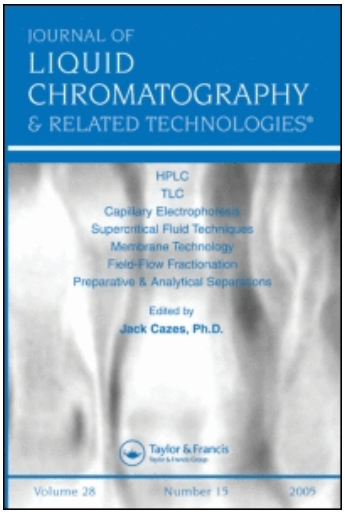

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CHEMICAL STUDIES ON TOBACCO SMOKE LXVII. QUANTITATIVE DETERMINATION OF ALKALOIDS IN TOBACCO BY LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC method was developed for the quantitative determination of individual alkaloids of the basic fraction of tobacco extracts. Two reverse phase RP-18 columns were used, eluting with a gradient of aqueous triethylamine phosphate buffer (pH 7.56) and acetonitrile.

The method was optimized and compared favorably with existing quantitative techniques in regard to time factors, sensitivity and accuracy.

INTRODUCTION

Tobacco alkaloids are regarded to be essential for man's habituation to tobacco products (1). Since nicotine elicits the greatest pharmacological effect of all of the tobacco alkaloids (2) and since it accounts for more than 85% of the alkaloids in tobacco, most analytical methods have been directed towards quantitating of nicotine (3), while fewer studies were concerned with total alkaloids in tobacco and its smoke (4), or with the concurrent determination of individual tobacco alkaloids (5,6,7). Preliminary data on the separation of individual alkaloids have been reported by Elmenhorst, using gas-liquid chromatography on capillary columns (8).

Recent studies on the correlation of alkaloids in tobacco and specific N-nitrosamines in processed tobacco and in mainstream and sidestream smoke (7,9) required the quantitative determination of individual alkaloids.

This paper represents the results of a study on the quantitative determination of nicotine and minor alkaloids in tobacco by reverse phase HPLC.

MATERIAL AND METHODS

Apparatus.

A Model ALC/GPC-204 liquid chromatograph (Waters Associates, Milford, Mass.) equipped with a Model 6000 solvent delivery system and a Model LC-25 UV/visible detector monitoring at 254 nm was employed throughout this study. The test samples were introduced through a Waters Model U6K septumless sample loop injector. The isolated alkaloids were identified by mass spectra obtained at 70 eV on a Hewlett-Packard Model 5982A GLC-MS system. Liquid scintillation counting was done with a Nuclear-Chicago Isocap 300 Scintillation system. Nicotine determination by GLC (4) was carried out with a Hewlett-Packard 5710A gas chromatograph with FID in addition to the spectrophotometric nicotine determination (FTC) for which a Carey Model 118 spectrophotometer was used (10).

Reagents.

All organic solvents were spectro-quality, the other chemicals of analytical reagent grade. Nicotine-methyl-^{14}C (50.4 mCi/mM), obtained from New England Nuclear, Boston, Mass., was purified and employed as an internal standard.

Reference Compounds.

Nicotine and 2,3'-dipyridyl were purchased from Aldrich Chemical Company, Milwaukee, Wisc. and anabasine from Fluka,

Basel, Switzerland. Nornicotine, cotinine, anatabine, myosmine, and N'-formyl-nornicotine were synthesized according to published methods (11-14).

Gas Liquid Chromatography.

The standard nicotine determination (4) was performed on a 6' stainless steel column packed with 2% KOH and 10% Carbowax 20M on 45-60 mesh Chromosorb W AW DMCS column temperature 165°C, detector and injector port 200°C. For the analysis of the basic fraction of the tobacco and for the GLC-MS analysis, we obtained the best results with a 12' glass column packed with 3% SP 2250 DB on 100-120 mesh Supelco port. After sample injection, the column temperature was kept at 120°C for 16 min and subsequently programmed to 240°C at a rate of 4°C/min.

Sample Preparation.

