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RAPID METHOD FOR THE ANALYSIS OF TOBACCO NICOTINE ALKALOIDS

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SUMMARY

A rapid glass capillary gas chromatographic (GC) method for the analysis of the nicotine alkaloids of tobacco, including nicotine, nornicotine, myosmine, anabasine, anatabine and 2,3′-dipyridyl, was developed. The use of glass capillary GC methodology, coupled with nitrogen-specific detection, provided rapid, unambiguous alkaloid analysis. About 25 mg of cured tobacco and 1 ml of 0.05 *M* methanolic potassium hydroxide (containing the internal standard, 2,4′-dipyridyl) were placed in a standard 2-ml autosampler vial, and the vial was capped and placed in an ultrasonic bath for 15 min. A 1-μl volume was analyzed by glass capillary GC operating in the split-injection mode on a wall-coated Superox™-4 column. This simple procedure is compared with the previously reported Soxhlet extraction–packed column GC method and the Griffith still–colorimetric method. The application of the method for the analysis of alkaloids in various cured and green, budworm-resistant, tobaccos is discussed.

INTRODUCTION

The quantitative determination of tobacco alkaloids has always been of great interest to tobacco scientists. In commercial flue-cured tobacco, nicotine constitutes about 98% of the total alkaloid fraction[1]. The important minor nicotine-type alkaloids are nornicotine, anabasine, anatabine and 2,3′-dipyridyl. Alkaloids contribute to the organoleptic properties of tobacco smoke, and alkaloid data have been used traditionally as an indicator of tobacco quality. Alkaloid determinations have shown that high levels of nornicotine in the cured tobacco produce an undesirable product[2]. Recent work indicates that nicotine and nornicotine are precursors of the

carcinogenic N-nitrosonornicotine. N-Nitrosoanatabine, another tobacco-specific nitrosamine, has also been identified in cured tobacco and in smoke[3]. Thus, data on levels of total alkaloids and individual components are important parameters in evaluating tobacco products for potential biological activity or marketing quality. It has also been reported that alkaloid levels may be responsible for resistance of some tobacco species to insects[4,5].

Commonly used methods for determining tobacco alkaloids are the steam distillation–spectrophotometric method of Griffith[6] (the Griffith still method) and the automated procedures of Harvey *et al.*[7] and Davis[8]. These methods yield excellent data for total alkaloids (calculated as nicotine), but additional steps are required to estimate the minor alkaloids.

Gas chromatography (GC) methods for quantitation of the individual alkaloids have employed unmodified or potassium hydroxide-modified liquid phases. Bush[9] extracted the alkaloids with a mixture of saturated aqueous barium hydroxide–benzene–chloroform and analyzed them by GC on a DC-550 column. Burns and Collins[10] used a 2-h Soxhlet extraction with methanol and directly analyzed the extract on a Carbowax 20M–potassium hydroxide column. Recently, Rosa[11] reported a pyrolysis–GC method for the estimation of nicotine and nornicotine. In this method, the alkaloids were directly volatilized from the tobacco sample on to the GC column in an injection-port pyrolyzer. The steps and time required for extraction in the first two GC methods and the inability to use an internal standard in the pyrolysis–GC method made these procedures undesirable for the screening of large numbers of samples in our quality, health-related and insect-resistance studies.

Consequently, we developed a method involving a brief sonification–extraction step with subsequent alkaloid separation and quantitation by glass capillary GC, with use of a nitrogen-selective detector. The method was applied to both cured and green tobacco samples, and extraction and analysis times were sufficiently decreased to permit the screening of numerous samples.

