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QUANTITATION OF MAJOR TOBACCO ALKALOIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JAMES A. SAUNDERS* and DENISE E. BLUME

United States Department of Agriculture, Science and Education Administration, Beltsville Agricultural Research Center, Tobacco Laboratory, Room 107, Building 001, Beltsville, MD 20705 (U.S.A.)

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SUMMARY

A high-performance liquid chromatographic technique was developed to quantitate the major alkaloids from both fresh green tissue and air dried tobacco leaf tissue. The procedure involves an aqueous extraction of the milled tissue followed by separation of the alkaloids on a reversed-phase C_{18} column with a mobile phase of 40% methanol containing 0.2% phosphoric acid buffered to pH 7.25 with triethylamine. This procedure provides quantitative analysis of the four major alkaloids in tobacco and can be partially automated to handle large numbers of samples.

INTRODUCTION

There are numerous reports in the literature on the detection and quantitation of tobacco related alkaloids from various tissues by a variety of methods. Nicotine, for example, has been analyzed in both animal and plant tissues by paper, thin-layer (TLC) and gas chromatography[1–8]. To provide reliable quantitative values these methods often involve extensive fractionation and extraction procedures including lengthy refluxing, dialysis, and/or multiple solvent extraction and irreversible derivatization of the alkaloids.

A convenient, rapid procedure for the quantitative analysis of tobacco alkaloids would be useful in a breeding program to control and monitor alkaloids in tobacco. Several automated procedures for the quantitation of nicotine in tobacco have been reported; however, these procedures also involve lengthy sample preparation and generally are not applied to other tobacco alkaloids[9–11]. Fejer-Kossey[12] has described the separation of the four major tobacco alkaloids, anatabine, anabasine, nicotine and nornicotine, by TLC of standards and Bush[6] reported the separation and quantitation of these same alkaloids by gas chromatography; however, both procedures would require time consuming sample preparation from tobacco leaf tissue and therefore be unsuitable for large numbers of samples.

Recently, interest in nicotine determinations in the urine of smokers has prompted the analysis of nicotine by high-performance liquid chromatography

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(HPLC)[13,14]. In addition, Jane[15] and Twitchett *et al.*[16] have described the separation of nicotine from common drugs of abuse by isocratic HPLC on silica gel columns.

The present paper now reports a procedure based on HPLC for the quantitative analysis of the four major alkaloids in *Nicotiana* namely nicotine, nornicotine, anabasine and anatabine. The procedure consistently gave reliable results, and if used with an automatic sample injector, can process large numbers of either green or air dried tobacco samples with a minimum of operator care.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters* (Milford, MA, U.S.A.) Model 710 automatic injector, an M-6000A solvent pump and a Model 440 detector equipped with a 254-nm filter. The tobacco alkaloids were quantitatively separated on a Waters μBondapak C_{18} reversed-phase column (30 cm × 4 mm) eluted with an isocratic mobile phase of 40% (v/v) methanol containing 0.2% (v/v) phosphoric acid buffered to pH 7.25 with triethylamine at a flow-rate of 0.5 ml/min. All solvents and samples were filtered through a 0.45-μm Millipore filter prior to use. Nicotine, nornicotine, anabasine and anatabine were quantitated by a Waters 730 Data Module which automatically integrated peak areas and compared them with those of authentic standards.

Chemicals

Nicotine was obtained from Eastman-Kodak (Rochester, NY, U.S.A.), anabasine and nornicotine were obtained from Pfaltz & Bauer (Flushing, NY, U.S.A.) and anatabine in the picrate form was a gift of Dr. E. Leete, University of Minnesota. Hydrolysis of the picrate crystals of anatabine was accomplished by a technique of Leete and Chedekel[17] in which crystals were dissolved in 2 *N* HCl, and the solution was extracted with diethyl ether; the aqueous phase was made basic with 6 *N* NaOH, extracted with diethyl ether and the etheral phase chromatographed on TLC silica gel plates with chloroform–methanol–NH_4OH (80:21:1). Free anatabine was recovered on the thin-layer plate with an R_F value of 0.7.

Chromatographic solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Procedure for alkaloid determinations

Air-dried leaves of *Nicotiana tabacum* USDA Tobacco Introduction Collection No. 266 were ground in a Wiley Mill with a 2-mm mesh, and the meal was oven dried at 60°C for 24 h to a constant dry weight. The milled samples were weighed into 0.5 g lots and extracted with 10 ml of 25 m*M* sodium phosphate buffer (pH 7.8) at 30°C for 24 h with constant agitation. The aqueous extract was filtered under reduced pressure through a Whatman No. 2 filter-paper and diluted ten-fold with water. Each extract was filtered through a 0.45-μm Millipore filter and sealed in a screw-capped septum vial to permit automatic injection of a 20-μl aliquot.

* Mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

One-gram samples of fresh tobacco were ground in 10 ml of 40% (v/v) methanol containing 0.1% (v/v) 1 *N* HCl with a Ten Broeck homogenizer. The homogenate was centrifuged at 500 *g* for 3 min and filtered through Whatman No. 2 filterpaper in a büchner funnel. Each sample was diluted four-fold and filtered through a 0.45-μm Millipore filter prior to automatic injection.

All quantitative determinations were made with duplicate injections and comparisons with authentic standards run intermittently with the unknown samples.

Gas chromatography

For comparative purposes authentic alkaloid standards and tobacco samples TI No. 266 were quantitated by gas chromatography after the techniques of Alworth *et al.*[18]. The gas chromatograph system consisted of a Varian Model 3700 gas chromatograph equipped with a Chromosorb W column coated with 5% OV-101 using helium as a carrier gas. The temperature was programmed to rise from 90 to 145°C at 4°C/min on the column and the alkaloids quantitated with an ion detector using a Shimadzu integrator.

RESULTS AND DISCUSSION

There have been very few reports on the quantitative determination of the four major alkaloids in tobacco by a technique which could be conveniently automated to survey large numbers of samples. Typically the technique for the analysis of alkaloids in dried tobacco entails the production of cigarettes, burning the material and collecting the smoke condensate on filter pads[11,19,20]. The pad would then be subjected to solvent elution and the alkaloids quantitated by colorimetric or chromatographic procedures. Nicotine, which is the major alkaloid of most varieties of *Nicotiana tabacum*, has been studied by a variety of investigators who were interested in its quantitative determination in smoking products and in biological fluids such as blood and urine[2–4,21–26]. In studies of this nature, the nicotine is characteristically extracted by several organic solvents and quantitated by gas chromatography.

The technique described in this paper utilizes the convenience of HPLC to quantitate the four major alkaloids of tobacco and can be used with either green or dried material. While there is little sample preparation involved in the analysis of each sample, several different related compounds of interest can be analyzed at the same time. The technique is rapid and reliable and the process can be adapted to handle large numbers of samples. Fig. 1 represents a typical HPLC elution profile of the major tobacco alkaloid standards nicotine, nornicotine, anabasine and anatabine when separated on a μBondapak C_{18} reverse phase column using a mobile phase of 40% methanol containing 0.2% phosphoric acid buffered to pH 7.25 with triethylamine. Retention times at a flow-rate of 0.5 ml/min under these conditions are 23.3 min for nicotine, 13.9 min for anatabine, 12.2 min for anabasine and 11.0 min for nornicotine. To determine the reproducibility of the HPLC assay over a range of concentrations of alkaloids, standard amounts of nicotine, nornicotine and anabasine were quantitatively recovered from various buffered systems (Table I). This table shows that the HPLC system could detect the alkaloids at the 40–50 ng level and that the quantitative recovery was reliable throughout the range tested.

Fig. 1 shows that nicotine exhibited a slight trailing effect under these condi-

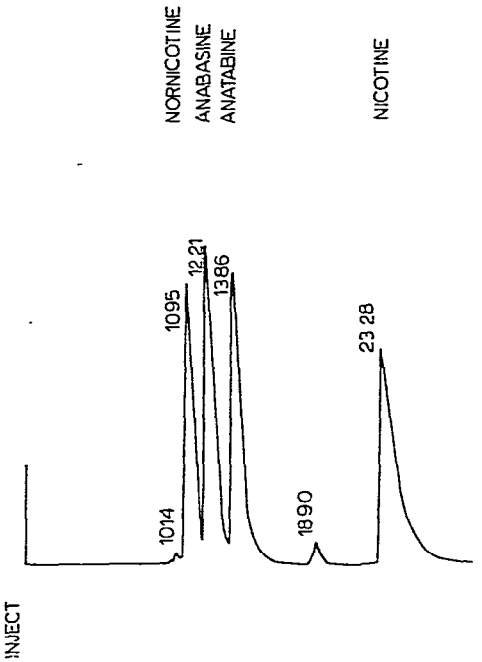

Fig. 1. Separation of standards of the four major alkaloids in tobacco by a HPLC system containing a μBondapak C_{18} reversed-phase column. The mobile phase consisted of 40% (v/v) methanol containing 0.2% (v/v) phosphoric acid buffered to pH 7.25 with triethylamine. At a flow-rate of 0.5 ml/min, the retention times for nornicotine, anabasine, anatabine and nicotine were 11.0 min, 12.2 min, 13.9 min and 23.3 min respectively.

