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COMPOSITION STUDIES ON TOBACCO—XXII.

THE NITROMETHANE-SOLUBLE, NEUTRAL FRACTION OF CIGARETTE SMOKE

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Abstract—A nitromethane-soluble residue from the neutral fraction of cigarette smoke was resolved into a number of components by column and gas chromatographic methods. Spectral analysis (i.r., u.v. and mass) indicated the presence of benzyl benzoate, benzyl cinnamate, myristicin, cinnamonitrile, carbazole, indole and skatole. Some evidence was obtained for the presence of other aromatic esters, a halogenated aromatic hydrocarbon and certain substituted indoles and carbazoles.

INTRODUCTION

EXTRACTION of the neutral fraction of cigarette smoke with nitromethane removes a subfraction rich in polynuclear aromatic hydrocarbons (PAH).[1] In view of the known characteristics of the tumor-initiating activity of smoke,[2] it is probable that the bulk of such activity is found in this nitromethane-soluble fraction. Also, since the observed tumor-initiating activity cannot be accounted for by the known PAH content of smoke,[3] it is possible that other unidentified carcinogenic compounds occur in this fraction. Although the neutral substances of smoke have been investigated extensively,[2,4] no compositional information on the nature of the nitromethane-soluble substances was available prior to the initiation of the present work. Since then, preliminary reports[5,6] have appeared on the isolation of several aromatic compounds from this fraction. The present report describes the isolation of other components from this fraction and gives details not provided in the preliminary publications.

RESULTS AND DISCUSSION

Smoke condensate was separated into acidic, basic and neutral fractions in the conventional manner (Fig. 1). The neutral fraction was further separated into substances soluble in cyclohexane, methanol–water (4:1), and nitromethane.

Resolution of the nitromethane-soluble extract was achieved by initial column chromatographic separations on silicic acid. Fraction A was eluted from silicic acid with 1:1 *n*-hexane–benzene and showed i.r. spectral characteristics of amino and/or hydroxyl, carbonyl, and

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[1] D. HOFFMANN and E. L. WYNDER, *Anal. Chem.* 32, 295 (1960).
[2] E. L. WYNDER and D. HOFFMANN, *Advan. Cancer Res.* 8, 249 (1964).
[3] E. L. WYNDER, *Acta Med. Scand., Suppl.* 369, 63 (1961).
[4] R. A. W. JOHNSTONE and J. R. PLIMMER, *Chem. Rev.* 59, 885 (1959).
[5] I. SCHMELTZ, R. L. STEDMAN, W. J. CHAMBERLAIN and C. D. STILLS, *Chem. Ind. (London)* 2009 (1965).
[6] I. SCHMELTZ, R. L. STEDMAN, J. S. ARD and W. J. CHAMBERLAIN, *Science* 151, 96 (1966).

aromatic groups. Gas–liquid chromatographic (GLC) analysis of this fraction on SE-30 indicated the presence of at least twenty components. Infrared spectral analysis of selected GLC peaks showed the probable presence of oxygenated aromatic compounds, indoles and carbazoles. Further purification of the silicic acid eluate by column chromatography on alumina and GLC analysis of the alumina column eluates resolved the fraction into aromatic esters, an aromatic ether, indoles and carbazoles.