EXPERIMENTAL*

Materials

Tobaccos used in the work were grown under conditions normally used for flue-cured tobacco at the Tobacco Research Laboratory (Oxford, NC, U.S.A.), Pee Dee Experiment Station (Florence, SC, U.S.A.) and the Border Belt Tobacco Research Station (Whiteville, NC, U.S.A.). Green tobacco-leaf samples were rapidly frozen with dry ice and crushed, the stems were removed, and the lamina was stored at −20°C. Normal, flue-cured tobacco was stemmed and ground in a Wiley mill (40-mesh screen). For moisture determinations, about 200 mg of ground cured tobacco was heated for 3 h at 98° ± 0.08°C in a vented oven[12], while about 10 g of green tobacco was dried overnight (15–18 h).

Nicotine (Eastman-Kodak, Rochester, NY, U.S.A.) was distilled before use. Anabasine (99+%; Tridom Chemical, Hauppauge, NY, U.S.A.), 2,3′-dipyridyl (98%; Aldrich, Milwaukee, WI, U.S.A.), and 2,4′-dipyridyl (97%, Aldrich) were used

* Reference to a company or product name does not imply approval or recommendation by the U.S. Department of Agriculture.

as received. A "tobacco alkaloid mixture" containing nicotine and comparable amounts of the minor alkaloids (nornicotine, myosmine, anabasine, anatabine, and 2,3′-dipyridyl) was obtained from the end cut (pot residue boiling above the boiling-point of nicotine) of the fractional vacuum distillation of commercially available nicotine (which is obtained from tobacco).

Sonification–extraction procedure

About 25 mg of ground cured tobacco were weighed into a 2-ml automatic sampler vial, 1 ml of internal standard (IS) stock solution (0.25 mg/ml of 2,4′-dipyridyl in 0.05 *N* methanolic potassium hydroxide) was added, and the vial was capped. After sonification for 15 min in an ultrasonic cleaning bath (Model SC-100, Ultrasonic Industries), the sample was analyzed by glass capillary GC. For the analysis of green leaf samples, 2 to 2.5 g of green leaf was placed in an 8-dram vial, 10 ml of IS stock solution were added, the vial was capped with a PTFE-lined cap, and the sample was sonified for 15 min. About 1 ml of the resulting solution was transferred to a 2-ml automatic sampler vial and analyzed by glass capillary GC.

Soxhlet extraction procedure

The Soxhlet extraction procedure was similar to that of Burns and Collin[10]. Briefly, about 2.5 g of ground cured tobacco were weighed into a cellulose extraction thimble and 25 mg of 2,4′-dipyridyl were weighed into the extraction flask. The tobacco was extracted under nitrogen with refluxing methanol (100 ml) for 2 h in a standard Soxhlet apparatus After cooling the extract, 10 ml of 0.5 *N* methanolic potassium hydroxide were added to the extraction flask (a precipitate formed), and a 1-ml portion of the liquid was removed for glass capillary GC analysis.

Glass capillary GC analysis

The samples were analyzed with a Hewlett-Packard Model 5840 gas chromatograph equipped with a Model 7672A automatic sampler and a nitrogen–phosphorus (NPD) flame ionization detector. The standard 5840 instrument was modified for glass capillary GC analysis as previously described[13]. The injection-port glass insert was deactivated by washing with 0.5 *N* methanolic potassium hydroxide. The alkaloids were analyzed by using 60:1 split-injection on a 35 m × 0.25 mm I.D. Carbowax wall-coated open-tubular (WCOT) column (temperature program 170–200°C at 2°C/min; linear flow-velocity, 20 cm/sec of He) and on a 15 m × 0.25 mm I.D. Superox™-4 WCOT column (temperature progron 130–200°C at 4°C/min; linear flow-velocity, 25 cm/sec of He). The columns were prepared according to Arrendale *et al.*[14,15]. The NPD was operated under hydrogen- and air-flow conditions as recommended by the manufacturer. The injection-port temperature was 200°C, the detector temperature was 280°C, and 1-μl samples were injected. The NPD-response curves were obtained by analyses of a series of standard solutions. Nicotine (25 mg) and anabasine (25 mg) were dissolved in a 25-ml volumetric flask with the IS stock solution (response stock solution). Aliquots of the response stock solution were diluted to 10 ml with the IS stock solution to yield a range of nicotine and anabasine concentrations from 0.05 to 1.00 mg/ml. Nornicotine, myosmine and anatabine were assumed to have response factors similar to the response factor of anabasine (pure standards of these components were not commercially available).

