Chemical Studies on Tobacco Smoke

I. The Quantitative Determination of Indoles in Cigarette Smoke*

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Studies with fractions of cigarette smoke condensate have demonstrated that the neutral subfraction B_1 exhibits the highest tumorigenic activity of all to mouse skin (3). This subfraction (0.6% of the whole condensate) contains practically all of the polynuclear aromatic hydrocarbons of cigarette smoke. Also terpenes, fatty acid esters of solanesol and aliphatic alcohols, small amounts of cyclic and acyclic alkanes, alkenes and some N-heterocyclic hydrocarbons were identified in B_1 . The highest concentration for a single component in B_1 was 6.9% for skatole (3-methylindole). Indole, 3-ethylindole and 3-n-propylindole were also identified.

The relatively high concentrations of these N-heterocyclic hydrocarbons in the subfraction B_1 suggest that the quantities of these compounds in cigarette smoke are higher than so far reported. Rodgman and Cook (5) isolated 1.4 μg . indole per cigarette and 4.3 μg . skatole from the smoke of Turkish cigarettes, in crystalline form. They found other alkylated indoles in 2 semisolid subfractions and tentatively identified these as admixtures of two or more dimethylindoles (0.8 μg .) and two or more trimethylindoles (0.5 μg .). Recently Schmeltz et al. (6, 7) reported the presence of certain heterocyclic aromatic compounds in cigarette smoke. These included indole, skatole, a monomethylindole other than skatole, dimethyl- and trimethylindoles.

The purpose of this communication is to report a method for the quantitative determination of indole and alkylated indoles in cigarette smoke.

METHODS AND MATERIAL

Cigarette Smoking

United States blended nonfilter cigarettes of 85 mm. length are conditioned in a humidity chamber at 58% R.H. and 21° C. and selected at an average weight of ± 20 mg. Six cigarettes are then smoked on one channel of the CMS-10 smoking machine (9) at a rate of one puff per minute of 2 seconds duration and 35 ml. volume; butt length is 23 mm. The smoke is drawn through three traps of about 100 ml. volume each. The first trap is cooled with ice water; the second is cooled with dry ice-acetone, and the third trap, a gas wash bottle, is filled with 50 ml. of methanol-water (4:1). If a quantitative determination of 3-n-propylindole is desired at least 16 cigarettes have to be smoked. The condensates from traps 1 and 2 are transferred into a 250 ml. separatory funnel with the 50 ml. methanol-water from trap 3. Fifty ml. of n-hexane followed by 15 ml. methanol-water (4:1) and 14 ml. n-hexane are used to rinse all materials out of the traps.

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Indole	C_M/C_H	C_H/C_W
Indole	6.7	2.0
2-Methylindole	3.8	3.2
3-Methylindole	3.2	5.0
4-Methylindole	3.3	5.0
5-Methylindole	3.2	5.0
6-Methylindole	3.3	4.9
7-Methylindole	3.5	4.3
2,3-Dimethylindole	2.6	13.3
3-Ethylindole	2.3	24.0
3-n-Propylindole	2.0	24.5

n-hexane and 65 ml. methanol-water (4:1). The Nuclear Chicago Scintillation System 720 counted at an efficiency of 72.6% for the unquenched C^{14} -labeled indole in toluene with 0.4% PPO (2,5-diphenyloxazole) and 0.005% POPOP (p-bis [2 (5-phenyloxazoly)] benzene) as scintillators.

TABLE 1
Partition coefficients of indoles (21° C.)

C_M Concentration in methanol-water 4:1.
 C_H Concentration in n-hexane.
 C_W Concentration in methanol-water 1:2.
 Coefficients were obtained after spectroscopic determination (UV) for concentrations of about 1 mg./liter.

Internal Standard

One μ g. of highly purified indole-2, 3- C^{14} (416,700 dpm; International Chemical and Nuclear Corp., City of Industry, California) in 1 ml. n-hexane is added to the 64 ml.