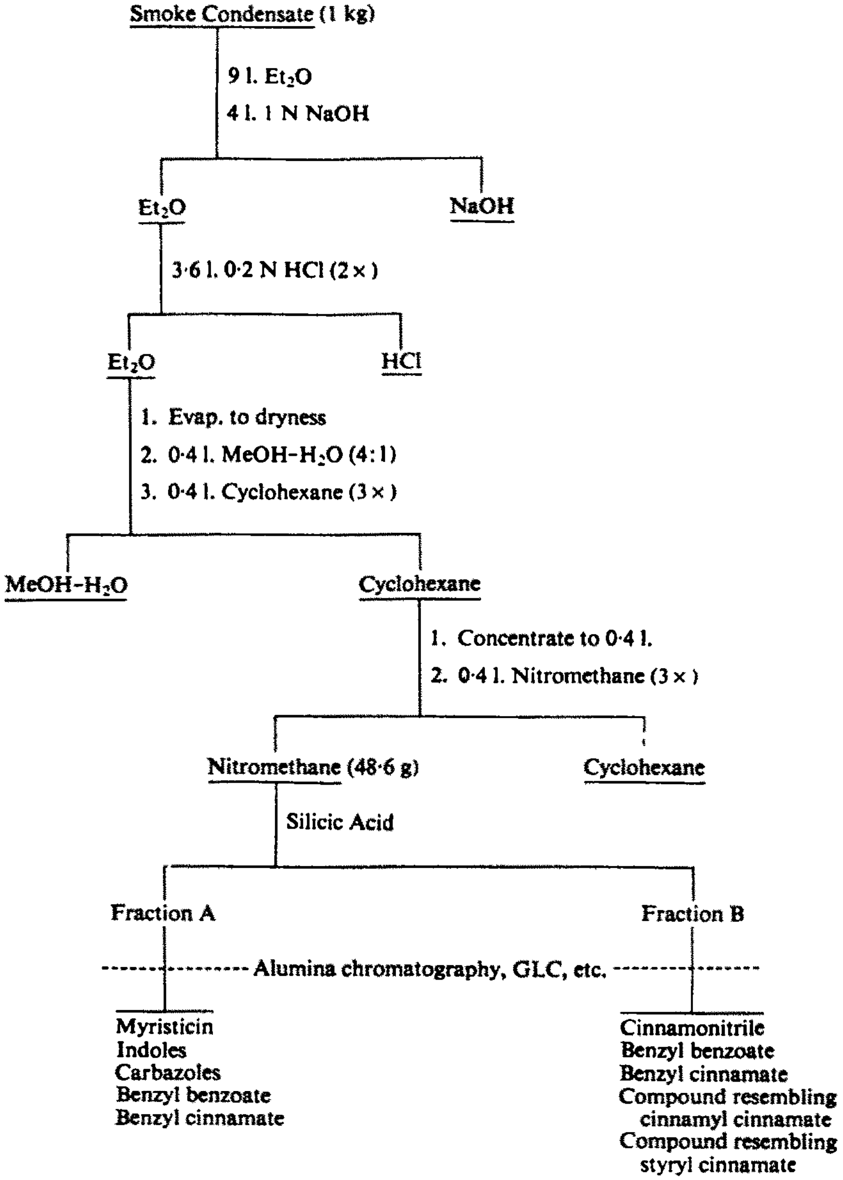

FIG. 1. FRACTIONATION OF SMOKE CONDENSATE.

Identification of the components was made in each case by collection of the GLC peak, assignment of general structural features by i.r. and u.v. spectral analysis, and confirmation of the structure by mass spectral examination. When authentic compounds were available, confirmation of identity was made by GLC and spectral comparisons of known compounds and the isolated substances. In this manner, the presence of benzyl benzoate, benzyl cinnamate, myristicin, skatole, indole and carbazole in Fraction A was demonstrated. When authentic compounds were unavailable for comparative purposes, an evaluation of probable structure was made on the basis of i.r., u.v. and mass spectral considerations. Thus, the presence in Fraction A of a methylindole (other than skatole) and a methylcarbazole was indicated, but the orientation of the methyl group in either case was not determined. The i.r. and mass spectra of other components in this fraction showed the probable presence of alkyl-substituted indoles and carbazoles, the exact structures of which were not determined.

Fraction B was eluted from silicic acid by 1 : 1 hexane–benzene subsequent to Fraction A. Further separation of this eluate on alumina yielded a fraction containing several components among which were benzyl benzoate and benzyl cinnamate. One of the remaining components was identified as cinnamonitrile by gas chromatographic, i.r. spectral and mass spectral characteristics. Conclusive identities of the other substances could not be established. One of these had i.r. spectra suggestive of aromatic esters with strong absorption at 6·12 μ and major fragment peaks at 250, 131 and 103 corresponding to the parent ion and possibly to cinnamoyl and styryl ions. To our knowledge, the existence of styryl cinnamate has never been shown either by isolation from a natural source or by synthesis. A second unknown compound gave similar absorption at 6·12 μ, a parent peak at mass 264 and a major fragment peak at mass 117, in addition to other unidentified peaks. Cinnamyl cinnamate has a molecular weight of 264 and should fragment to yield cinnamyl ion, mass 117.