Alkaloid identification

The components in the "tobacco alkaloid mixture" and in the tobacco extracts were identified by GC–mass spectrometry (MS) and by GC retention values[11]. GC–MS data were obtained on a Hewlett-Packard Model 5930A mass spectrometer coupled to a Hewlett-Packard Model 5710 gas chromatograph equipped with 1.8 m × 2 mm I.D. glass column packed with 10% Carbowax 20M–2% potassium hydroxide on Chromosorb W AW (80–100 mesh) and temperature-programmed from 180–200°C at 4°C/min. For confirmatory identification by glass capillary GC, the alkaloids were isolated by preparative GC on a packed Carbowax 20M column, and the retention times of the isolated alkaloids were determined on the capillary columns.

RESULTS AND DISCUSSION

GC analysis

The most successful GC methods for the analysis of nicotine and the minor tobacco alkaloids have involved use of Carbowax–potassium hydroxide columns[11]. The basic nature of this substrate prevents adsorption and tailing of the more basic components, such as nornicotine. However, when conventional flame ionization detection is used, the presence of neutral compounds, such as neophytadiene and aliphatic hydrocarbons, in the tobacco extract may cause errors in quantitation of the minor alkaloids. However, the use of a nitrogen-selective detector eliminates such interfering peaks and produces a very clean alkaloid profile (Fig. 1). Even so, the Carbowax column, like other packed columns[10,11], still fails to separate all the components.

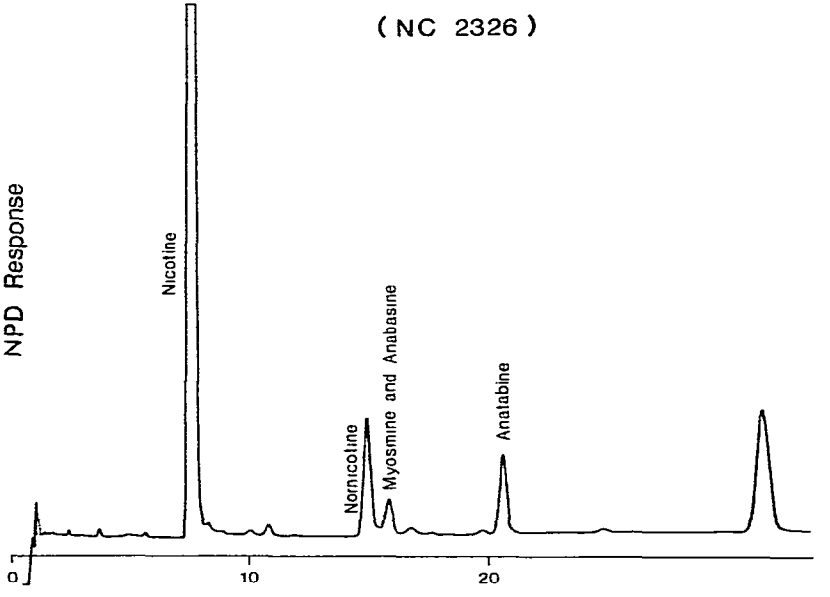

Fig. 1. Gas chromatogram of tobacco alkaloids on a packed Carbowax 20M–potassium hydroxide column using the NPD.

