

Determination of Secondary, Tertiary, and Total Alkaloids in Tobacco*

The results of last year's studies (1) indicated that certain changes in the Cundiff-Markunas and in the so-called "tentative" methods were needed. Subsequently, the Cundiff-Markunas method (2) was modified (3) to increase the recovery of nornicotine from tobacco and to eliminate the necessity for potentiometric titration when high nornicotine tobaccos were encountered. These modifications, made by the authors of the original paper, were: (a) the use of sodium hydroxide in place of barium hydroxide to liberate the alkaloids, and (b) the addition of glacial acetic acid to the acetylated sample before titration with perchloric acid. Also, the 1958 studies showed that Griffith's method was a little more efficient in recovering alkaloids than the tentative method; consequently, a new procedure was written specifying the distillation conditions of Griffith (4) but permitting the use of any of the conventional apparatus, including Griffith's.

The two modified methods and five tobacco samples were sent to collaborators with the

request that samples be analyzed in duplicate and that all data be reported on forms supplied. In addition to having a choice of stills in the distillation method, the collaborators were allowed to choose between the gravimetric and spectrophotometric methods for measuring the alkaloids distilled. Four of the five samples, the flue cured, Turkish, Maryland, and cigar filler, were the same tobaccos used in 1958, but the burley tobacco was replaced by a new sample with a higher alkaloid level. The samples, which had been ground to pass a 1 mm sieve and had been mixed well, were equilibrated in air and sealed in screw-capped glass jars. The samples were to be analyzed "as received" and the data reported on the same basis.

METHODS

A. DISTILLATION PROCEDURE

Total Alkaloids (As Nicotine)

Apparatus

(a) *Distillation apparatus*.—AOAC 5.106, Griffith-Jeffrey (5), Griffith (4) (available from Consolidated Glass Products Co., Kingsport, Tenn.) or other suitable steam distillation apparatus may be used.

(b) *Spectrophotometer*.—Beckman DU or other instrument capable of accurately measur-

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² American Tobacco Co., Richmond 24, Va.

³ Phillip Morris, Inc., Richmond 15, Va.

⁴ Consolidated Cigar Corporation, Glastonbury, Conn.

ing absorbance in 200-300 $m\mu$ range, equipped with 1 cm quartz cells.

Reagents

(a) *Alkali-salt solution*.—Dissolve 300 g NaOH in 700 ml H_2O and saturate with NaCl.

(b) *Silicotungstic acid solution (for gravimetric determination only)*.—Dissolve 120 g $SiO_2 \cdot 12WO_3 \cdot 26H_2O$ in H_2O and dilute to 1 L. (Solution should be clear and free from green color.)

Distillation

Weigh accurately 2-5 g tobacco sample and transfer to distillation flask or apparatus. (If final determination of nicotine is gravimetric, use sample which will give at least 0.1 g alkaloids; if spectrophotometric, use sample of at least 2 g.) (If Griffith still is employed, use 0.05-0.20 g sample; if Griffith-Jeffrey still, 0.2-0.5 g.) Place 25 ml HCl (1 + 4) in suitable receiver (1 L volumetric flask is desirable) and place receiver so that condenser tube dips into HCl solution. (With smaller stills, use 10 ml HCl (1 + 4) in 250 ml volumetric flask). Add 50 ml alkali-salt solution to distillation flask so that sample is rinsed into bottom of flask. (With smaller stills, use 5 ml alkali-salt solution.) If large 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 current of steam as can be condensed efficiently. Effluent condensate should not be above room temperature. Apply heat to distillation flask to prevent steam condensate from diluting alkali-salt solution. Use burner, mantle, or other heat source to keep volume in flask approximately constant. Collect about 900 ml of condensate (or distill additional 100 ml after condensate shows no nicotine by silicotungstic acid test). Collect 225 ml with smaller stills. Dilute distillate to volume.

Determination

(a) *Spectrophotometric*.—Dilute aliquots of distillate (if necessary) with 0.05N HCl so that absorbance at 259 $m\mu$ is 0.5-0.8 and read absorbance at 236, 259, and 282 $m\mu$. Calculate corrected A'_{259} which equals 1.059 [observed $A_{259} - 1/2 (A_{236} + A_{282})$] after correcting all observed A values to original distillate volume basis. Concentration, c , of alkaloids as nicotine in g/L is given by $c = (A'_{259}/34.3) \times b$, where b is the length of the cell in cm. Calculate % alkaloid (as nicotine) = $c \times$ volume distillate $\times 100/g$ of sample.

(b) *Gravimetric*.—Determine alkaloids in distillate as in 5.106, but double amount of silicotungstic acid specified, i.e., 2 ml for each 10 mg alkaloids suspected.