An interesting halogen compound was isolated from a silicic acid eluate just prior to Fraction A. The i.r. spectrum of the compound was identical to that reported by others for 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene (TDEE) which has been previously isolated from cigarette smoke[7, 8]. The mass spectrum showed the presence of three chlorine atoms and a molecular weight of 282. TDEE is believed to be a pyrolysis product of the pesticide, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (TDE), which has been found in harvested tobacco.[9]

Table 1 lists the substances and approximate levels thereof isolated from the nitromethane-soluble residue in the present study. Myristicin, cinnamonitrile and the benzyl esters of benzoic and cinnamic acids have not been reported in tobacco smoke or leaf prior to the present work. Indole, skatole, methylindoles, and alkylated carbazoles have been reported,[10] but the positions of substitutions were not determined in the last two groups of compounds. The levels of the heterocyclic compounds found in the present study vary somewhat from those reported by others[10], but none of the studies published to date, including the present one, has been quantitative. The presence of myristicin may be of special importance since this compound is believed[11] to be the component in nutmeg oil responsible for the pharmacological activity of the oil. A closely related compound, safrole, is a weak hepatic carcinogen.[12] The possible presence of elemicin (I), an analog of myristicin, in tobacco leaf has been

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[8] T. G. Bowery, P. E. Gatterdam, F. E. Guthrie and R. L. Rabb, *J. Agr. Food Chem.* 13, 356 (1965).

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[10] A. Rodgman and L. C. Cook, *Tobacco Sci.* 6, 176 (1962).

[11] A. T. Weil, *Econ. Botany* 19, 194 (1965).

[12] E. L. Long, A. A. Nelson, O. G. Fitzhugh and W. H. Hansen, *Arch. Pathol.* 75, 595 (1963) (from *Biol. Abstp.* 45, 60335 (1964)).

indicated by Onishi,[13] and this compound may serve as a precursor of myristicin (II) by the loss of a molecule of methane during pyrolysis.

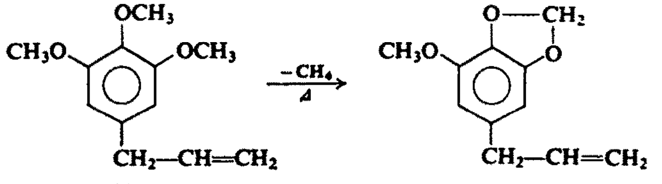
Table 1. Nitromethane-soluble substances from the neutral fraction of cigarette smoke. Levels* of some of the major components

Fraction	Compound	μg/100 cigarettes
A	Skatole	198·8
	Indole	12·2
	Methylindole (not Skatole)	18·0
	Substituted indoles†	87·0
	Carbazole	206·6
	Methylcarbazole	110·6
	Substituted carbazoles†	38·8
	Myristicin	64·0
	Benzyl benzoate‡	40.0
	Benzyl cinnamate‡	40·0
B	Cinnamonitrile	13·2

* Levels represent amounts isolated, and do not take into consideration operational losses.

† Substitution may be dimethyl, trimethyl, ethyl, methylethyl or propyl.

‡ These esters were also found in Fraction B.



(I) (II)

EXPERIMENTAL

Smoking Conditions

Commercial, U.S. made cigarettes were smoked on an automatic cigarette smoking machine under standard conditions (puff duration, 2 sec; puff volume and rate, 35 ml/min). The smoke was condensed in traps cooled in dry ice–acetone. The condensate was washed from the traps with acetone; the acetone was removed *in vacuo* and the residue was partitioned[14] among several solvents (Fig. 1).