We felt that, because of their separation efficiencies, WCOT glass-capillary columns could be utilized to resolve the alkaloids and to shorten analysis time. Fig. 2a shows the chromatogram of the "tobacco alkaloid mixture" (obtained from the distillation of nicotine) on a Carbowax 20M WCOT column. Even though the column was very efficient (>3000 effective plates per meter), it did not resolve myosmine and anabasine. Peak shapes in this glass capillary gas chromatogram indicated a lack of surface activity. More importantly, the decrease in the relative peak areas of nornicotine, compared with that obtained on the packed column, indicated that considerable adsorption of nornicotine was occurring. The large decrease in the nornicotine peak indicated a high degree of acidity of the Carbowax 20M column. The acidic sites were neutralized by several injections of ethylenediamine, as indicated by the size of the nornicotine peak in the resulting chromatogram (Fig. 2b).

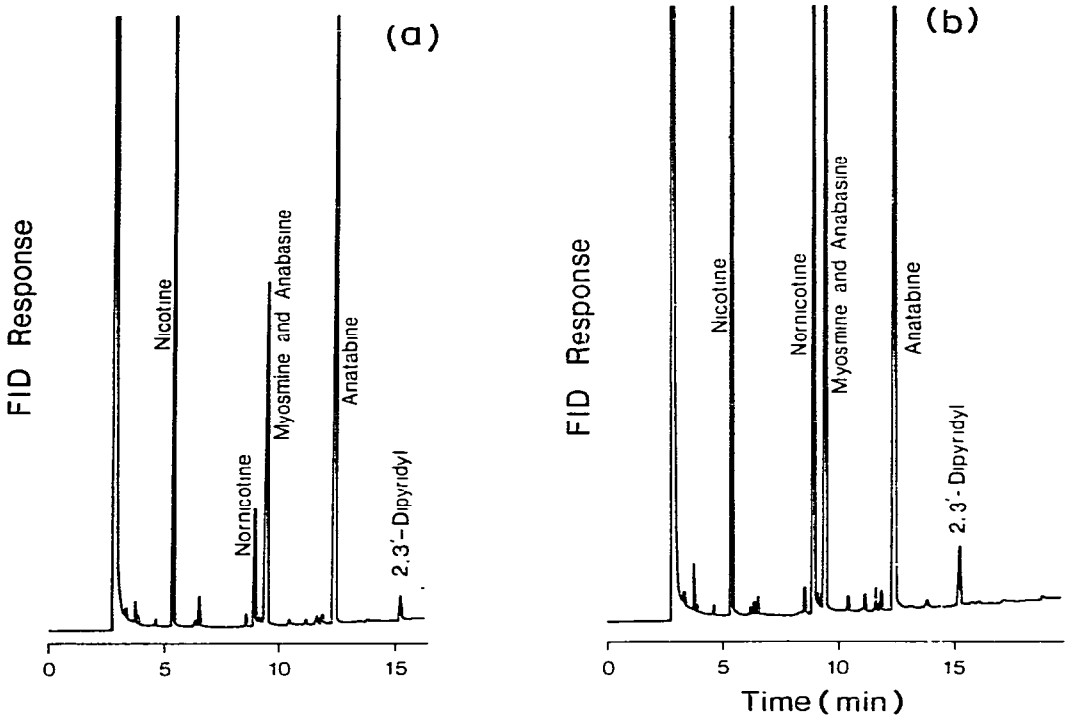

Fig. 2. Glass capillary gas chromatograms of tobacco-alkaloid mixture on a Carbowax 20M column using the FID: (a), before treatment with ethylenediamine; (b), after treatment with ethylenediamine.

After careful deactivation of its glass surface[14], a capillary column was coated[15] with Superox™-4 (a new liquid phase from Alltech; 4,000,000 mol.wt. polyethylene glycol). It did not absorb nornicotine and separated myosmine and anabasine (Fig. 3). Although it has been reported that traces of water are deleterious to Superox columns[16], we have analyzed over 400 tobacco samples without noticeable loss of column efficiency (cured tobacco contains 5–12%, and green leaf about 90% of water). Figs. 4a and 4b show the glass capillary gas chromatograms of methanol extracts of a commercial flue-cured tobacco variety (NC 2326), and a high-nornico-

