotine by the silicotungstic acid test). Dilute distillate to volume.

(a) *Spectrophotometric determination of nicotine.*—Dilute aliquots of distillate (if necessary) with 0.05N HCl so that the absorbance at 259 m μ is between 0.5 and 0.8 and read the absorbance at 236, 259, and 282 m μ .

Calculate corrected A'_{259} by following equation: $A'_{259} = 1.059 [\text{obs. } A_{259} - \frac{1}{2}(A_{236} + A_{282})]$, after correcting all observed A values to original volume basis. Concentration, c , of nicotine in g/L is given by: $c = A'_{259}/34.3b$, where b is the length of the cell in cm. Calculate % alkaloid (as nicotine) by: % alkaloid = $c \times \text{vol. distillate (L)} \times 100/\text{g sample weight}$, when distillate is 1 L and cell is 1 cm. % alkaloid = $A'_{259} \times 2.915/\text{g sample weight}$.

(b) *Gravimetric determination.*—Determine nicotine in distillate as in A.O.A.C. Method, but double the amount of silicotungstic acid specified, i.e., use 1 ml per each 5 mg nicotine suspected.

Method IV.—Each collaborator used his own method and supplied a copy of the procedure with his results

Table 1. Percentage of total alkaloid as nicotine by the Cundiff-Markunas Method

| Coll. | Sample 1 | Sample 2 | Sample 3 | Alkaloid, % Sample 4 | Sample 5 | Sample 6 | s |
|----------------|----------|----------|----------|-------------------------|----------|-------------------|---------|
| 2 | 2.86 | 1.08 | 3.68 | 2.34 | 0.68 | 1.42 | 0.013 |
| 4 | 3.03 | 1.15 | 3.76 | 2.48 | 0.74 | 1.52 | 0.009 |
| 5 | 3.06 | 1.15 | 3.77 | 2.51 | 0.70 | 1.55 | 0.010 |
| 6 ^a | 2.91 | 1.11 | 3.87 | 2.90 | 0.70 | — | — |
| 7 ^b | 3.12 | 1.16 | 3.90 | 2.81 | 0.67 | 1.64 | — |
| 8 | — | — | — | — | — | 1.49 | — |
| 9 | 2.78 | 1.06 | 3.51 | 2.32 | 0.67 | 1.42 | 0.008 |
| 11 | 3.04 | 1.14 | 3.82 | 2.59 | 0.73 | 1.52 | 0.028 |
| 12 | 2.91 | 1.06 | 3.69 | 2.27 | 0.66 | — | 0.005 |
| 13 | 2.97 | 1.12 | 3.78 | 2.46 | 0.72 | 1.54 | 0.031 |
| 14 | 2.93 | 1.12 | 3.74 | 1.95 | 0.65 | 1.52 | 0.042 |
| \bar{x} | 2.95 | 1.11 | 3.72 | 2.37 | 0.69 | 1.50 ^c | (0.018) |
| s_m | 0.096 | 0.043 | 0.097 | 0.199 | 0.034 | 0.051 | |

^a Method of Jeffrey and Eoff, *Anal. Chem.*, 27, 1903 (1955). Sample 4 contained 0.4% myosmine, all others 0.0.

^b Method used distillation followed by paper chromatography and spectrophotometric analysis (method in development).

^c Theoretical = 1.63% total alkaloid as nicotine.

Results

The per cent total alkaloids as nicotine obtained for the six samples by those collaborators who used Method I are shown in Table 1. The values reported are the averages of duplicate determinations. The data obtained by Collaborators 6 and 7 are also included in this table, although the methods used were not those of Cundiff and Markunas. The data, however, were not included in the calculation of interlaboratory means or standard deviations. These were placed in Table 1 because the methods used measured nicotine and normicotine in addition to total alkaloids. Both methods employed paper chromatography to separate the alkaloids, but they differed in that Collaborator 6 obtained the total alkaloids by extraction whereas Collaborator 7 used distillation.