B. CUNDIFF-MARKUNAS PROCEDURE

This method, which determines total alkaloids (as nicotine), tertiary alkaloids (as nicotine), and secondary alkaloids (as nornicotine), is described in detail in the article, "Modification of the Extraction Procedure for the Determination of Alkaloids in Tobacco," by R. H. Cundiff and Peter C. Markunas (3).

Results

Twelve collaborators reported 14 sets of values for the five samples by the distillation procedure. Two laboratories determined the alkaloids by both the spectrophotometric and gravimetric methods. The data are shown in Table 1, where s is the intralaboratory standard deviation calculated from the difference between duplicates, \bar{x} is the mean of collaborators' averages, and s_m is the interlaboratory standard deviation. Good precision was obtained within all laboratories, with only one having an s value as high as 0.1. This was presumably due to the abnormal behavior of one sample. The interlaboratory precision is considered acceptable but not as good as desired.

Four collaborators used the Griffith still and four used the AOAC apparatus. A comparison of data from these two groups shows excellent agreement between interlaboratory means, but shows better precision, in general, between those using the Griffith still. Nine sets of data were obtained by the spectrophotometric method and four sets by the gravimetric method. Of the nine, four used Griffith stills, two Griffith-Jeffrey, two AOAC, and one the Kjeldahl apparatus modified to admit steam. The four who used the gravimetric method also used the AOAC or similar Kjeldahl still; thus, comparing results by the AOAC and Griffith stills is similar to comparing results by the gravimetric and spectrophotometric methods. Thus it is not possible to separate the effect of the AOAC still from that of the gravimetric method.

There are three sets of data, 13, 15, and

Table 1. Percentage total alkaloids (as nicotine) by the distillation method

Collab. No.	Sample ^a					s	Still	Grav. or Spectro.	
	1	2	3	4	5				
1	1.10	4.68	0.72	2.73	3.95	0.044	Griffith	—	x
2 ^b	1.10	4.64	0.73	2.41	4.01	0.100	Griffith	—	x
3	1.04	4.35	0.70	2.66	3.49	0.064	AOAC	x	—
4	1.10	4.78	0.69	2.50	4.03	0.040	AOAC	x	—
5	1.12	4.71	0.73	2.77	3.95	0.008	Griffith	—	x
6	1.10	4.67	0.71	2.61	3.81	0.020	G-J ^c	—	x
7	1.15	4.82	0.75	2.80	4.04	0.006	Griffith	—	x
9	1.00	4.64	0.66	2.62	3.87	0.018	Griffith	—	x
11	1.16	4.85	0.76	2.67	4.11	0.041	AOAC	x	—
13	1.11	4.71	0.66	2.86	3.73	0.035	Kjeld. ^d	—	x
14	1.13	4.84	0.75	3.04	3.98	0.050	G-J	—	x
15	1.10	—	0.74	2.86	3.94	0.025	AOAC	—	x
11a	1.12	4.84	0.72	2.83	4.03	0.027	AOAC	—	x
13a	1.04	4.61	0.64	(1.74)	3.66	0.116	Kjeld.	x	—
\bar{x}	1.10	4.71	0.71	2.75	3.89				
s_m	0.046	0.140	0.038	0.145	0.173				
Griffith \bar{x}	1.10	4.71	0.72	2.73	3.95				
AOAC \bar{x}	1.10	4.70	0.72	2.70	3.92				
Griffith s_m	0.065	0.100	0.039	0.076	0.070				
AOAC s_m	0.044	0.237	0.029	0.160	0.248				
Grav. \bar{x}	1.08	4.65	0.69	2.61	3.83				
Spectro. \bar{x}	1.10	4.74	0.72	2.79	3.92				
Grav. s_m	0.058	0.223	0.058	0.078	0.290				
Spectro. s_m	0.042	0.077	0.035	0.120	0.102				

^a Sample 1, flue-cured; Sample 2, Burley; Sample 3, Turkish; Sample 4, Maryland; Sample 5, cigar filler.

^b Data arrived too late to be included in the calculation of \bar{x} and s_m values.

^c Griffith-Jeffrey still (5).

^d Modified to admit steam.

11a, obtained with the AOAC or similar still and the spectrophotometric method. The \bar{x} and s_m values for these three sets agree well with those of the other six sets where the Griffith or Griffith-Jeffrey still and the spectrophotometric method were used. This would indicate that interlaboratory differences are due more to methods than to stills. If this is true the gravimetric method produced slightly lower values for all five samples and poorer interlaboratory precision for the burley and cigar filler tobaccos.

Of the two collaborators who used both methods for determining alkaloids, one obtained higher values by the spectrophotometric method, and the other obtained higher

values by the gravimetric method for all samples except No. 4, which was the Robinson strain of Maryland tobacco. The spectrophotometric method should give higher total alkaloid values as nicotine for this sample because the alkaloids are dominantly nornicotine, which has a higher absorptivity than nicotine.

