Quantitative Determination of Individual Alkaloids in Tobacco by Gas Chromatography

LOUIS D. QUIN and NICHOLAS A. PAPPAS[1]

Department of Chemistry, Duke University, Durham, N. C.

Methods based on gas chromatography have been developed for the determination of nicotine and nornicotine in tobacco. In their present state, the new methods are useful where the amount of alkaloid exceeds about 0.2% of the tobacco. The time for an analysis is short, and accurate results are obtained. The greatest advantage of the gas chromatographic technique is its specificity for the individual alkaloid. Gas chromatography has also been successfully used in the procurement of small samples of the pure alkaloids from tobacco.

Previous reports (*4*) from this laboratory have described the successful use of gas chromatography in separation, identification, and determination of a number of tobacco alkaloids as found in cigarette smoke. It appeared that this technique might also be used in determination of the alkaloids in tobacco itself. There is a definite need at present for analytical methods which are specific for nicotine or nornicotine and which are sufficiently precise and rapid to be valuable in routine work on tobacco samples. A gas chromatographic procedure should in principle offer a solution to this problem. Successful translation into practice of this proposal is the subject of this paper.

Experimental

Gas Chromatographic Equipment. A Perkin-Elmer Vapor Fractometer Model 154-B, modified as previously described (*4*), was used in conjunction with a Leeds and Northrup AZAR recorder set at 1-mv. full scale deflection. Throughout this work, the voltage to the detector was held at 8.0 volts. Column packings, temperature, and helium flow rate (determined with a soap-bubble meter at the vent) were selected to meet the specific demands of the analysis and will be described below. It is important that the instrument selected for use give at its maximum sensitivity a response equal to or greater than that of the instrument used here; with instruments of different sensitivity, modification of the sample sizes or concentrations specified herein will be necessary.

Purification of Nicotine. Commercial nicotine was found gas chromatographically to contain a number of other minor compounds, probably alkaloidal, and was not considered suitable for use in preparing standard solutions. Its purification was accomplished by gas chromatographing several 0.3- to 0.4-ml. aliquots on a 1-meter × 14 mm. in O.D. glass U column of propylene glycol (PPG, molecular weight 1025) on firebrick, 1:4, at 180° C. and with 120 ml. of helium carrier per minute. Nicotine, emerging in a broad, slightly asymmetric peak at 32 minutes, was well separated from other impurities and was collected at the vent in a dry ice–chilled U-tube. Trapped material was then rechromatographed and collected for use in the preparation of standard solutions.

Preparation of Nornicotine. Since nornicotine was unavailable commercially, its isolation from an appropriate tobacco was performed. About 200 grams of ground, cherry red tobacco, the main alkaloid of which has been reported to be nornicotine (*2*), was wetted with a 5% acetic acid solution, treated with 3 liters of benzene-chloroform, 1 to 1, v./v., 500 ml. of 36% sodium hydroxide solution, and then shaken intermittently over a 12-hour period. Celite filter aid was added and the mixture filtered on a Büchner funnel. The organic layer was separated and concentrated in vacuo to a dark oil, essentially solvent-free. Aliquots of the oil were gas chromatographed on a 1-meter × 14 mm. in O.D. column of polybutylene glycol (PBG, molecular weight 1500) on firebrick, 1:4, at 180° C. and with 120 ml. of helium carrier per minute. The nornicotine peak emerged at 63 minutes and was well separated from a substantial amount of forerunning nicotine as well as from later peaks. The collected material was somewhat red and was rechromatographed as above. About 2 ml. of a pale-yellow product was then obtained; it was free of impurities by gas chromatography and was used without further purification. To prevent fouling of the injection system in such isolation work, it would be desirable to distill in vacuo the concentrated tobacco extract prior to gas chromatography.