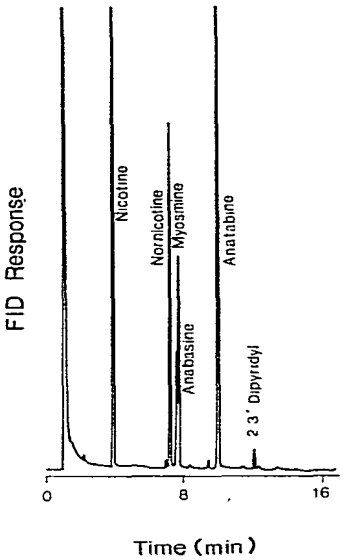

Fig. 3. Glass capillary gas chromatogram of tobacco-alkaloid mixture on a Superox™-4 column using the FID.

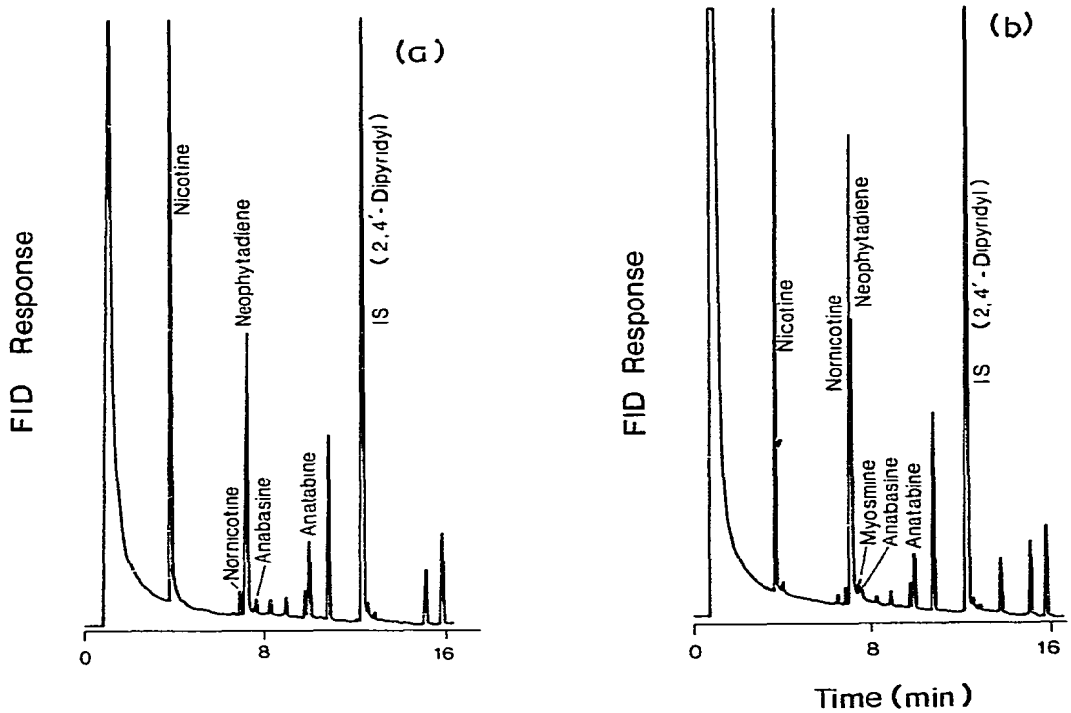

Fig. 4. Glass capillary gas chromatogram of methanol extracts of tobacco on a Superox™-4 column using the FID: (a), NC 2326 tobacco; (b), Cherry Red tobacco.