The intralaboratory standard deviations,

s values, were all reasonably good, the highest being 0.04. Interlaboratory standard deviations, s_m values, for all samples except 4, Maryland tobacco, were less than 0.1, showing generally good agreement between laboratories. The mean of all collaborators except No. 7 was lower than the theoretical value (1.63%) for the total alkaloids in Sample 6. As noted above, Collaborator 7 liberated the alkaloids by distillation whereas all others used extraction.

Five collaborators employed the method of Griffith (2) in analyzing the five tobacco samples. Their average values and standard deviations are shown in Table 2. The interlaboratory mean, \bar{x} and the standard deviation, s_m , for each sample are included in the table. Again the s_m value for Sample 4 was much greater than that for the other samples. The rather high s_m of 0.183 for Sample

Table 2. Percentage of total alkaloids as nicotine by the Griffith method

| Coll. | Sample 1 | Sample 2 | Alkaloid, % Sample 3 | Sample 4 | Sample 5 | s |
|----------------|----------|----------|-------------------------|----------|----------|---------|
| 1 ^a | 3.00 | 1.14 | 3.99 | 2.78 | 0.71 | 0.023 |
| 2 | 3.12 | 1.11 | 3.98 | 2.85 | 0.70 | 0.033 |
| 4 | 2.90 | 1.02 | 3.57 | 2.06 | 0.68 | 0.017 |
| 5 | 3.06 | 1.12 | 4.04 | 2.88 | 0.66 | 0.024 |
| 7 | 3.12 | 1.16 | 3.90 | 2.81 | 0.67 | 0.009 |
| 9 | 3.02 | 1.11 | 3.83 | 2.33 | 0.71 | 0.010 |
| \bar{x} | 3.04 | 1.10 | 3.86 | 2.59 | 0.68 | (0.019) |
| s_m | 0.091 | 0.051 | 0.183 | 0.370 | 0.022 | |

^aData arrived too late to include in calculation of \bar{x} and s_m values.

Table 3. Percentage of total alkaloids as nicotine by the tentative method

| Coll. | % Alkaloid in Sample | | | | | | Still | Data |
|----------------|----------------------|-------|-------|-------|-------|---------|------------------|-------|
| | 1 | 2 | 3 | 4 | 5 | s | | |
| 1 ^a | 3.01 | 1.11 | 3.85 | 0.99 | 0.75 | 0.068 | AOAC | grav. |
| 2 | 3.10 | 1.14 | 4.05 | 2.37 | 0.70 | 0.078 | Griffith | spec. |
| 3 | 2.98 | 1.08 | 3.93 | 2.26 | 0.59 | 0.196 | AOAC | spec. |
| 4 | 2.97 | 1.10 | 3.87 | 2.15 | 0.73 | 0.030 | AOAC | grav. |
| 5 | 3.06 | 1.16 | 3.87 | 2.59 | 0.76 | 0.027 | AOAC | spec. |
| 6 | 3.03 | 1.10 | 3.76 | 2.73 | 0.67 | 0.019 | Griffith-Jeffrey | spec. |
| 9 | 2.80 | 1.01 | 3.50 | 1.94 | 0.63 | 0.003 | — | spec. |
| 11 | 3.11 | 1.16 | 3.97 | 2.60 | 0.75 | 0.032 | AOAC | grav. |
| 13a | 2.89 | 1.05 | 3.80 | 2.59 | 0.67 | 0.032 | — | spec. |
| 13b | 3.02 | 1.12 | 3.95 | 2.39 | 0.69 | 0.067 | — | grav. |
| \bar{x} | 3.00 | 1.10 | 3.86 | 2.40 | 0.69 | (0.041) | | |
| s_m | 0.100 | 0.050 | 0.160 | 0.253 | 0.055 | | | |

Data arrived too late to include in calculation of \bar{x} and s_m values.