Analytical Extraction of Alkaloids from Tobacco. Nicotine Determination. Two grams of cured tobacco passing a 40-mesh sieve was accurately weighed and then wetted with 10 ml. of a 5% acetic acid solution; exactly 20 ml. of benzene-chloroform solution, 1 to 1 (v./v.), saturated with a 36% sodium hydroxide solution was added, followed by 10 ml. of 36% sodium hydroxide. The mixture was shaken for 20 minutes and then one spoonful of Celite was added. About 2 ml. of the organic solution was collected by filtration and used directly, in 50-μl. aliquots, for gas chromatography. If the tobacco contains less than about 0.5% of nicotine, a concentration step is necessary.

Nornicotine Determination. The same procedure was used with the following quantities: 3.0 grams of tobacco, 15 ml. of acetic acid, 50 ml. of benzene-chloroform, 10 ml. of sodium hydroxide. Exactly 15 ml. of the organic solution was then collected by filtration and concentrated to 5 ml. with a stream of nitrogen. For gas chromatography, 50-μl. aliquots were taken.

Determination of Nicotine by Gas Chromatography. A 1-meter × 6 mm. in O.D. glass U-tube packed with a 1:4 mixture of polypropylene glycol (molecular weight 1025) on firebrick (Fisher, Columpak, 30 to 60 mesh, prewashed with 2% alcoholic potassium hydroxide) was used. At 190° C. and a helium flow of 75 ml. per minute, nicotine was eluted at 5.0 minutes from this column. Resolution of the peak from the solvent was not perfect (Figure 1), but was satisfactory. If time is not of importance in the analysis, operation at 180° C. and 50 ml. per

[1] Present Address, Tobacco Institute, Drama, Greece.

Table I. Calibration Data[a] for Nicotine and Nornicotine

Concn., Mg./Ml.	Peak Height, Cm. Nicotine[b]	Nornicotine[c]
0.50	1.20	0.35
1.00	2.75	0.95
2.00	5.75	2.05
3.00	8.75	3.25

[a] Average of two determinations.
[b] Maximum sensitivity, 50 μl. on PPG 1025 at 190° C.
[c] One-half sensitivity, 50 μl. on PBG 1500 at 180° C.

Table II. Alkaloid Content of Several Tobaccos

	Nicotine, Wt. %							
	Burley		Bright		Commercial Cigarette		Cigar Filler	
Sample	G.C.[a]	C.-M.[b]	G.C.[a]	C.-M.[b]	G.C.[a]	C.-M.[b]	G.C.[a]	C.-M.[b]
1	2.04	2.07	1.68	1.70	1.28	1.42	0.59	0.59
2	2.06	2.07	1.72	1.62	1.34	1.42	0.62	0.59
3	2.06	2.12	1.68	1.65	1.24	1.42	0.56	0.59
4	1.93	...	1.67	...	1.25	...	0.59	...
5	1.93	...	1.67	...	1.31	...	0.62	...
Av.	2.00	2.09	1.68	1.66	1.29	1.42	0.60	0.59
Std. dev.	0.07	0.03	0.02	0.04	0.04	0.00	0.03	0.00

	Nornicotine, Wt. %			
	N. sylvestris		Cherry Red N. tabacum	
	G.C.[a]	C.-M.[c]	G.C.[a]	C.-M.[c]
1	1.53	1.62	1.41	1.31
2	1.56	...	1.41	1.34
3	...	...	1.41	...
Av.	1.55	1.62	1.41	1.33

[a] Gas chromatographic method, conditions as described in experimental section.
[b] Cundiff-Markunas method for total tertiary alkaloids, expressed as nicotine (1).
[c] Cundiff-Markunas method for total secondary alkaloids, expressed as nornicotine.

minute gives a peak at 10 minutes that is perfectly resolved from the solvent. The former conditions were used in this study.

A calibration curve was prepared by chromatographing 50-μl. aliquots of several solutions of nicotine in benzene. For the tobacco used here, solutions over the range 0.5 to 3.0 mg. per ml. were satisfactory. Microdipper pipets were used for sample injection throughout the analytical work. The height of the nicotine peak was used as quantitative measure. Typical data for calibration with the instrument at maximum sensitivity appear in Table I. A straight line is easily drawn through these points. The calibration curve should be checked occasionally during a day's operation.

