Anal. Calcd. for $C_{23}H_{28}O_8$: C, 63.88; H, 6.53; O, 29.60. Found: C, 63.78; H, 6.81; O, 29.36.

Evaporation of the mother liquors from the first recrystallization yielded 0.5 g. of noncrystalline material λ_{max}^{EtOH} 234–238 mμ, log ε 3.88 whereas combination and evaporation of the mother liquors from the following two recrystallizations gave *ca.* 0.2 g. of crystals λ_{max}^{EtOH} 276–280 mμ, log ε 3.96. This latter substance was then adsorbed on 6 g. of silica gel from a solution of methylene chloride. Elution of the column with methylene chloride–acetone (9:1) led to 60 mg. of crystals which were recrystallized four times from acetone thus providing 1α,2α-dihydroxy-Δ^6-dehydrocortisone acetate (V) m.p. 235–238°, $[\alpha]_D$ +230° (pyridine), λ_{max}^{EtOH} 280 mμ, log ε 4.32. The poor analytical results cannot be ascribed to dehydration since the substance gave no color with ferric chloride and the ultraviolet absorption spectra was not altered by addition of alkali.

Anal. Calcd. for $C_{23}H_{28}O_8$: C, 63.88; H, 6.53. Found: C, 64.92; H, 6.83.

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Alkaloids of Tobacco Smoke. I. Fractionation of Some Tobacco Alkaloids and of the Alkaloid Extract of Burley Cigarette Smoke by Gas Chromatography[1]

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An alkaloid extract from Burley tobacco cigarette smoke was separated by gas chromatography on polyglycol columns. It was necessary to perform the separation under three sets of conditions to overcome difficulties associated with the wide boiling range of the mixture and the relatively massive amount of nicotine present. There appears to be a minimum of sixteen alkaloidal or basic compounds, in addition to nicotine, boiling above 150–170° in the extract. Besides its analytical features, the gas chromatographic method is valuable in isolation and purification of the alkaloids.

It has been known for many years that other alkaloids in small quantities accompany nicotine in tobacco smoke,[2] but much uncertainty exists as to the identity and amounts of these compounds. The recent paper chromatographic work of Kuffner, Schick, and Bühn[3] has done much to clarify in a qualitative manner the alkaloidal content of cigar smoke. They were able to show that many of the alkaloids in the smoke were present in the tobacco itself. However, definitive studies are lacking on cigarette smoke, which differs from cigar smoke in several respects. We have recently undertaken a study of the alkaloids in a continuation of our work on the chemical composition of cigarette smoke.[4]

It was anticipated that the tobacco alkaloids, generally boiling in the range 200–300°, might be subject to separation by the versatile technique of gas chromatography. This hope was realized and in a preliminary communication[5] we reported the successful application of gas chromatography to these compounds. It was found that good separation of a majority of the alkaloids studied could be achieved at moderate temperatures (about 190°) on 1 meter columns containing certain polyglycols as the stationary liquid phase. The list of known alkaloids studied has been extended since this initial report; a complete list with the retention times on three different columns is provided as Table I.

TABLE I

GAS CHROMATOGRAPHY OF INDIVIDUAL TOBACCO ALKALOIDS

Liquid Phase	Columns and Conditions: Polypropylene glycol[a]	Polybutylene glycol[b]	Polyethylene glycol[c]
Temp., °C.	190	180	190
He flow, ml./min.	45	50	48
	Retention Time, Min.		
3-Pyridyl methyl ketone	4.3	3.1	4.3
3-Pyridyl ethyl ketone	6.1	5.0	5.3
3-Pyridyl *n*-propyl ketone	8.1	7.0	6.6
Nicotine	8.6	8.2	5.2
Nornicotine	16.1	14.3	12.3
Myosmine	16.4	14.7	13.4
Anabasine	19.4	18.1	13.8
Nicotyrine	21.0	18.3	19.4
Metanicotine	23.5	20.9	16.5
Anatabine	25.2	22.5	21.1
2,3′-Dipyridyl	31	26	29
N-Methyl nicotinamide	42	30	64
Nornicotyrine	73	55	101
Cotinine	79	63	85

[a] Mol. wt. 1025. [b] Mol. wt. 1500. [c] Mol. wt. 20,000.