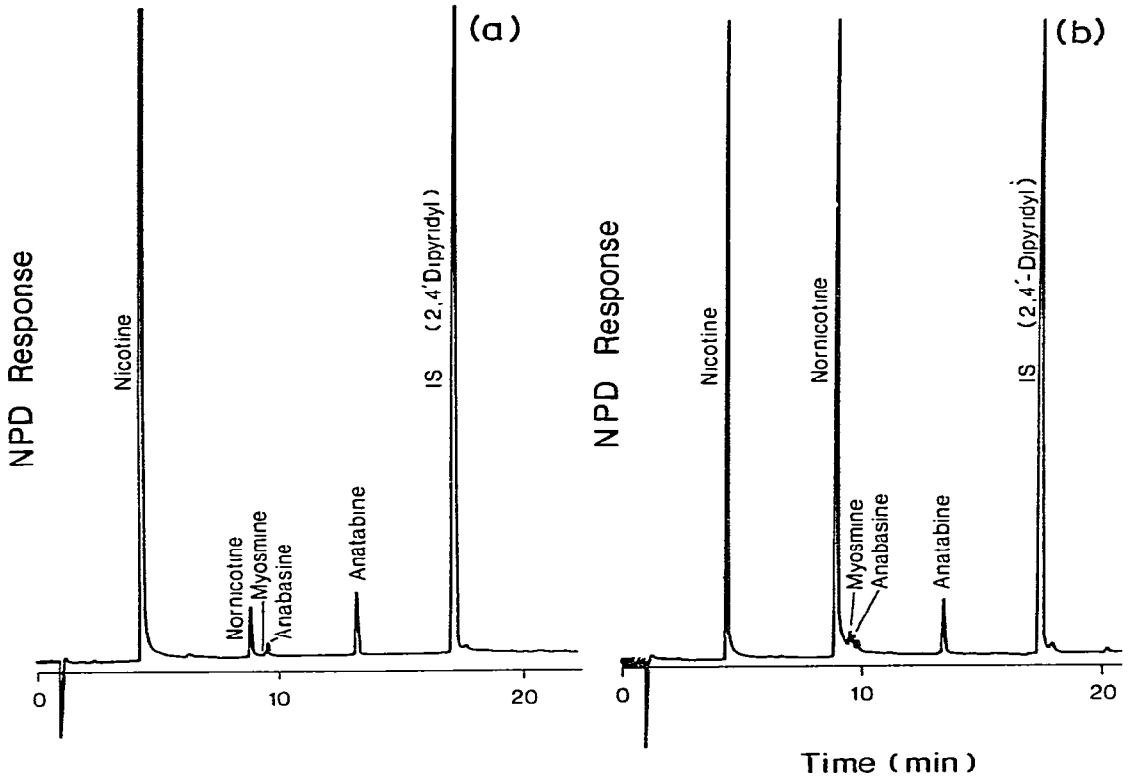

Fig. 5. Glass capillary gas chromatogram of methanol extracts of tobacco on a Superox™-4 column using the NPD: (a), NC 2326 tobacco; (b), Cherry Red tobacco.

tine-containing tobacco (Cherry Red), respectively. As in packed-column analyses, the neophytadiene and hydrocarbons of tobacco interfered in the analyses. As before, these interferences were readily eliminated by using the nitrogen-selective detector (Fig. 5).

NPD linearity

During our early work, we found that, for packed-column separations, the NPDs supplied with the instrument failed to respond linearly over the alkaloid concentration range 0.05 to 1.0 mg/ml. In contrast, linear and very reproducible response was obtained in glass capillary GC for the same detector (Table I). However, when the detectors were replaced, linear response was again not obtained. It was found that each NPD produced a different calibration curve, but showed no appreciable change in curve shape during its lifetime. Thus, for each detector, a relative response *versus* concentration curve (Fig. 6) is needed.