3 was caused by one average value being markedly lower than the other four. Collaborator 5 analyzed Sample 6 by Methods II and III and obtained 1.60% and 1.62%, respectively, as compared with the theoretical value of 1.63%.

The tentative method, III, was tested by nine collaborators using a variety of stills and either the spectrophotometric or gravimetric procedure in the final step. The data obtained, still used, and method of measuring the alkaloids are shown in Table 3. The s , \bar{x} and s_m values are reported here as in Tables 1 and 2. The intralaboratory standard deviations were not as good as for the other two methods but were satisfactory in most cases. The high value for Collaborator 3 was caused by one poor pair of duplicates. The interlaboratory standard deviations were

again reasonably good for Samples 1, 2 and 5 but not for 3 and 4. The average alkaloid content for the three who used the gravimetric method was a little higher than the average for those who used the spectrophotometric method for 4 of the 5 samples, but the differences were not significant statistically.

Eight collaborators analyzed the samples by procedures other than the three under test. The data obtained are shown in Table 4 with a reference or comment on the methods used. Agreement among the methods is generally good except for Sample 4, which contains large amounts of nornicotine. This is to be expected because some of the methods used were designed to try to determine only nicotine. The methods were ranked for each sample by assigning a rank of 1 to

Table 4. Percentage of total alkaloids as nicotine by collaborator's chosen procedure

| Coll. | % Alkaloid in Sample | | | | | | Method |
|-----------------|----------------------|-------|-------|-------------------|-------|---------|--|
| | 1 | 2 | 3 | 4 | 5 | s | |
| 2 | 3.12 | 1.14 | 3.98 | 2.85 | 0.69 | 0.035 | Similar to II |
| 3 | 2.81 | 0.97 | 3.75 | 1.10 ^b | 0.63 | 0.021 | <i>Ind. Eng. Chem.</i> , 29, 45 (1937) |
| 4 | 2.84 | 1.07 | 3.64 | 1.21 ^b | 0.67 | 0.044 | AOAC |
| 6 | 2.93 | 1.13 | 3.88 | 2.92 | 0.71 | 0.010 | Similar to III ^a |
| 9 | 3.02 | 1.10 | 3.88 | 2.33 | 0.68 | 0.019 | Similar to III |
| 10 | 3.10 | 1.11 | 4.05 | 2.48 | 0.71 | 0.015 | AOAC |
| 11 | 2.94 | 1.08 | 3.84 | 0.87 ^b | 0.68 | 0.016 | <i>Ind. Eng. Chem.</i> , 29, 45 (1937) |
| 12 | 2.84 | 1.08 | 3.67 | 0.37 ^b | 0.62 | 0.038 | <i>Anal. Chem.</i> , 25, 1784 (1953) |
| 15 ^c | 3.03 | 1.08 | 3.96 | 2.26 | 0.68 | 0.016 | <i>Japan Analyst</i> , 2, 364 (1956) |
| \bar{x} | 2.95 | 1.09 | 3.84 | 2.65 | 0.67 | (0.025) | |
| s_m | 0.120 | 0.053 | 0.143 | 0.247 | 0.033 | | |

^a 0.150 g sample, 4 ml 30% NaOH satd with NaCl, 250 ml distillate.

^b Not included in \bar{x} and s_m values because method was designed primarily to measure only nicotine.

^c Data not included in calculation of \bar{x} and s_m values.