(1) Supported by a grant from the Damon Runyon Memorial Fund. Presented at the Twelfth Tobacco Chemists' Research Conference, October 23, 1958, Durham, N. C.

(2) The literature has been recently reviewed: A. I. Kosak in *The Biologic Effects of Tobacco*, ed. by E. L. Wynder, Little, Brown and Co., Boston, Mass., 1955, p. 15; A. I. Kosak, *Experientia*, **10**, 69 (1954); L. Marion in *The Alkaloids*, ed. by R. H. F. Manske and H. L. Holmes, Acedemic Press, N. Y., N. Y., 1950, Vol. 1, p. 228.

(3) F. Kuffner, K. Schick, and H. Bühn, *Monatsh.*, **87**, 749 (1956).

(4) Preceding paper: L. D. Quin and M. E. Hobbs, *Anal. Chem.*, **30**, 1400 (1958).

(5) L. D. Quin, *Nature*, **182**, 865 (1958).

The coverage of known tobacco alkaloids is seen to be quite broad. Only one alkaloid studied, oxynicotine, failed to be eluted under the conditions employed. The eluted substances in every case were shown by their ultraviolet absorption spectra to be identical with the starting compounds.

In the present paper, we describe the application of gas chromatography to the separation of the alkaloid-containing fraction of cigarette smoke. In the following paper,[6] the identification of a number of the alkaloids in this fraction is described.

Nicotine appears to account for 90% or more of the alkaloid fraction of cigarette smoke under consideration here;[7] the several other alkaloids are consequently present as trace constituents. It has been found expedient to perform the gas chromatographic separation of the gross mixture under three different sets of conditions to provide adequate overall resolution and overcome the interference of nicotine in the elution of neighboring peaks. Polypropylene glycol (mol. wt. 1025)) as the stationary liquid phase has given the most complete resolution of the mixture and was used in this study.

At a column temperature of 140–150°, the alkaloids or other bases emerging before nicotine were resolved on a 1 m. by 6 mm. column (Fig. 1).

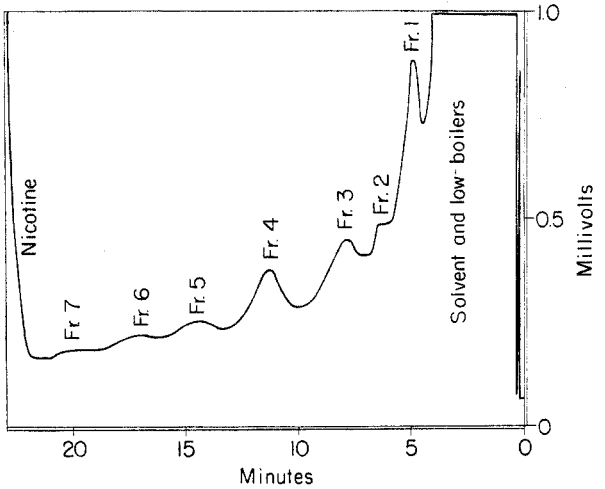

Fig. 1. Gas chromatogram of alkaloid fraction from smoke of 4.4 cigarettes, showing pre-nicotine peaks. Temp., 145°C. He flow, 47 ml. per min. Detector voltage, 8.0. Sensitivity, 1/2. Column, 1 m. × 6 mm. P.P.G. 1025

Seven peaks were noted (Fractions 1–7). These appear to be due to substances boiling above about 170°, as suggested by the elution of 2,4-lutidine (b.p. 157°) and collidine (b.p. 172°), when run separately under the same conditions, in shorter retention times than those of any of these peaks. Lower boiling bases are possibly present but are obscured here by the large solvent peak. At this relatively low temperature, the column is overloaded with nicotine, resulting in the elution of this compound in a broad, asymmetric peak. The run is terminated when nicotine breaks through.