TABLE I

LINEARITY OF ANALYSIS FOR ALKALOIDS STANDARDS

Each value represents the average of duplicate injections.

Alkaloid injected (ng)	*Recovery (ng)*		
	Nicotine	*Nornicotine*	*Anabasine*
50	45	44	54
100	99	90	99
200	210	195	213
400	412	401	425
600	620	611	606
800	789	820	802
1000	1046	1060	1037
2000	2032	2150	2227
3000	3080	3089	3383
5000	4960	5121	5321
7000	6970	6905	6780

tions, however, the quantitative integration of the area under the peak was not affected and the tailing did not increase with the age of the column. Nornicotine, as purchased commercially, contained at least two major contaminants which represented as much

as 40% of the product. The lesser contaminant had a retention time between that of anatabine and nicotine and did not interfere with the analysis of the four alkaloids of interest, however, the greater contaminant had a retention time close to that of anatabine and could interfere with its quantitation when anatabine was run simultaneously with nornicotine standards. Therefore, these contaminants of nornicotine were separated from the authentic standard prior to quantitation of the alkaloids using the HPLC system and peak collection.

Fig. 2 represents a typical HPLC elution profile of the alkaloids extracted with the standard buffer from the dried leaves of *Nicotiana tabacum*, USDA TI No. 266. This particular variety of tobacco was collected in Mexico and had an unusually high level of nornicotine as compared to most commercial lines. A prominant nicotine peak and small anatabine peak are evident. In this particular variety of tobacco the anabasine, if present, was below detectable limits; however, anabasine is present in higher concentrations in other tobacco varieties. Nicotine and nornicotine, the two alkaloids in highest concentration in tobacco, were well separated by this chromatophic system, even when the columns had been used for over 800–1000 injections. Phenolics and tannins which chromatograph in the earlier part of the elution profile

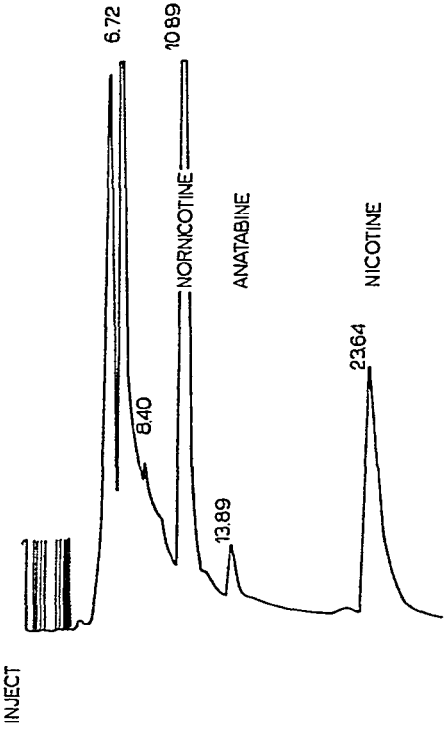

Fig. 2. HPLC elution profile of alkaloids in *Nicotiana tabacum*, USDA Tobacco Introduction Collection No. 266 extracted for 24 h with 25 m*M* phosphate buffer (pH 7.8) and Millipore filtered. Nicotine and nornicotine with retention times of 23.6 min and 10.9 min, respectively, represent the two major alkaloids in this variety and were clearly separated from other peaks absorbing at the same wavelength. A trace of anatabine which has a retention time of 13.9 min is also present in this particular variety of tobacco.

were partially extracted by the aqueous extraction system; however, these compounds do not interfere with the quantitative analysis of the major alkaloids in tobacco. Preliminary sample clean-up on both Amberlite XAD-2 resins and Waters Sep-paks did not improve the resolution or quantitation of the tobacco alkaloids. By prefiltering all samples through Swinnex Millipore filters, our laboratory found that column life would last a minimum of 500–600 injections without significant decrease in the resolution of standards. The efficiency of a column could be roughly determined by its ability to resolve nornicotine and anabasine standards by more than 30–40 sec. When the resolution between these two compounds fell below this level, the column was discarded. Guard columns which are often used to extend the useful range of the main columns were not used on the reverse phase μBondapak C_{18} system due to the decrease in resolution associated with the precolumns. If the reverse-phase chromatographic system was designed for nicotine and nornicotine quantitation alone, the decrease in resolution by precolumns would be minimal, therefore, the useful life of HPLC column presumably could be extended with the application of a guard column.