For analysis of tobacco, the benzene-chloroform extract is injected in 50-μl. aliquots. The column is ready to receive a new sample after each nicotine peak is eluted, as no other peaks emerge during prolonged elution. However, this point has not been checked for more concentrated solutions than those used here. A typical chromatogram is shown as Figure 1; the method of peak height measurement is apparent from it. Results for several replicate samples of four different tobacco samples are presented in Table II. Values for total tertiary alkaloids were obtained with the revised Cundiff-Markunas method (1) and also appear in this table.

The ability of the method to recover nicotine was checked by adding 40 mg. of nicotine (in benzene solution) to each of two 2.0-gram samples of burley tobacco. The total nicotine content would be 80 mg. for each sample. By gas chromatography, 81.4 and 80.6 mg. of nicotine were found for the two samples.

Determination of Nornicotine by Gas Chromatography. The polypropylene glycol column was satisfactory for resolving nornicotine from nicotine, but not from anabasine. A 1-meter × 6 mm. in O.D. column of polybutylene glycol (molecular weight 1500) 1 to 4 on firebrick was found useful for this analysis. Figure 2 demonstrates resolution of these three alkaloids in a mixture produced by addition of pure anabasine to cherry red tobacco. A column temperature of 180° C. and a helium flow of 50 ml. per minute gave a retention time of 10 minutes for nornicotine.

A calibration curve was prepared from several benzene solutions of nornicotine of 0.5 to 3.0 mg. per ml. An instrumental sensitivity setting of 0.5 was used, in anticipation of a need for a curve capable of handling a variety of samples including some of higher nornicotine content than those reported here. Full sensitivity could have been used in this work. Again a straight line was easily drawn from a plot of peak height *vs.* milligrams per milliliter. Typical data are given in Table I.

Extracts from cherry red *N. tabacum* and from *N. sylvestris* were injected in 50-μl. aliquots for analysis. Results appear in Table II, along with Cundiff-Markunas values for total secondary alkaloids, expressed as nornicotine.

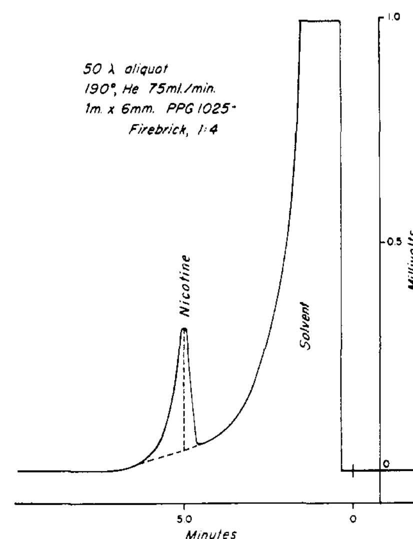

Figure 1. Gas chromatogram of alkaloid extract from bright tobacco

Discussion

The utility of gas chromatography in the quantitative determination of nicotine and nornicotine in tobacco is clearly demonstrated by this work. Under proper conditions, sharp elution peaks can be obtained for each compound in a suitable tobacco extract. The nicotine peak can be readily obtained free of interference by other alkaloids, as shown in earlier work (4). The nornicotine determination requires a column especially selected to give good resolution from anabasine, a minor alkaloid generally present in trace amounts that is eluted close to nornicotine. As seen in Figure 2, this resolution can be accomplished on a polybutylene glycol column. One other alkaloid, myosmine, also requires special care for resolution from nornicotine. While the above column is not satisfactory for this purpose, a 2-meter column of polyethylene glycol (molecular weight 20,000) operated at 150° C. gives sufficient resolution to detect any myosmine present in tobacco extract to an extent that might interfere with nornicotine determination. However, no evidence for the presence of myosmine in the tobacco used here has been found. Hence, the gas chromatographic method gives values specific for the two alkaloids under consideration. The method could be readily extended to include other volatile alkaloids; in fact, anabasine has been determined in one sample of *N. glauca*, in which it is the main alkaloid, by using conditions established for nornicotine determination.