Table 5. Summary of means, standard deviations, and coefficients of variation for the four methods and five tobacco samples

| Method | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|-------------------------------|-------------|-------------|-------------|-------------|-------------|
| Means | | | | | |
| I | 2.95 | 1.11 | 3.72 | 2.37 | 0.69 |
| II | 3.04 | 1.10 | 3.86 | 2.59 | 0.68 |
| III | 3.00 | 1.10 | 3.86 | 2.40 | 0.69 |
| IV | 2.95 | 1.09 | 3.84 | 2.65 | 0.67 |
| Standard Deviations | | | | | |
| I | 0.096 | 0.043 | 0.097 | 0.199 | 0.034 |
| II | 0.091 | 0.051 | 0.183 | 0.370 | 0.022 |
| III | 0.100 | 0.050 | 0.160 | 0.253 | 0.055 |
| IV | 0.120 | 0.053 | 0.143 | 0.247 | 0.033 |
| Coefficients of variation (%) | | | | | |
| I | 3.25 | 3.87 | 2.61 | 8.40 | 4.93 |
| II | 2.99 | 4.64 | 4.74 | 14.3 | 3.24 |
| III | 3.33 | 4.55 | 4.14 | 9.32 | 7.97 |
| IV | 4.07 | 4.86 | 3.72 | 9.32 | 4.93 |

the method giving the highest value, 2 to the second highest, etc. The total of the rankings for the four top methods is as follows: 9 for the method of Collaborator No. 2, 10½ for No. 10, 13 for No. 6 and 19 for No. 9. The method employed by Collaborator 2 was similar to Method II which is designed to determine total alkaloids. The second ranking method was the A.O.A.C. procedure, and the third and fourth were similar to Method III.

The results obtained by the four methods for determining alkaloids in tobacco are summarized in Table 5. Agreement between the means obtained by the four methods is generally good. The variations for Sample 4 were again the greatest, with Sample 3 next. Analysis of variance, methods versus collaborators, was made by using the data obtained by the four collaborators who used Methods I, II, and III. An analysis of variance was made by using the data for Samples 1 and 4, and the average of all five samples. No critical *F* values were obtained, although the variance due to collaborators was markedly higher than that due to methods in each case. An analysis of variance also was made by using the data from the six collaborators who used both Methods I and III. The average for the five samples was used and when these averages were arranged in descending order it was noted that the same collaborator was high for both methods and the same was low for both. An *F* value for collaborators of 4.68 (*F*.05=

5.05) was obtained as compared with 0.185 for methods.

To put the interlaboratory standard deviations for the different samples and methods on a comparable basis, coefficients of variation have been calculated and listed in Table 5. The interlaboratory precision for the different methods is quite uniform for all samples except 4 whose coefficients average more than twice those for the other samples.

Determination of Nicotine and Nornicotine

The Cundiff-Markunas procedure, I, was used by eight collaborators to determine the nicotine and nornicotine in the six samples submitted. In addition, two collaborators, 6 and 7, determined these constituents by other methods. Collaborator 8 analyzed only Sample 6 because he did not receive the other samples in time for analysis. The data obtained and their statistical evaluation are shown in Table 6. The data for Collaborators 6 and 7 were not included in the calculations.

Intra- and interlaboratory standard deviations were about the same in this study as for the total alkaloid measurements; that is, they were reasonably good except for the interlaboratory agreement on Sample 4. The average value, \bar{x} for nicotine in the synthetic Sample 6, was 0.03% above the amount added, whereas the nornicotine \bar{x} value was 0.14% low and the total alkaloid value shown in Table 1 was low by 0.13%.

The most logical explanation for these low

Table 6. Percentage of nicotine and nornicotine found by the Cundiff-Markunas Method