Tobacco analyses

The recent successful use of an ultrasonification–extraction technique for tobacco hydrocarbons and neophytadiene[17] led us to develop similar extractions for the tobacco alkaloids. As shown in Table II, we found that a simple sonification extrac-

TABLE I

COMPARISON OF RESPONSE FACTORS (K)*,** OF SELECTED ALKALOIDS TO FLAME IONIZATION (FID) AND NITROGEN-PHOSPHORUS (NPD) DETECTORS

	$K \pm$ rel. S.D.	
	*NPD****	*FID*§
Nicotine	1.63 ± 1.8	1.04 ± 2.5
Anabasine	1.45 ± 1.0	0.94 ± 1.8
2,3'-Dipyridyl	0.95 ± 1.6	0.98 ± 0.6

* Relative to 2,4'-dipyridyl on Superox™-4 column.
** K = (response of X/wt. of X)/(response of IS/wt. of IS).
*** Average of four determinations.
§ Average of three determinations.

tion yielded results identical with those obtained by the more laborious Soxhlet-extraction methods of Burns and Collin[10]. Since Burns and Collin had shown that the Soxhlet method quantitatively extracted all nicotine alkaloids, no work was needed to determine the efficiency of sonification extraction. The total-alkaloid levels obtained by our sonification–glass capillary GC method were also compared with values obtained by the widely used Griffith still method[6]. Again, good agreement between the two methods was obtained over a wide range of alkaloid levels (Table III).

Our primary aim in developing a rapid method for the analysis of tobacco alkaloids was to evaluate different breeding lines in our efforts to produce safer tobacco products for U.S. consumers. One of these efforts involves the development of pest-resistant tobaccos. Since pesticide residues on tobacco are a possible health risk for consumers, the production of insect-resistant tobacco varieties is highly desirable. Several tobacco introductions (TIs) have been identified as having resistance to the tobacco budworm[18,19], one of the most damaging insects. Green and cured leaf samples of non-resistant and resistant tobaccos were analyzed for alkaloid levels (Table IV). Since budworm damage occurs in the early developing leaf (bud leaf) of

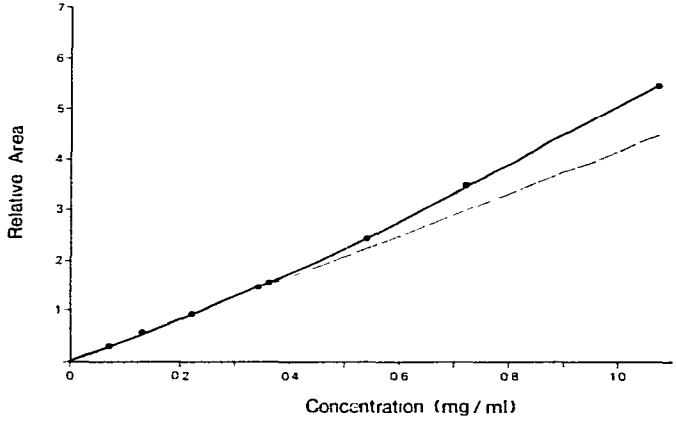

Fig. 6. Calibration curve for anabasine for a NPD.

TABLE II

COMPARISON OF DATA FROM SONIFICATION-GLASS CAPILLARY GC AND SOXHLET EXTRACTION METHODS

Alkaloid	*Distribution (%)**									
	Sonification						*Soxhlet*			
	Run:	*1*	*2*	*3*	*4*	*5*	*Av. ± rel. S.D.*	*1*	*2*	*3*	*Av. ± rel. S.D.*
Nicotine		94.4	96.5	94.6	94.8	94.9	95.0 ± 0.84	95.3	96.6	95.4	95.8 ± 0.72
Nornicotine**		2.5	2.0	2.0	2.2	1.9	2.1 ± 0.24	1.7	1.5	1.5	1.6 ± 0.12
Anabasine		0.5	0.5	0.7	0.6	0.6	0.6 ± 0.08	0.5	0.4	0.4	0.4 ± 0.07
Anatabine**		2.7	2.7	2.7	2.4	2.6	2.6 ± 0.13	2.7	2.7	2.8	2.7 ± 0.07
	*% Leaf****										
Total alkaloids		2.66	2.56	2.61	2.49	2.54	2.57 ± 0.07	2.54	2.66	2.61	2.60 ± 0.06

* Percentage of total alkaloids (NC 2326 tobacco; Oxford, NC, U.S.A., 1978).