Concentrating of the Indoles

After thorough shaking, n-hexane and methanol-water will clearly separate. Two additional extractions of the n-hexane layer with equal volumes of methanol-water (4:1, preequilibrated

with n-hexane) suffice to extract at least 97% of the indoles from the upper layer (Table 1). The combined methanol-water layers contain about 80% of the original condensate. Water is added to the combined methanol-water extracts so that the methanol-water ratio changes from 4:1 to 1:2 (about 280 ml. H_2O to 200 ml. extract). Four extractions with 500 ml. of n-hexane (preequilibrated with methanol-water, 1:2) suffice to bring at least 98% of all indoles into the n-hexane layers (Table 1). The combined hexane extracts are concentrated to 30–50 ml. by vacuum distillation (12 mm. Hg.) at water bath temperatures below 30° C. The hexane phase (its residue amounts to about 10% of original condensate) is twice extracted with 20 ml. 2-N acetic acid and washed with water once. The hexane layer is dried over desiccated sodium sulfate, filtered, and concentrated to 3–5 ml. The residue contains 4–6% of the smoke condensate (from the smoke of one cigarette about 1.5 mg.).

FIGURE 1

Separation scheme for indoles from cigarette smoke condensate*

The Indole-Fraction is separated into individual components by gas chromatography.

*The weights given are average values from several separations.

Column Chromatography

The indole concentrate in *n*-hexane is placed on a column (1 × 40 cm.) with 15 g. of Florosil, mesh 60–100 (Fisher Scientific Co., New York). The column is developed with 100 ml. *n*-hexane and subsequently eluted with *n*-hexane-benzene (4:1). The first 50 ml. of the *n*-hexane-benzene eluate are free of indole (no β -activity), fractions 2–5 (each 50 ml.) contain indole, the next two fractions (100 ml.) contain sometimes alkylated indoles. Fractions 2–7 are concentrated to a few milliliters under reduced pressure (12 mm. Hg.) with water bath temperatures not exceeding 40° C. The concentrate is transferred into a small vial (3 ml.). After adding 200 μ l. of toluene, the solution is freed from *n*-hexane and benzene by evaporation in nitrogen at room temperature. The residue in this concentrate contains less than 1% of the original condensate; see separation scheme Fig. 1.

Gas Chromatography

Two to three microliters of the concentrate are injected into a gas chromatograph, in our case a Perkin-Elmer Model 800 with hydrogen flame ionization detector. The separation of the indoles is achieved on a 2 m. stainless steel column with an i. d. of 3 mm., filled with 20% Apiezon L on Gas Chrom P, mesh 60–80 (Applied Science Laboratories, State College, Pennsylvania). The carrier gas is helium; its inlet pressure 2.5 atm. The separation is achieved isothermally at 155° C.; the temperature of the injection port stays at 265° C. The detector temperature is kept at 280° C. in order to avoid condensation of less volatile components from the concentrate, such as carbazoles. A Leeds & Northrup recorder ranging from 0.0 to 1.0 m. v. is employed; with the attenuation at 20, about 1.2 μ g. of skatole reach the full scale of the recorder chart. A typical gas chromatogram as obtained from the indole concentrate is shown in Fig. 2. According to the retention times obtained with reference components, the major peaks are indole, skatole, and possibly 3-ethylindole, and 3-*n*-propylindole (Table 2).

Identification of Indoles

For the identification of the smoke indoles, a large scale separation beginning with 50 g. condensate was carried out. The indoles were concentrated as outlined, however, without addition of an internal standard. An Aerograph Gas Chromatograph 1525 with thermoconductivity detector that permits the collection of the effluents corresponding to the maxima in the chromatogram was utilized in this case. The actual trapping was achieved by condensing the materials in a capillary tube. A stainless steel column of 2 m. length with an inside diameter of 6 mm. filled with 5% SE-30 on Gas Chrom P was used for the separation with the following temperature profile: For 10 minutes 135° C., then linear temperature programming at a rate of 4° C. per minute up to 250° C. The temperature of the injection port was kept at 265° C., that of the detector at 285° C. Under these conditions a single gas chromatogram could carry up to 5 mg. of indole concentrate. The retention times observed under these conditions are given in Table 2. The first two large peaks (7 and 11 minutes) delivered white crystalline materials; the combined materials from several separations were freed from impurities, deriving from the liquid phase (SE-30), by chromatography on a small Florosil column. Both purified effluents yielded white crystals. The effluents, obtained at 13 minutes yielded a light yellow oil which was combined from several sepa-