The moisture content of the tobacco was determined according to Bethmann *et al.* (15). Five grams of tobacco (dry weight) were stirred for 24 hours in the dark and at room temperature in 250 ml of distilled water to which nicotine-^{14}C (0.2 µg in solution) had been added as an internal standard. After adjustment to pH 1-1.5 with HCl and filtration on Celite (545 Johns Manville), the filtrate was extracted twice with 100 ml of dichloromethane. The pH of the aqueous phase was then adjusted to 11 with concentrated NaOH and subsequently extracted four times with 100 ml each of dichloromethane to yield the basic fraction of the tobacco extract. The dichloromethane was evaporated and the sample volume was adjusted with methanol to 2 ml (Fig. 1). In order to verify that neither N'-formyl-nornicotine nor cotinine was artifactually formed from nicotine or from nornicotine during the work up, a mixture of nicotine and nornicotine standards was submitted to the complete analysis. No peak was detected where N'-formyl-nornicotine or cotinine would have been expected. Also substituting ethyl acetate for dichloromethane as an extracting sol-

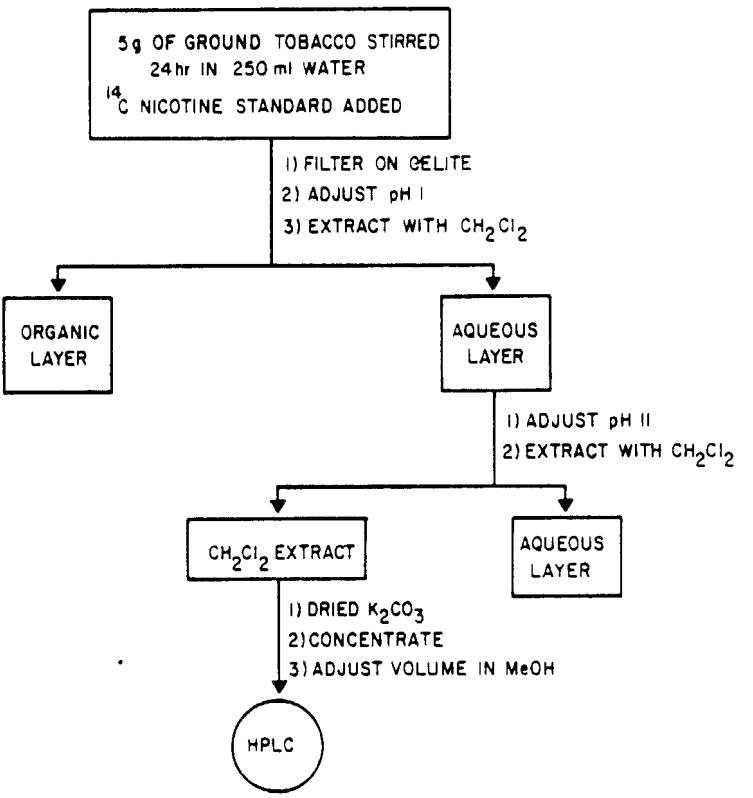

FIGURE 1. Sample preparation scheme.

vent did not yield any difference in the result of the anaylsis. Recovery of the ^{14}C-nicotine ranged between 85 and 95%. A 10 µl sample was then subjected to analysis by HPLC.

HPLC-Analysis.

All tests were carried out at room temperature. The most satisfactory chromatographic system was found to be a short Bonda-pak Corasil C-18 precolumn followed by two Lichrosorb RP-18 columns. The separation was optimized by varying column types, mobile phases, buffer pH and concentration, flow and elution gra-

dient. The best separation of the main tobacco alkaloids was obtained with an aqueous buffer of 0.07 M triethylamine adjusted to pH 7.56 with H_3PO_4 (as solvent A) and acetonitrile (as solvent B), and at a flow rate of 1.5 ml/min with a linear gradient from 0 to 25% of solvent B in 90 minutes. Identification of all major peaks of the tobacco extract chromatograms was ascertained by GC-MS of collected fractions and by matching HPLC retention times with those of authentic samples. For quantitations, peak areas were measured using the peak height x width at half height method, which was found to give very consistent results. The standard calibration curves were linear (correlation coefficient >0.999) for all individual tobacco alkaloids in the concentration ranges used throughout this study. Detection limit was better than 1 µg/injection.