Fractionation of Smoke Condensate

The nitromethane solution from the separation of 1 kg condensate (from 50,000 cigarettes) was evaporated to dryness, and the residue (48·6 g) was dissolved in 100 ml of 4:1 (v/v) *n*-hexane–benzene. This solution was chromatographed on a 60 × 4·4 cm column of methanol-washed, activated (150° for 16 hr) silicic acid (906 g). The column was eluted successively

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[14] I. Schmeltz, C. D. Stills, W. J. Chamberlain and R. L. Stedman, *Anal. Chem.* **37**, 1614 (1965).

with 95:5 and 1:1 *n*-hexane–benzene (5·9 and 6·0 l., respectively), benzene (4·5 l.), 3:1 and 1:1 benzene–ethyl ether (6·0 and 3·5 l., respectively), ethyl ether (1·5 l.), 95:5 and 9:1 ethyl ether–methanol (2·0 and 3·0 l., respectively) and methanol (3·0 l.). Generally, 0·5 l. fractions were collected, evaporated to dryness, weighed and screened by i.r. spectral analysis. Individual fractions were pooled on the basis of residue weights and i.r. spectral characteristics.

Fraction A

The first 3 l. of the 1:1 *n*-hexane–benzene eluate yielded 4·8 g of a dark-brown oil. Gas chromatography of a portion of this oil on a column (1·2 m × 0·64 cm) packed with glass beads containing 0·25% SE-30 (programmed from 65° to 300° at 4°/min, injector: 325°, detector: 315°, bridge current: 150 mA, helium flow rate: 60 ml/min) indicated the presence of at least twenty components. Two major peaks eluting at 141° and 180° were collected in glass U-tubes cooled in dry ice–acetone, and were shown by spectral analysis to be skatole and benzyl benzoate, respectively. Other collected gas chromatographic peaks showed i.r. spectral characteristics of indoles and carbazoles.

Two grams of Fraction A were placed on a 14 × 2·2 cm column of methanol-washed, activated (170° for 16 hr) neutral alumina (50 g). The column was eluted successively with petroleum ether (0·1 l.), 95:5 and 1:1 petroleum ether–benzene (0·1 and 0·8 l., respectively), benzene (0·5 l.), 3:1 and 1:1 benzene–ethyl ether (0·5 and 0·3 l., respectively), ethyl ether (0·3 l.), 95:5 ethyl ether–methanol (0·3 l.) and methanol (0·3 l.). One hundred milliliter fractions were collected, dried, weighed, screened by i.r. spectroscopy and pooled on the basis of superficially similar characteristics.

The fractions thus obtained were chromatographed (2·4 m × 0·64 cm column of 20% SE-30 on Chromosorb W, detector: 325°, injector: 325°, flow rate: 60 ml helium/min, column temperature: 180° for 15 min and then programmed at 8°/min to 265°). Identities of the substances isolated from the pooled fractions, were as follows (retention times in parentheses):

Fractions 1–3:	aliphatic hydrocarbons
Fractions 4–5:	benzyl benzoate (25·8 min)
Fraction 6:	myristicin (13·3 min), benzyl benzoate and benzyl cinnamate (34·0 min)
Fractions 7–10:	methylindole (not skatole) (7·6 min), another substituted indole (11·0 min) and benzyl cinnamate
Fraction 11:	indole (5·8 min), skatole (9·8 min) and a substituted indole (13·9 min)
Fraction 12:	skatole
Fractions 13–15:	carbazole (27·5 min)
Fractions 16–17:	carbazole (10·0 min) and substituted carbazoles (14·0 and 18·5 min)

Pooled fractions 16–17 were gas chromatographed under the following conditions: 3·1 m × 0·64 cm column of 20% SE-30 on Chromosorb W, detector: 290°, injector: 260°, flow rate: 60 ml/min, column temperature: 240°.

Fraction B

The final 3 l. of the 1:1 *n*-hexane–benzene eluate off the silicic acid column gave 2·6 g of a dark-brown oil. Chromatography on alumina and development by the flowing technique yielded a fraction which was eluted with 1:1 petroleum ether–benzene. This fraction contained eleven peaks on GLC analysis (2·4 m × 0·64 cm column of 20% SE-30 on Chromosorb W, detector: 325°, injector: 325°, flow rate: 60 ml/min, column temperature: 165° for 15 min and then programmed at 4°/min to 280°) having identities or structural characteristics as follows:

GLC Retention time (min)	Identity
9	Cinnamonitrile
37·6	Benzyl benzoate
52·0	Benzyl cinnamate
59·0	Compound resembling styryl cinnamate

A fraction eluting subsequent to the above yielded a compound having the i.r. spectrum and molecular weight of cinnamyl cinnamate. The retention time, at twice the above programming rate, was 46·5 min.

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