At 190°, the alkaloids immediately following nicotine are resolved (Fig. 2). A column of wider diameter (10 mm. o.d.) was desirable for this portion of the separation, as it permitted a relatively large nicotine load to be placed on the column without an asymmetric, tailing peak resulting. The increased size of the column necessitated faster helium flow rates to obtain sample elution in reasonable times. Six peaks (Fractions 8–13) were readily detected after the nicotine had been eluted. The peaks appearing before nicotine (not shown on Fig. 2) differ somewhat in number and relative

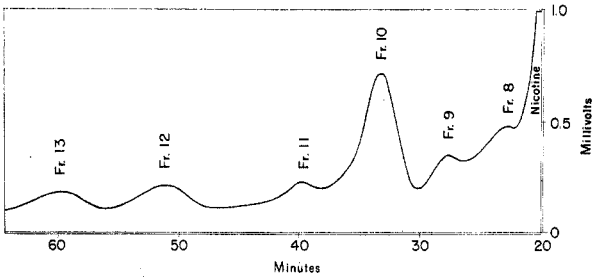

Fig. 2. Gas chromatogram of alkaloid fraction from smoke of 4.4 cigarettes, showing immediate post-nicotine peaks. Temp., 190°C. He flow, 73 ml. per min. Detector voltage, 8.0. Max. sensitivity. Column, 1 m. × 10 mm. P.P.G. 1025

position from those obtained in the previous separation as Fractions 1–7. Large differences in temperature, helium flow, and column diameter exist between the two separations, and some or all of these variables may be responsible for this fact.

The higher boiling alkaloids are more readily examined on the smaller diameter column; retention times are of a more desirable magnitude, and no interference by the nicotine occurs with these outlying peaks. At 190°, three peaks (Fig. 3, Fractions 14–16) appeared after the final peak recorded in the preceding portion of the separation.

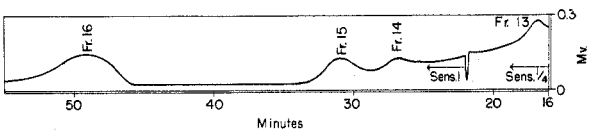

Fig. 3. Gas chromatogram of alkaloid fraction from smoke of 4.4 cigarettes, showing late post-nicotine peaks. Temp., 190°C. He flow, 72 ml. per min. Detector voltage, 8.0. Column, 1 m. × 6 mm. P.P.G. 1025

Tentatively assuming homogeneity of each peak, it is seen that a minimum of sixteen substances accompany nicotine in the alkaloid fraction of cigarette smoke. The possibilities exist that other

(6) L. D. Quin, *J. Org. Chem.*, 24, 914 (1959).

(7) The isolation procedure is specifically designed for the study of the high-boiling bases; low boilers such as pyridine, etc., are probably lost. It should be noted that all of the bases discovered in this work have not yet been established as being truly alkaloidal in the sense that they are pyridine derivatives. However for convenience this fraction will be referred to as the alkaloid fraction.

substances are present but in concentrations too small to be detected, or that some substances originally present are destroyed in the isolation or chromatographic procedures. It is also possible that alkaloids are present of too little volatility to be gas chromatographed under the conditions used so far.

The resolution provided by the above conditions is generally inadequate for obtaining pure samples of the eluted compounds. However, by re-chromatographing a collected eluate, a specimen free of forerunning compounds can generally be obtained for other studies. Even in an extreme case, such as Fraction 8, which is riding on the nicotine tail, collection and re-chromatography provided a nicotine-free sample (Fig. 4). In some cases, re-chromatography on a different column might be advantageous.

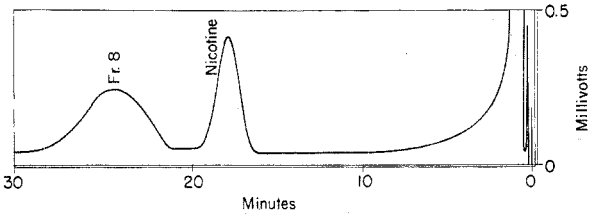

Fig. 4. Gas chromatogram of Fraction 8. Temp., 190°C. He flow, 72 ml. per min. Detector voltage, 8.0. Max. sensitivity. Column, 1 m. × 10 mm. P.P.G. 1025

The conditions developed in this study are subject to modification and improvement. Longer columns would improve the resolution of neighboring peaks. Also, it is likely that column packings having greater stability and better selectivity will be discovered. It is felt, however, that the present method provides satisfactory separation of a complex mixture of tobacco alkaloids, and should be useful in the study of such mixtures from sources other than cigarette smoke. It is also possible that certain other alkaloid families having slight volatility and good thermal stability will be separable by this or a related gas chromatographic technique.