RESULTS AND DISCUSSION

Due to the high efficiency of the dual column system, the presence of a guard column had no noticeable effect on the separation. Therefore, despite the simplified sample clean-up and the basic pH of the solution, no appreciable alteration of the columns was observed, even after several months of continuous use. The repetitive analysis of basic fractions of varied tobacco brands showed the HPLC analysis to be quite reproducible, provided the pH of the buffer was kept constant ($\pm$ 0.01 pH unit). A typical chromatogram of such a mixture is shown in Figure 2. One of the main problems of the tobacco alkaloid analysis is to achieve a sufficient separation between the predominant nicotine peak and those of the other alkaloids, and to minimize its broadening and tailing. This requirement excludes the use of a Partisil 10/ODS-2 type column which gave considerable broadening of most of the alkaloid bands. The pH of the buffer was found to have a significant effect on the separation and on the order of elution of the main peaks. Below pH 7.5, the nicotine peak broadening becomes excessive and pH above 8 would drastically shorten the life of

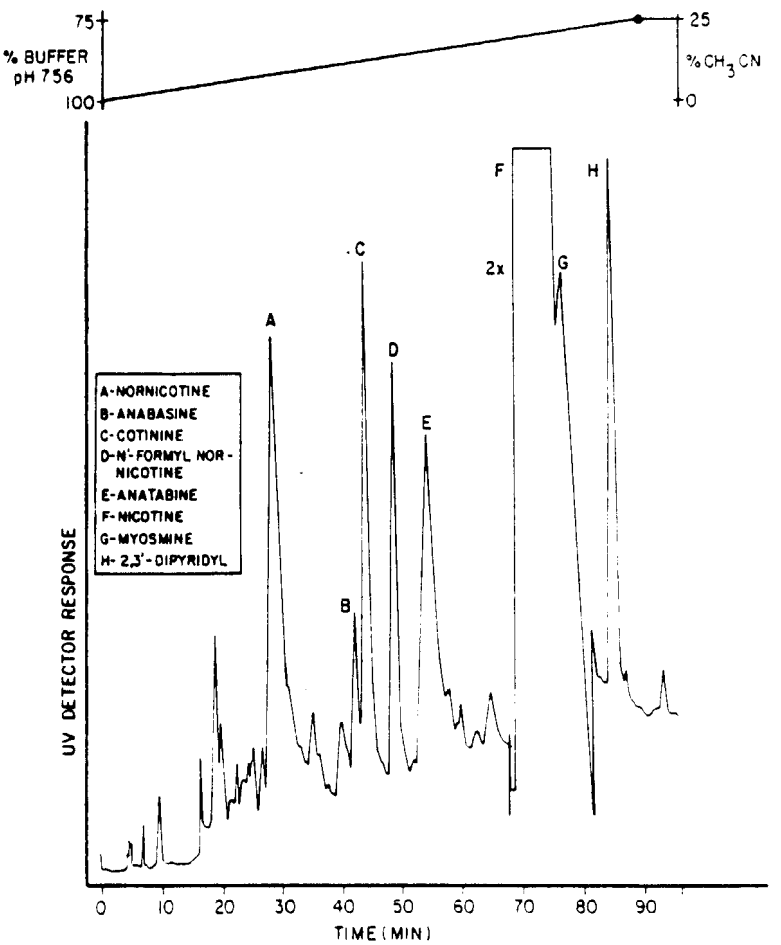

FIGURE 2. HPLC chromatogram of the basic fraction of tobacco extract. UV detector 254 nm, flow rate 1.5 ml/min; Lichrosorb RP-18 2 x 25 cm.