The data for the 1958 and 1959 studies are summarized in Table 2. Only for the Maryland tobacco sample is there any marked difference between \bar{x} and s_m values. The method used this year gave a higher total alkaloid value for this sample, and better precision, than either the Griffith or tentative method used last year.

Total alkaloids as nicotine were determined by 8 collaborators using the modified Cundiff-Markunas method. The results, as shown in Table 3, are summarized in Table 2 along with results from last year's study and those obtained by the distillation method. Except for one collaborator, intralaboratory standard deviations were all very low. The interlaboratory standard deviations were satisfactory but were not as good as last

year, except for the sample of Maryland tobacco. The modifications in the Cundiff-Markunas method were designed to improve results on this type of sample and apparently were successful. The mean, instead of being lower than that by the distillation method is now higher, and interlaboratory precision is improved. The reason extraction gives higher values than distillation for this sample is not known and it should be pointed out that

Table 2. Summary and comparison of data from 1958 and 1959 alkaloid studies

		Flue-Cured	Turkish	Maryland	Cigar Filler	Burley
Distillation Methods						
<i>Total Alkaloids</i>						
1959	\bar{x}	1.10	0.71	2.75	3.89	4.71
	s_m	0.046	0.038	0.145	0.173	0.140
1958 (Griffith)	\bar{x}	1.10	0.68	2.59	3.86	
	s_m	0.051	0.022	0.370	0.183	
1958 (Tent.)	\bar{x}	1.10	0.69	2.40	3.86	
	s_m	0.050	0.055	0.253	0.160	
Cundiff-Markunas Method						
<i>Total Alkaloids</i>						
1959	\bar{x}	1.09	0.67	2.98	3.86	4.81
	s_m	0.085	0.054	0.137	0.160	0.082
1958	\bar{x}	1.11	0.69	2.37	3.72	
	s_m	0.043	0.034	0.199	0.097	
<i>Tertiary Alkaloids (nicotine)</i>						
1959	\bar{x}	1.05	0.63	0.94	3.63	4.44
	s_m	0.103	0.075	0.164	0.170	0.071
1958	\bar{x}	1.05	0.64	0.86	3.62	
	s_m	0.034	0.068	0.192	0.110	
<i>Secondary Alkaloids (normicotine)</i>						
1959	\bar{x}	0.04	0.03	1.90	0.17	0.32
	s_m	0.046	0.029	0.105	0.067	0.047
1958	\bar{x}	0.06	0.05	1.33	0.09	
	s_m	0.012	0.040	0.354	0.036	

Table 3. Percentage of total alkaloids (as nicotine) by the modified Cundiff-Markunas method

Collab. No.	Sample ^a					s
	1	2	3	4	5	
1	1.07	4.89	0.77	3.03	3.91	0.026
2 ^b	1.10	4.77	0.62	3.02	3.90	0.099
4	1.09	4.81	0.69	2.83	4.05	0.048
5	1.24	4.90	0.64	3.09	3.97	0.141
9	1.00	4.67	0.67	3.10	3.84	0.031
11	1.08	4.86	0.65	3.11	3.95	0.023
13	1.07	4.81	0.58	2.98	3.83	0.019
14	1.18	4.75	0.67	2.94	3.80	0.031
15	0.99	—	0.66	2.73	3.52	0.043
\bar{x}	1.09	4.81	0.67	2.98	3.86	—
s_m	0.085	0.082	0.054	0.137	0.160	—

^a Sample 1, flue-cured; Sample 2, Burley; Sample 3, Turkish; Sample 4, Maryland; Sample 5, cigar filler.
^b Data arrived too late to be included in calculation of \bar{x} and s_m values.

there is a possibility that this difference is not real since with two of eight collaborators the order was reversed. The modified method also raised the total alkaloid value for the cigar filler tobacco to that obtained by the distillation procedure. For the other four samples the agreement between \bar{x} and s_m values for the distillation and Cundiff-Markunas methods is very good, and shows that for the usual tobacco sample, the two methods are equally satisfactory.

The tertiary and secondary alkaloid data, as nicotine and nornicotine respectively, obtained by the eight collaborators who used the Cundiff-Markunas method, are shown in Table 4. Although the data are not used in calculating \bar{x} and s_m , the values reported by two collaborators (6 and 7) who used paper chromatography to separate the two alkaloids are included in the table.