EXPERIMENTAL

Isolation of alkaloid fraction of cigarette smoke. One hundred 70 mm. cigarettes of Burley tobacco, without additives, were humidified over a saturated sodium bromide solution for several days and smoked five at a time on an automatic machine to a butt length of 20 mm. Puffs of 2 seconds duration and 35 ml. volume were taken every minute. The smoke was passed through a 4.5 cm. circular glass-fiber filter of the type described by Wartman and Harlow.[8] The filter was replaced after each cycle. The particulate-free gas from the filter was passed through a trap chilled in Dry Ice-ethanol. The filters, trap, and connecting tubing were thoroughly washed with 1*N* HCl (400 ml. total) and this solution then continuously extracted with benzene for 3 days. The benzene was changed each day, stripped to 1 ml., and examined by gas chromatography to monitor removal of volatile acidic or neutral material. No evidence for such material was found after the third day of extraction. The acid solution was then made *p*H 11 with solid sodium hydroxide and the benzene extraction continued for three days. The extract was concentrated to 1.0 ml. and stored in the refrigerator for later use. This solution contains about 0.5 g. of total alkaloids.

Gas chromatographic equipment. The Perkin-Elmer Vapor Fractometer Model 154-B was used. A Fenwall Thermoswitch No. 17502 was installed to improve high-temperature stability of the instrument. The heater on the sample injection block was placed on line voltage to permit a temperature giving prompt sample vaporization. The vent line outside the thermostatted chamber was shortened to 6 in. and fitted with a Nichrome wire heater to prevent sample condensation therein. A Leeds and Northrup Speedomax type G recorder set at 1 mv. full scale deflection detected the elution peaks.

Samples were injected with either a syringe or the Perkin-Elmer "Micro dipper" pipets. For collection of eluted samples, 3 mm. o.d. glass U-tubes of 30 cm. total length were immersed in a Dry Ice bath and attached directly to the vent line.

The carrier gas was helium. The flow rate was measured with a soap-bubble meter.

Columns were U-tubes, 1 m. long, of 6 mm. or 10 mm. o.d. glass tubing. They were packed with mixtures of the stationary liquid phase on Firebrick (Fisher "Columpak") in the weight ratio of 1 to 4. Acidic material was first removed from the Firebrick by washing with 2% alcoholic potassium hydroxide; it was then washed with absolute alcohol and dried at 110°.

Column packings. Several materials were screened for suitability as the stationary liquid phase. The following gave sharp, symmetrical elution peaks for the alkaloids and were sufficiently stable and nonvolatile to permit their continued use for 2–3 weeks or more: polypropylene glycol, mol. wt. 1025 or 2025 (Union Carbide Chemicals Co.); polybutylene glycol, mol. wt. 1500 (Dow Chemical Co.); polyethylene glycol, mol. wt., 20,000 (Dow), or 4000 (Carbide). Polystyrene glycol, mol. wt. 750 (Dow) was found to be too volatile, and Hyprose SP 80 (Dow, octakis (2-hydroxypropyl) sucrose) too unstable, at 190°, but both gave satisfactory peaks and may find use at lower temperatures. Flexol R2H (Carbide, a polyester) and Apiezon M grease gave tailing peaks and poor selectivity.

Gas chromatography of individual alkaloids. Twenty μl. aliquots of benzene solutions each containing 5–10 mg. per ml. of a known alkaloid were chromatographed on three different columns. Conditions and retention times are given in Table I. The alkaloids were collected as eluted and dissolved in 95% ethanol to obtain solutions suitable for ultraviolet spectral analysis. The spectra, obtained on a Warren Spectracord, checked in every case with those of the known alkaloids run simultaneously.

Gas chromatography of cigarette smoke alkaloids. Samples of the concentrated benzene extract were run under numerous conditions during this study. Since the best resolution was obtained with a stationary liquid phase of polypropylene glycol, mol. wt. 1025, this material was used in the procedures finally adopted. Chromatography was performed under three sets of conditions on different aliquots of the benzene extract. Some typical conditions are summarized in Table II. In each case the injected sample was 50 μl. of the extract, representing about 25 mg. of total alkaloids, or the smoke of 4–5 cigarettes. All peaks were reproduced in different smoke preparations; typical chromatograms are presented as Figs. 1–3.