** Assumed to yield NPD response identical to that of anabasine.

*** Based on dry weight.

TABLE III

COMPARISON OF DATA FROM SONIFICATION–GLASS CAPILLARY GC AND GRIFFITH STILL METHODS

*Tobacco variety**	*Alkaloid content (%): field replication***		
LN 53	0.40*** (0.37)§	0.28*** (0.29)§	0.18*** (0.15)§
Coker 139	1.49 (1.61)	1.10 (1.15)	1.68 (1.56)
SC 58	3.28 (3.13)	3.34 (3.24)	3.89 (3.64)
Rustica Line No. 137	4.84 (4.77)	5.73 (5.85)	4.22 (4.29)

* Grown and cured at Oxford, NC, U.S.A., 1979.
** Samples obtained from different locations in the same field.
*** Values from sonification glass capillary GC method.
§ Values from Griffith still method[6].

TABLE IV

COMPARISON OF ALKALOID LEVELS AND DISTRIBUTIONS IN BUDWORM-RESISTANT AND NON-RESISTANT TOBACCOS

*Tobacco type and stalk position**	*Total alkaloids (%)***	*Distribution (%)*				
		Nicotine	*Nornicotine*	*Myosmine*	*Anabasine*	*Anatabine*
NC 2326 (NR)						
Green-bud***	0.21	95.6	3.4	–	1.0	–
Green-middle***	0.86	96.8	1.3	–	0.4	0.5
Green-bottom***	0.71	96.6	1.6	–	0.4	1.4
Cured composite§	3.21	96.2	0.7	–	0.5	2.7
TI-1112 (R)						
Green-bud***	0.24	93.7	4.2	–	0.4	1.7
Green-middle***	0.33	63.8	33.4	–	0.3	2.4
Green-bottom***	0.39	64.0	33.4	–	0.5	2.4
Cured composite§	4.29	17.1	72.7	2.4	2.6	5.2
TI-804 (R)						
Green-middle***	0.74	87.6	9.6	–	0.8	2.0
Cured composite§	4.11	92.9	3.2	0.1	1.0	3.0
TI-163 (R)						
Green-bud§§	0.67	96.2	0.3	–	2.4	1.0
Cured composite§	4.29	94.9	1.5	0.1	0.6	3.0
TI-165 (R)§						
Green-bud§§	1.04	98.1	0.3	–	0.7	0.9
Cured composite§	5.81	95.2	2.6	0.1	0.6	2.4
TI-168 (R)						
Green-bud§§	1.00	97.8	0.2	–	1.1	0.9
Cured composite§	5.40	95.2	1.5	0.1	0.6	2.7
TI-170 (R)						
Green-bud§§	0.72	98.2	–	–	0.1	0.8

* NR = Non-resistant tobacco; R = resistant tobacco.
** Based on dry weight.
*** Whiteville, NC, U.S.A., at onset of flower, 1978.
§ Oxford, NC, U.S.A., 1979.
§§ Florence, SC, U.S.A., 1979.

the plant, we analyzed various green-leaf positions at the susceptible growing stage. The data indicated that alkaloid levels were not significant factors in resistance. The most resistant tobacco (TI-1112) was a nicotine converter, a tobacco that produces a high level of undesirable nornicotine in the final cured product. Other resistant TIs had acceptable alkaloid levels and distributions.

We have developed a simple and rapid method, based on sonification and modern glass capillary GC analytical techniques, for determining major tobacco-alkaloid levels and distributions. This short procedure permits a trained technician, using one automated glass capillary GC system, to analyze about 45 samples per day. This permits the analyses of sufficient samples for the method to be useful in the evaluation of numerous tobacco products for quality and consumer acceptability. Also, this method should have wide applicability to the analysis of volatile alkaloids in other plant extracts or organic mixtures.

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