The gas chromatograms obtained for extracts of the concentration prescribed in the experimental section are extremely simple. When nicotine is the main alkaloid, no peaks other than the large

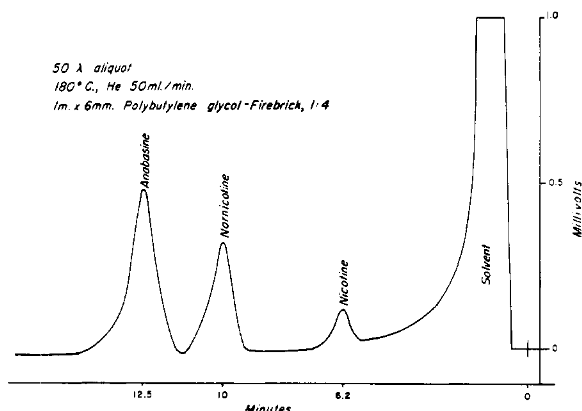

Figure 2. Gas chromatogram of alkaloid extract of cherry red tobacco with added anabasine

solvent peak and the nicotine peak itself are observed. This is a very significant point, for it is then possible to inject a new sample immediately after nicotine has been eluted. The experimental conditions were purposely selected to provide rapid elution of nicotine (5 minutes). It would thus be possible to run approximately 10 to 11 samples per hour on one instrument. The method then is well suited for rapid, routine analysis. Furthermore, it is likely that conditions giving even faster nicotine elution will be developed. An occasional shutdown to clean the injection assembly is necessary, as nonvolatile residues develop therein. Also, replacement of the column may become necessary after prolonged use. However, neither of these requirements represents a major detraction from instrumental operating time.

The nornicotine determination is almost as rapid. The nornicotine peak has been eluted in the present analytical study in 10 minutes, following nicotine at about 6 minutes. No other peaks are observed, except for a minor one for anabasine. However, it is not necessary to wait for this peak before injecting the next sample. Thus under present conditions about five determinations of nornicotine could be made per hour. The nicotine content can also be determined from this chromatogram when reference to a proper calibration curve is made.

Calculation of the per cent of alkaloid present is simple. Experimental conditions were purposely employed to give elution peaks sharp enough to be measured by height rather than by area. The base line is readily established, particularly for nornicotine, where it is almost perfectly horizontal to the chart line. With a proper tool, the height can be determined to three figures. It is important, however, to prepare extracts of alkaloid concentration sufficient to give, at maximum instrument sensitivity, peaks at least 1 cm., and preferably 2 to 4 cm., in height to minimize errors in height measurement. The peak height must be related to a calibration curve to determine the alkaloid content. The calibration curve is prepared from solutions whose concentration is expressed in milligrams per milliliter. With proper selection of sample size, the value obtained for milligrams of alkaloid per milliliter of extract can be numerically the same as per cent alkaloid in tobacco. Thus, the per cent alkaloid can be simply obtained from a measurement of peak height and reference to a calibration curve. The curve itself is actually a straight line, obtained by plotting peak height *vs.* concentration of known solutions. The relation is not one of direct proportionality. Since peak height is influenced by gas flow and temperature, the curve is useful only as long as these instrumental variables are held constant. In general, a curve is prepared once a day, prior to analytical work; its validity can be checked readily by running one or two known solutions.

The reproducibility of the method for nicotine is good, as is apparent from examination of the data provided in Table II. The standard deviation is of the same order as that characteristic of the Cundiff-Markunas and other methods (*3*), although more data are needed to verify this point. Fewer data were obtained for the nornicotine determination, but it appears that this method is also adequately reproducible.

That the new method is actually giving correct values was established by performing the widely accepted Cundiff-Markunas determination, as revised recently (*7*), on the same tobacco specimens. This method, based on titration in nonaqueous media of the alkaloids, provides values for total tertiary alkaloids, expressed as nicotine, and total secondary alkaloids as nornicotine. For the particular tobacco samples used in this study, it is probable that little contribution to each value is made by alkaloids other than the one used for expressing the results. That the gas chromatographic and the Cundiff-Markunas methods give essentially the same values for nicotine and nornicotine is apparent from the data in Table II. It is thus concluded that the new method gives reliable figures for the two alkaloids.