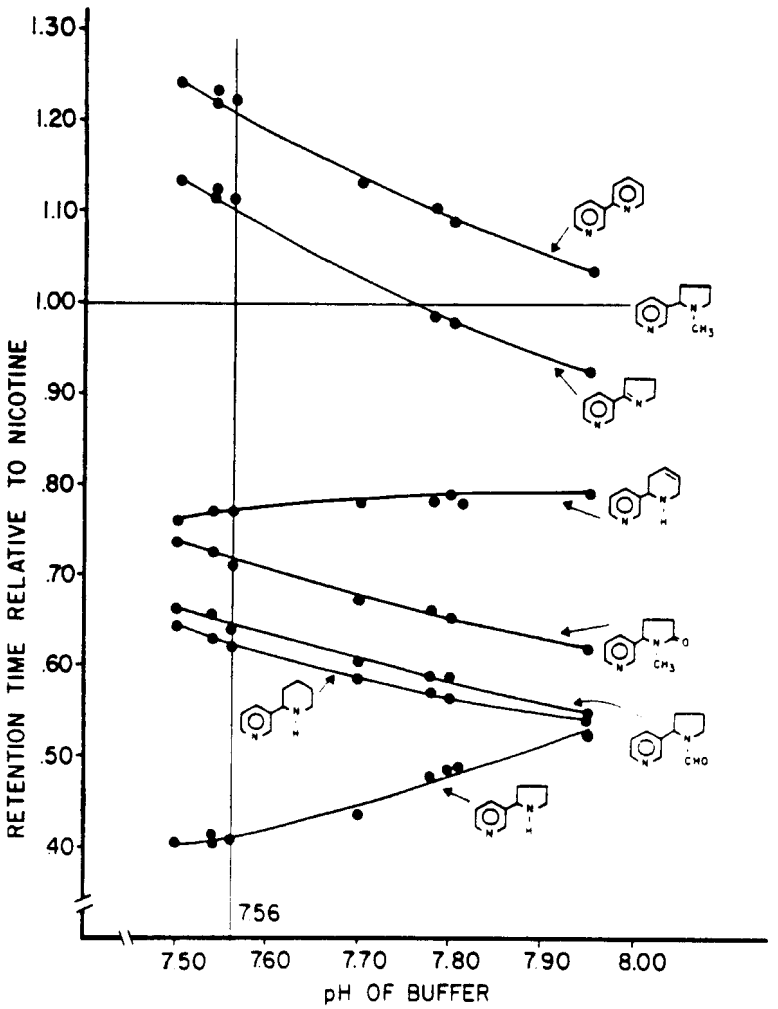

FIGURE 3. Retention times of the main tobacco alkaloids relative to nicotine as a function of the pH of the buffer.

TABLE 1.

Alkaloid Content of Various Tobacco Brands

µg/g - dry basis

	Dark Tobacco Commercial		Burley	Bright	Kentucky Ref.
	A	B			(IR1)
Nicotine	11,500	10,000	15,400	12,900	21,100
Nornicotine	550	200	630	210	630
Anatabine	360	380	570	600	930
Anabasine	140	150	90	150	190
Cotinine	195	140	90	40	80
Myosmine	45	50	60	30	85
2,3'-Dipyridyl	100	110	30	10	30
N'-Formyl-nornicotine	175	210	140	40	100

these columns. Figure 3 shows the effect of eluent pH on the relative rentention times of the main tobacco alkaloids in that range. The optimum was found to be at pH 7.56. However, if a precise quantitation of myosmine is required, a buffer pH over 7.8 is preferable because myosmine then elutes before nicotine.

The alkaloid content of various tobacco brands, as determined by HPLC analysis, is presented in Table 1. In order to ascertain the validity of the method, nicotine quantitation by HPLC was compared to the usual spectrophotometric Coresta and the GC standard nicotine determinations, on the same brand of reference tobacco. The HPLC and GC determinations were performed on the same sample extracts; the spectrophotometric determinations were performed on different tobacco samples from the same batch, and represent the total alkaloid content of the tobacco. The results are summarized in Table 2 and demonstrate good agreement between the HPLC data and those from the GLC and spectrophotometric methods.(Results of the spectrophotometric analysis were expected to be somewhat higher since the total alkaloid content is quantitated).

In conclusion, reverse phase HPLC offers an accurate, reproducible and sensitive method of quantitating tobacco alkaloids which compares favorably with the preexisting methods. This technique can also be extended to the semi-preparative scale.

TABLE 2

Nicotine Determinations of IR1 Kentucky Ref. Tobacco

mg/g

Sample #	Spectrophotometric (Coresta)	GC	HPLC
1	20.8	21.0	20.8
2	21.5	21.5	21.4
3	21.3	21.2	21.0
Means	21.2 $\pm$ 0.2	21.2 $\pm$ 0.2	21.1 $\pm$ 0.2

ACKNOWLEDGEMENT

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