Purification by re-chromatography is illustrated with Fraction 8. This fraction was collected from three consecutive 50 μl. smoke extract samples, the trap rinsed with 1 ml. of benzene into a 3-in. testtube, and the solvent evaporated to

(8) W. B. Wartman and E. S. Harlow, presented before the Division of Agricultural and Food Chemistry, 133rd National Meeting of the American Chemical Society, San Francisco, April 15, 1958.

TABLE II

FRACTIONATION OF CIGARETTE SMOKE ALKALOIDS BY GAS CHROMATOGRAPHY

Separation Performed:	Pre-nicotine	Immediate Post-nicotine	Late Post-nicotine
Column[a]	1 m. × 6 mm.	1 m. × 10 mm.	1 m. × 6 mm.
Temp., °	145	190	190
He rate, ml./min.	47	75	60
Retention time of nicotine, min.	24[b]	18	5.5
Retention time of other peaks	4.9, 6.2, 7.9, 11.3, 14.4, 17.0, 20	23, 28, 33, 39, 51, 60	27, 31, 49

[a] Packed with polypropylene glycol, mol. wt. 1025, on Firebrick, 1:4. [b] On separately run sample.

about 50 μl. with a stream of nitrogen. This solution was then chromatographed under the same conditions used in collection of the original sample. The chromatogram is shown as Fig. 4.

Acknowledgment. The author expresses appreciation to Dr. Marcus E. Hobbs for his advice and interest during this work. The technical assistance of John M. Flowers, Jr., during portions of the work is acknowledged. Generous samples of tobacco alkaloids were supplied by the American Tobacco Co., Dr. R. F. Dawson of Columbia University, and Drs. R. N. Jeffrey and T. C. Tso of the U. S. Department of Agriculture. The cooperation of the Research Laboratory of the Liggett and Myers Tobacco Co. is acknowledged.

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Alkaloids of Tobacco Smoke. II. Identification of Some of the Alkaloids in Burley Cigarette Smoke[1]

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By combinations of gas and paper chromatography and ultraviolet spectroscopy, myosmine, nornicotine, anabasine, anatabine, 2,3′-dipyridyl, and cotinine have been conclusively identified in Burley tobacco cigarette smoke. Rough determinations were made for the quantities of these substances and of nicotine in the smoke.

In the preceding paper,[2] a gas chromatographic method for the separation of an alkaloid extract obtained from Burley tobacco cigarette smoke was described. A number of the compounds producing the observed chromatographic peaks have been identified, some for the first time in cigarette smoke. Approximate figures for the amounts of the identified alkaloids were also obtained by the gas chromatographic method, providing the first published data on the secondary alkaloid content of tobacco smoke.

The gas chromatographic technique of separation was advantageous in that it easily provided crude samples of the individual alkaloids from a mixture by simply condensing them from the gas stream as they were eluted. These samples can then be purified by re-chromatography to remove impurities eluted in close proximity and collected simultaneously. Only when compounds are eluted at essentially the same time does this procedure fail to provide a pure specimen. This procedure avoided the time consuming and tedious operations of fractional crystallization, extraction, or distillation employed in the past for alkaloid separations. The specimens obtained generally contained a trace of the material used as the stationary liquid phase in the column, but this did not interfere with the subsequent work. This impurity could probably be removed if necessary.

The retention time of each gas chromatographic fraction from the alkaloid extract of cigarette smoke was observed on columns of polypropylene glycol (mol. wt. 1025) and of polyethylene glycol (mol. wt. 20,000). These were compared with the retention times of known alkaloids obtained under identical conditions. Checks on both columns between a known and a compound of smoke origin provided a tentative identification. The different retentive ability of the two columns for the various alkaloids[3] made this a reliable cross-checking procedure. In one case, this technique failed to provide distinction between two compounds; myosmine and nornicotine were eluted more or less together on

(1) Supported by a grant from the Damon Runyon Memorial Fund. Presented at the Twelfth Tobacco Chemists' Research Conference, October 23, 1958, Durham, N. C.

(2) L. D. Quin, *J. Org. Chem.*, **24**, 911 (1959).

(3) See Table I, ref. 2.