FIGURE 2

Chromatography of indole concentrate from cigarette smoke

Conditions:
2 m. stainless steel column i.d. 3 mm., filled with 20% Apiezon L on Gas Chrom P.
Column temperature: 155° C.
Carrier gas: helium (2.5 at.).
Detector: hydrogen ionization detector.

rations and was purified by chromatography on small Florosil columns. Ultraviolet absorption, infrared absorption, and mass spectra were taken from the very light yellow oil. The combined materials from 10 large-scale separations of about 5 mg. concentrate each, which emerged after 16 minutes, were rechromatographed as described before, and finally purified on Florosil. They yielded a light yellow oil, which was subjected to ultraviolet absorption, infrared absorption spectroscopy and mass spectrometry. The minor peaks with retention times between 13 and 16 minutes were collected for ultraviolet absorption spectra only.

The ultraviolet absorption spectra were prepared from cyclohexane solutions in 2.0 cm. cells with

TABLE 2 Retention times*

Indole	Retention times of gas chromatography systems (in minutes)	
	I	II
Indole	4	7
Monomethylindoles**	7	11
2,3-Dimethylindole	11	13
3-Ethylindole	11	13
3-n-Propylindole	16	16

*System I: 155°C. isotherm. 2 m. column with i.d. 3 mm., liquid phase 20% Apiezon L on Gas Chrom P (mesh 60-80). Carrier gas helium, inlet pressure 2.5 atm.

System II: Temperature profile: isotherm for 10 min. at 135°C. Thereafter, linear temperature programming at a rate of 4°C. per min. up to 250°C. 2 m. column with i.d. 6 mm., liquid phase 5% SE-30 on Gas Chrom P (mesh 60-80). Carrier gas helium, inlet pressure 2.5 atm.

**In both systems slight differences were observed for the retention times between some of the six C-monomethylindoles; however, we did not succeed in separating admixtures of monomethylindoles.

the literature (m. p., ultraviolet absorption and infrared absorption spectra; 1, 8). The syntheses were based on the Fischer method, using the phenylhydrazones of n-butyraldehyde or n-valeraldehyde respectively. Zinc chloride was chosen as catalyst and naphthalene as solvent for the condensation and ring formation. The yields for the pure components were in both cases below 10%, but the objective was to obtain highly pure material rather than a good yield. All reference components were purified on Florosil; their purities were determined by gas chromatography and infrared absorption spectra.

Reference Components

Indole; 2-, 3-, 4-, 5-, 6- and 7-methylindoles and 2,3-dimethylindole were obtained from the K & K Laboratories (Plainview, New York); 3-ethylindole and 3-n-propylindole were synthesized according to the method of the "Gesellschaft für Teerverwertung" (2) on a 10 g. scale. The highly purified components were identical with the materials described in

Quantitative Determination

Three samples of 2 μ l. each of the indole concentrates obtained after column chromatography are injected into the gas chromatograph and three 50 μ l. samples were placed into the counting vials with scintillator solution. Since only 50 μ l. of the yellow concentrate in toluene are added to 20 ml. scintillator solution, the quenching effect remains within the experimental variation of the counting method, which is better than $\pm 1\%$.

RESULTS

Qualitative Analysis

The large scale separation of the indoles from the concentrate of about 50 g. of cigarette smoke condensate resulted in the isolation of specimens of crystalline indole and skatole, which were identified by melting point, ultraviolet absorption and infrared absorption spectra. According to the mass spectrum the third major component was likely to be 3-ethylindole (parent ion $m/e = 149$; the major fragments $m/e = 130$ and 115). Ultraviolet absorption and infrared absorption spectra were identical with the synthesized 3-ethylindole and with data in the literature (4).