The extraction method used for nicotine determination has been essentially that developed by Cundiff and Markunas (*7*), although other procedures leading to a nonaqueous solution of the free alkaloids could be used. One difference is in the amount of organic extractant used; this work uses 20 ml. of solvent for 2.0 grams of tobacco, whereas 100 ml. is used for 2.5 grams of tobacco in the Cundiff-Markunas method. This reduction in solvent was necessary to provide an extract sufficiently concentrated in nicotine to permit direct use in gas chromatography. This concentration can be obtained from tobaccos having nicotine in excess of about 0.5%; an evaporation step would be necessary for low nicotine tobaccos. Use of the reduced amount of solvent in extraction is validated by the fact that the anticipated results for nicotine were obtained. Furthermore, in experiments where pure nicotine was added to tobacco, total recovery of nicotine was quantitative. Finally, a Cundiff-Markunas titration run on an extract with the reduced volume of solvent gave, within experimental error, the same value for total tertiary alkaloids as for extracts of the normal size. For nornicotine determination, a less drastic solvent reduction, to 50 ml. for 3.0 grams of tobacco, was employed, and no concern was felt about the occurrence of an incomplete extraction. This extract is suitable for direct analysis, if the content of nornicotine in the tobacco exceeds about 1%, although in the present work a concentration step (a 15-ml. aliquot taken to 5 ml.) was employed.

The methods as they now stand do not provide for the determination of very small amounts of nicotine or nornicotine, but are most useful when the amount of the alkaloid exceeds about 0.2% of the tobacco. If the value is much lower than this, a concentration of the extract must be performed to provide a sample giving sizable gas chromatographic peaks. In the case of highly concentrated solutions, peaks for compounds other than alkaloids may appear and may complicate the chromatograms. It will be recalled that the extraction procedure would eliminate acidic, but not neutral, compounds; it is indeed fortuitous that no extraneous elution peaks for the neutral compounds are observed in the present nicotine and nornicotine analyses. Another limitation of the new method as it now stands is that trace amounts of nornicotine, as in domestic varieties of *N. tabacum* such as bright and burley, cannot be determined accurately. The relatively large amount of nicotine in these varieties

tends to overload the column, and the nicotine interferes with the much smaller nornicotine peak. Determination of the area of the latter is then difficult. A practical value for the limiting ratio of nicotine to nornicotine because of this effect is about 20–30 to 1. However, this problem could probably be overcome by using more sensitive detecting devices, such as those based on ionization phenomena. A much more dilute solution could then be used and the broadening of the nicotine peak due to column overloading in the present work would be avoided. No experience with tobacco samples having a large nornicotine to nicotine ratio has yet been obtained.

Of more than passing interest is the use of gas chromatography to obtain sizable amounts of pure alkaloid, notably nornicotine. The columns used here are sufficiently selective to eliminate undesired volatile alkaloids, and with the use of a wide-diameter column processing of individual samples up to 0.5 gram has been realized. No significant reduction in resolution was noted with use of large columns. It has thus become possible to procure, with little difficulty, nornicotine from an appropriate tobacco, such as *N. sylvestris*, *N. glutinosa*, or cherry red *N. tabacum*, in a state free from other alkaloids. In a similar manner, pure anabasine can be obtained from a source such as *N. glausa*. Such operations would be even more feasible with the use of gas chromatographs specially designed for preparative scale work at high temperatures.

Acknowledgment

Cooperation of the Research Laboratories of the Liggett and Myers Tobacco Co., and The American Tobacco Co. is gratefully acknowledged. Samples of cherry red *N. tabacum* and *N. sylvestris* were kindly supplied by J. A. Weybrew, North Carolina State College.