Two sets of \bar{x} and s_m values are reported; the first includes all data by the proposed method except those of collaborator 2 (whose data arrived late) and the second includes all values except those reported by collaborators 1, 2, and 4. Collaborator 1 suggested that his data not be used because he thought that his glacial acetic acid was not

Table 4. Percentage of tertiary and secondary alkaloids as nicotine and nornicotine respectively by the modified Cundiff-Markunas method

Collab. No.	Sample					s
	1	2	3	4	5	
Nicotine						
1	1.01	4.72	0.40	2.33	3.80	0.034
2	1.11	4.40	0.66	0.91	3.61	0.080
4	0.99	3.94	0.46	0.75	3.29	0.089
5	1.12	4.54	0.61	1.16	3.86	0.165
6 ^a	all	3.75	all	0.15	all	—
7 ^b	0.89	3.98	0.64	0.25	3.58	0.036
9	0.97	4.39	0.62	1.11	3.55	0.036
11	1.05	4.50	0.61	0.90	3.72	0.030
13	0.97	4.39	0.51	0.80	3.60	0.012
14	1.23	4.40	0.74	0.92	3.68	0.160
15	0.99	—	0.66	0.75	3.36	0.034
\bar{x}^c	1.04	4.41	0.58	1.09	3.61	—
s_m	0.087	0.240	0.120	0.524	0.203	—
\bar{x}^d	1.05	4.44	0.63	0.94	3.63	—
s_m	0.103	0.071	0.075	0.164	0.170	—
Nornicotine						
1	0.06	0.16	0.34	0.65	0.11	0.019
2	0.0	0.34	0.0	1.94	0.26	0.023
4	0.09	0.80	0.22	1.97	0.69	0.074
5	0.11	0.33	0.03	1.76	0.11	0.044
6 ^a	trace	0.13	trace	1.9	trace	—
7 ^b	0.08	0.39	0.03	2.21	0.21	0.015
9	0.03	0.25	0.06	1.82	0.27	0.019
11	0.03	0.34	0.03	2.02	0.21	0.023
13	0.09	0.38	0.07	1.99	0.21	0.017
14	0.0	0.32	0.0	1.84	0.11	0.145
15	0.0	—	0.0	1.96	0.14	0.031
\bar{x}^c	0.05	0.37	0.09	1.75	0.23	—
s_m	0.042	0.203	0.116	0.454	0.190	—
\bar{x}^d	0.04	0.32	0.03	1.90	0.17	—
s_m	0.046	0.047	0.029	0.105	0.067	—

^a Method of Jeffrey and Eoff, *Anal. Chem.* 27, 1903 (1955). Sample 4 contains 0.1–0.2% myosmine plus traces of anatabine and anabasine.

^b Unpublished method using distillation, paper chromatography, and spectrophotometry.

^c Data for collaborators 2, 6, and 7 not included.

^d Data for collaborators 1 and 4 also omitted because No. 1 questioned whether his acetic acid was glacial and No. 4 had trouble determining the end point of the acetylated sample.

glacial; this would introduce water and cause low nornicotine and high nicotine results in samples containing relatively large amounts of nornicotine. Another collaborator commented that he had difficulty in determining the endpoint when titrating the acetylated sample. The directions for titrating the acetylated sample have been revised in an attempt to clarify this operation. Both collaborators obtained good values for total alkaloids so their trouble was with acetylation or titration of the acetylated sample.

Intralaboratory precisions were generally very good; however, the interlaboratory standard deviations for all except the Maryland sample were not as low as last year. The same was true for total alkaloids by this method; this raises the question as to the cause for the higher s_m values. This could be due to lack of experience with the modified method or the modification itself. If the latter is true, the original method could be used for normally low nornicotine samples and the modified method for tobaccos with appreciable amounts of the secondary alkaloids. Based on the precisions obtained in the two years' studies, such a system would seem reasonable, but if the \bar{x} values are compared with those from the distillation method, the modified method appears to be better than the original.

A point of further concern is the low nicotine values obtained for the Maryland sample by the two who used chromatographic separation techniques. These values, 0.15 and 0.25%, are drastically different from the \bar{x} of 0.94% obtained by the non-aqueous titration procedure. This same difference was observed in the data obtained last year for the same sample. The question is: Does a high nornicotine content cause erroneously low nicotine values by the chromatographic technique, or is there a relatively large amount of tertiary nitrogen-containing material extracted from the Maryland sample which is not nicotine?

The modified Cundiff-Markunas method gives results for total alkaloids as nicotine which agree reasonably well with those obtained by the distillation method, and is better than the original method for tobaccos with high nornicotine contents.

Recommendations

The Referee recommends¹—

- (1) That the distillation method tested this year be adopted as first action.
- (2) That the modified Cundiff-Markunas method tested this year be adopted as first action, and
- (3) That studies of this method be continued to determine if the interlaboratory precision will improve with usage and to test further its accuracy on samples of abnormally high nornicotine content.

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¹ These recommendations were approved by Subcommittee A and adopted by the Association. See *This Journal*, **43**, 126 (1960).