The mass spectrum of the last major component from both gas chromatographic systems had the parent ion $m/e = 159$. The fragments obtained by the bombardment of the specimen with electrons of 70 e. v. were $m/e = 144, 130, 116, 103, 91$ and 78. With the relative abundance of 100 for the major ion 159 the other peaks had abundances of 24(144), 30(130), 4(116), 2.9(103), 2.6(91), and

5.2(78). The molecular weight corresponds to that of trimethylindoles, methylethylindoles, i-propylindoles, and n-propylindoles. However, the greatest abundance after $m/e = 159$ is observed for $m/e = 130$ indicating the structure of propylindole(s). The U.V. spectrum of the unknown compound was identical with that of synthesized n-propylindole (maxima at 292, 284, 280, and 274 m μ .); however, this would not exclude certain other alkylated indoles. In the infrared absorption spectrum the isopropyl-bands between 1385–1380 and 1370–1365 cm.⁻¹ were absent; the spectrum was practically identical with that of synthesized 3-n-propylindole. The isolated specimen contained a small impurity as indicated by the parent ion $m/e = 173$ which could be a higher homolog of the alkylated indoles.

Quantitative Analyses

The results of five quantitative analyses of the smoke were 13.9 ± 0.5 μ g. per cigarette for indole and 14.0 ± 0.9 μ g. per cigarette for skatole (Table 3). The average values per cigarette from 3 analyses of 16 cigarettes each were 4.2 μ g. for 3-ethylindole and 0.21 μ g. for 3-n-propylindole. The sum of the dimethylindoles was tentatively estimated to be less than 0.03 μ g. per cigarette. The yield of the indoles from cigarette smoke in the final concentrate varied in the five analyses between 85 and 90%.

TABLE 3 Indole and skatole in cigarette smoke*

Number of analyses**	Indole (μ g. per cigarette)	Skatole (μ g. per cigarette)
1	13.5	13.7
2	14.6	13.4
3	13.5	14.3
4	14.3	15.5
5	13.4	13.0
Average ($\bar{m}$)	13.9	14.0
Standard variations (S_v)	0.5	0.9
Variation coefficient ($\frac{S_v}{\bar{m}} \times 100$)	4.4 %	6.4 %

*85 mm. nonfilter United States blended cigarette; smoking conditions: one puff per minute, 35 ml. puff volume, 2 seconds puff duration, butt length 23 mm.

**Each analysis is completed with six cigarettes.

DISCUSSION

The described analysis permits the quantitative determination of cigarette smoke within the experimental variations of $\pm 4\%$ for indole and $\pm 6.5\%$ for skatole, those for 3-ethylindole and 3-n-propylindole were below $\pm 10\%$. These relatively large variations for the alkylated indoles may be due, in part, to variations in the smoking pattern (for condensate $\pm 5\%$) and to the method of smoke condensate collection. The use of tritiated 3-ethylindole or 3-n-propylindole as second internal standard, thus affording dual-channel counting, may reduce the experimental deviation for the alkylated indoles by several percent.

The values for the indoles reported here appear relatively high compared with those by Rodgman and Cook (5). However, these authors fractionated the condensate from Turkish cigarettes with the main objective of isolating new components from the smoke rather than that of quantitative analysis. Therefore, their data should be considered only in terms of isolated amounts.

Like Rodgman and Cook (5) we did not find monomethylindoles in cigarette smoke other than 3-methylindole (skatole). But, it is possible that minute traces of other methylindoles are present (< 0.1 μ g. per cigarette) and are covered in the gas chromatograms and infrared spectrum by the excess of 3-methylindole. Extensive efforts would have to be made in order to isolate very small amounts of such monomethylindoles and seemed as unjustified at this time as the isolation of the traces of dimethylindoles. It appears theoretically unlikely that ethylindoles or n-propylindoles with alkyl substitution other than in the 3-position are present (4).