| Coll. | % Nicotine in Sample | | | | | | | % Nornicotine in Sample | | | | | | |
|----------------|----------------------|-------|-------|-------|-------|-------------------|---------|-------------------------|-------|-------|-------|-------|-------------------|---------|
| | 1 | 2 | 3 | 4 | 5 | 6 | s | 1 | 2 | 3 | 4 | 5 | 6 | s |
| 2 | 2.73 | 1.02 | 3.50 | 0.88 | 0.60 | 1.24 | 0.055 | 0.12 | 0.05 | 0.16 | 1.34 | 0.07 | 0.16 | 0.040 |
| 4 | 2.85 | 1.09 | 3.67 | 1.23 | 0.72 | 1.28 | 0.018 | 0.16 | 0.06 | 0.08 | 1.16 | 0.02 | 0.23 | 0.012 |
| 5 | 2.86 | 1.07 | 3.66 | 1.00 | 0.64 | 1.26 | 0.041 | 0.18 | 0.07 | 0.10 | 1.38 | 0.06 | 0.27 | 0.046 |
| 6 ^a | 2.85 | 1.1 | 3.85 | 0.4 | 0.7 | — | — | 0.06 | 0.01 | 0.02 | 2.1 | 0.0 | — | — |
| 7 ^b | 2.81 | 1.02 | 3.50 | 0.28 | 0.62 | 1.23 | — | 0.09 | 0.04 | 0.13 | 2.12 | 0.04 | 0.38 | — |
| 8 | — | — | — | — | — | 1.19 | — | — | — | — | — | — | 0.28 | — |
| 9 | 2.63 | 1.01 | 3.41 | 0.83 | 0.63 | 1.12 | 0.020 | 0.14 | 0.05 | 0.10 | 1.54 | 0.03 | 0.28 | 0.024 |
| 11 | 2.90 | 1.08 | 3.75 | 0.63 | 0.70 | 1.30 | 0.025 | 0.13 | 0.05 | 0.06 | 1.78 | 0.02 | 0.19 | 0.030 |
| 12 | 2.75 | 1.01 | 3.64 | 0.71 | 0.62 | — | 0.009 | 0.14 | 0.05 | 0.04 | 1.43 | 0.04 | — | 0.004 |
| 13 | 2.80 | 1.08 | 3.68 | 0.88 | 0.70 | 1.24 | 0.024 | 0.16 | 0.04 | 0.10 | 1.44 | 0.02 | 0.26 | 0.026 |
| 14 | 2.67 | 1.03 | 3.57 | 0.70 | 0.51 | 1.25 | 0.061 | 0.18 | 0.07 | 0.11 | 0.57 | 0.14 | 0.24 | 0.036 |
| \bar{x} | 2.77 | 1.05 | 3.62 | 0.86 | 0.64 | 1.24 ^c | (0.031) | 0.15 | 0.06 | 0.09 | 1.33 | 0.05 | 0.24 ^c | (0.027) |
| s_m | 0.096 | 0.034 | 0.110 | 0.192 | 0.068 | 0.057 | — | 0.022 | 0.012 | 0.036 | 0.354 | 0.040 | 0.044 | — |
| Coeff. var. | 3.47 | 3.24 | 3.04 | 22.3 | 10.8 | 4.60 | — | 14.7 | 20.0 | 40.0 | 26.6 | 80.0 | 18.3 | — |

^a Method of Jeffrey and Eoff, *Anal. Chem.*, 27, 1903 (1955). Sample 4 contained 0.4% myosmine, all other alkaloids 0.0
^b Method used distillation followed by paper chromatography and spectrophotometric analysis (in development).
^c Theoretical Nicotine = 1.21%, Nornicotine = 0.38%.

values seems to be that the nornicotine picrate was not completely extracted from the Filtercel. Incomplete acetylation of the nornicotine possibly contributed to the low value for this alkaloid and the slightly high value for nicotine. The only procedure which obtained the theoretical amount of nornicotine was that of Collaborator 7 in which the alkaloids are isolated by distillation. A further indication that nornicotine when in relatively high concentrations is not extracted completely or is bound by Celite or Filtercel is the difference of 0.22% in total alkaloids found by Methods I and II for Sample 4. Also the higher nornicotine obtained by the distillation procedure of No. 7 for Sample 4 indicates that there is something inherent in the extraction procedure of Cundiff and Markunas which leads to low values for nornicotine in tobacco.

It is recommended* that the study be continued.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 42, 18 (1959).