Literature Cited

(1) Cundiff, R. H., Markunas, P. C., *J. Assoc. Offic. Agr. Chemists* **43,** 519 (1960).
(2) Jeffery, R. N., Tso, T. C., J. Agr. Food Chem. **3,** 680 (1955).
(3) Ogg, C. L., Bates, W. W., Jr., Cogbill, E., Harrow, L. S., Peterson, E. L., *J. Assoc. Offic. Agr. Chemists* **43,** 524 (1960).
(4) Quin, L. D., *J. Org. Chem.* **24,** 911, 914 (1959).

Received for review February 27, 1961. Accepted May 18, 1961. Taken from a thesis submitted by N. A. Pappas in partial fulfillment of the requirements for the A.M. degree, Duke University. The National Tobacco Board of Greece provided N. A. Pappas with financial aid in this study.

MOLECULAR STRUCTURE AND ACTIVITY

Effect of Alpha-Methoxylation and Nitrogen Acetylation on Absorption and Translocation of a Plant Regulator, Methyl Indole-3-acetate

JOHN W. MITCHELL and PAUL J. LINDER
Crops Research Division, U. S. Department of Agriculture, Beltsville, Md.

Alpha-methoxylation of several plant growth–regulating chemicals enhanced their absorption and translocation by plants. Alpha-methoxylation has now been extended to methyl indole-3-acetate, a growth regulator of the indole type; absorption and translocation of this regulator were also enhanced. Alpha-methoxylation together with nitrogen acetylation, however, resulted in greater translocatability than did methoxylation alone.

Substitution of a methoxy group for one of the hydrogen atoms of the alpha-carbon of several plant regulators of the phenylacetic acid type resulted in compounds that were more readily absorbed and translocated than phenylacetic acid itself (*3*). In addition, some of these methoxy derivatives were exuded from roots of plants in biologically detectable amounts (*4*, *5*). Absorption and translocation of regulators of the carbamate type were enhanced by similar structural modifications (*2*).

This article describes similar enhancement of absorption and translocation of methyl 3-indoleacetate, through alpha-methoxylation, and the effect of nitrogen acetylation on absorption and translocation of this indole compound.

Methods

Bean plants of the Pinto variety (*Phaseolus vulgaris* L.) with primary leaves approximately 4 cm. across and trifoliolate leaves folded in the terminal buds were selected for uniformity. A standard mixture of indole-3-acetic acid was prepared for comparative purposes as follows: 2 mg. of the acid was placed in a microbeaker and dissolved in 0.25 ml. of 95% ethyl alcohol. The microbeaker containing the solution was placed in 3.75 ml. of water mixed with sufficient ethyl alcohol to give a final concentration of 8% of alcohol. Forty milligrams of Tween 20 was then dissolved in the resulting solution to make the final concentration of growth regulator 0.05%, alcohol 8%, and Tween 20 1%. The related indole compounds under investigation were prepared in a similar way to give molecular concentrations equivalent to the concentration of indole-3-acetic acid in the standard mixture. Leaf treatments were made by application of 0.01 ml. of the preparation containing 5 μg. of indole-3-acetic acid or a molar equivalent amount of the related compounds as a narrow band across the upper surface of each primary leaf near the petioles of five plants. The liquid was distributed over the surface of each leaf with a glass rod (*1*). Treatment in this manner resulted in application of a total of 10 μg. of a compound to each plant.

In stem treatments, 0.01 ml. of the indole-3-acetic acid or related compounds was applied as a band approximately 1 cm. wide around the first internodes of each of five test plants. The mixture was confined to a designated area of stem surface by lanolin rings that extended around the stem (*1*). Response to leaf or stem treatments was evident as stem curvatures. These were measured and expressed as degrees.

Derivatives of indole-3-acetic acid studied were methyl 3-indoleacetate, mehyl *N*-acetyl-3-indoleacetate, methyl α-methoxy-3-indoleacetate, and methyl α - methoxy - *N* - acetyl - 3 - indoleacetate. One experiment involving the acetylated acetate, both with and without methoxylation, was carried out followed by two additional experiments in which these two indole compounds, together with the remaining ones listed above, were used.