To investigate the possibility of unknown contaminants co-chromatographing with the alkaloids in the solvent system, tobacco leaf extracts were also chromatographed in three additional solvent systems including methanol–2-propanol–water–acetic acid (2:1:1:0.01), methanol–water–acetonitrile–acetic acid (1:1:1:0.01) and dioxane–2-propanol–NH_4OH (80:4:0.3). In addition, the alkaloids peaks were individually recycled through the HPLC system for three replications without additional peaks appearing.

Previous reports on the quantitate extraction of nicotine from dried tobacco material have used organic as well as aqueous solvents techniques[5,9,10,20,27–30]. To determine the efficiency of various solvents in the extraction of alkaloids from tobacco, air-dried samples of *N. tabacum* USDA TI No. 266 were subjected to a variety of extraction systems, including the standard solvent 25 m*M* phosphate buffer, various concentrations of methanol, ethanol, acetone, 2-propanol, water, acid and the standard extracting buffer (Table II). In addition, various buffers (not shown in the table) at pH ranging from 3 to 10 were also tested at extraction times from 1 to 30 h. Finally, the standard buffer was tested at several temperatures from 30 to 80°C. The extracts were then analyzed by the HPLC system described, and the levels of each alkaloid quantitated. The standard 25 m*M* phosphate buffer compared favorably with all of the other conditions tested (Table II). The use of 0.6 *N* hydrochloric acid as a solvent only marginally increased the yield of nicotine while significantly decreasing the amount of nornicotine. The organic solvents tested, *i.e.*, 100% acetone, methanol, and ethanol showed that aqueous mixtures were necessary for the efficient extraction of the alkaloids from the dried leaf samples. When 50% methanol was used as the extracting solvent, the yield of nicotine was slightly higher than that obtained with the standard buffer, however, the yields of nornicotine and anatabine were not significantly changed. The various other buffers tested (pH 3–10) were no more effective than the standard buffer (data not shown), and the standard buffer was equally effective whether used at 30°C or higher. When the tobacco sample was spiked with known amounts of each alkaloid, nicotine and nornicotine were recovered with yields better than 95% and anatabine and anabasine were recovered at approximately a 90% yield.

Fresh tobacco material from either leaf or root tissue can also be analyzed for

TABLE II

THE EXTRACTION OF NICOTINE, NORNICOTINE AND ANATABINE FROM AIR-DRIED LEAVES OF *NICOTIANA TABACUM* USDA TOBACCO INTRODUCTION NO. 266

Extracting solvent	*Nicotine (mg/gram dry weight)*	*Nornicotine (mg/gram dry weight)*	*Anatabine (mg/gram dry weight)*
25 m*M* Phosphate buffer, pH 7.8	16.5	21.2	0.7
25 m*M* Phosphate buffer, pH 7.8, heated 15 min at 80°C	16.5	22.4	0.8
Water	16.8	22.0	0.7
0.6 *N* HCl	17.6*	5.3*	0.9*
100% Methanol	14.3*	16.3*	0.5*
100% Methanol with 0.1% *N* HCl	14.9*	17.1*	0.5*
50% Methanol	17.6*	22.4	0.6
40% Methanol	16.5	21.6	0.6
100% Ethanol	5.5*	5.2*	0.1*
50% Ethanol	17.0	21.8	0.7
100% Acetone	1.9*	0.9*	0*
40% Acetone	16.8	22.2	0.7
40% 2-Propanol	16.7	21.3	0.7

* Significant difference at 0.01 probability level from 25 m*M* phosphate buffer control.

the alkaloid content using this HPLC system. Fresh root or leaf tissue can be treated as described under Experimental and the analysis run within the hour. There is no need to submit the material to preliminary solvent extractions to remove chlorophyll, fatty acids or other contaminating substances. In the case of leaf tissue, 40% methanol containing 0.1% 1 *N* HCl was found to be slightly advantageous over the aqueous buffer system.

For comparative purposes, *Nicotiana tabacum* TI No. 266 was analyzed for nicotine and nornicotine by both the HPLC technique described herein and by accepted gas chromatographic procedures[18]. The data for the gas chromatographic determination of nicotine and nornicotine in tobacco (15.4 mg/gram dry weight nicotine, 23.0 mg/gram dry weight nornicotine) shows that comparable results are obtained on similar samples whether analyzed by gas chromatography or HPLC (Table II).

The detection and quantitation of the major alkaloids in tobacco leaf can be of importance in the development of breeding programs designed to influence the levels of nicotine in the final product. We have developed a HPLC system which when coupled to an automatic sample injector can process large numbers of samples from either fresh or dried leaf material with consistant reliability and few operator manipulations. The HPLC method has been used to analyze quantitatively the USDA Tobacco Introduction Collection of over 1200 samples[31].

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