Another point of concern is the quantitative ratio between indole and skatole. With 13.9 ± 0.5 μ g. per cigarette for indole and 14.0 ± 0.9 μ g. for skatole we found a ratio of 1:1. Rodgman and Cook (5) reported 1.4 μ g. indole and 4.3 μ g. skatole as isolated from one cigarette, giving a ratio of about 1:3. We found that the ratio changed in favor of skatole after condensates had "aged" for several months or years, in the case of one condensate, aged for several years, even up to 1:10. The condensates were usually kept refrigerated and in the dark. The ratios of concentrations between skatole and 3-ethylindole as well as skatole and 3-n-propylindole were found to be stable within the experimental deviation of the analysis. One possible explanation for this observation is the

higher chemical reactivity of the non-alkylated indole compared with that of skatole (4). However, this hypothesis requires experimental proof.

The concentrate of the indoles from cigarette smoke contains also carbazole and alkylated carbazoles. The retention times of these components in the gas chromatogram were greater than those of the alkylated indoles. The quantitative analysis was developed for indoles only. Systems for the analysis of carbazole and alkylated carbazoles are under study.

SUMMARY

A method for the quantitative determination of indole and alkylated indoles in cigarette smoke is described. The analysis consists of distributions between n-hexane/methanol-water (4:1); n-hexane/methanol-water (1:2); column chromatography on Florosil and gas chromatographic separation on 20% Apiezon L at 155° C. Quantitative data are secured by use of C¹⁴-labeled indole as internal standard.

In the smoke of an 85 mm. blended United States cigarette without filter tip were determined 13.9 µg. indole, 14.0 µg. skatole, 4.2 µg. 3-ethylindole, and 0.21 µg. 3-n-propylindole. The presence of an admixture of dimethylindoles was tentatively established; their concentration was below 0.03 µg. per cigarette.

ZUSAMMENFASSUNG

Eine Methode zur quantitativen Bestimmung von Indol und alkylierten Indolen in Cigarettenrauch wird beschrieben. Die Analyse umfaßt Verteilungen zwischen n-Hexan/Methanol-Wasser (4:1) und n-Hexan/Methanol-Wasser (1:2), Säulenchromatographie an Florosil und Gaschromatographie an 20% Apiezon L bei 155° C. Die quantitative Auswertung erfolgte mit Hilfe von C¹⁴-markiertem Indol als innerer Standard.

Im Rauch einer 85-mm-U.S.-Cigarette ohne Filter wurden gefunden: 13,9 µg Indol; 14,0 µg Skatol; 4,2 µg 3-Äthylindol und 0,21 µg 3-n-Propylindol. Es gilt weiterhin als wahrscheinlich, daß Cigarettenrauch auch ein Gemisch von Dimethylindolen enthält; ihre Gesamtkonzentration wird unterhalb 0,03 µg pro Cigarette geschätzt.

RÉSUMÉ

Une méthode de détermination quantitative de l'indol et d'indols alkylés contenus dans la fumée de cigarettes est décrite. L'analyse comporte le partage entre n-hexane/méthanol-eau (4:1) et n-hexane/méthanol-eau (1:2), la chromatographie sur colonne (Florosil) et la chromatographie gazeuse (Apiezon L, 20%) à 155° C. Les dosages sont affirmés moyennant l'indol marqué au ¹⁴C qui sert de standard interne.

Les auteurs ont mis en évidence dans la fumée d'une cigarette (85 mm) sans filtre fabriquée aux Etats-Unis la présence de 13,9 µg d'indol, de 14,0 µg de scatol, de 4,2 µg de 3-éthyl-indol et de 0,21 µg de 3-n-propyl-indol. Ils présumant que la fumée de cigarettes contient, au surplus, un mélange de diméthyl-indols dont la concentration est estimée au-dessous de 0,03 µg par cigarette.

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