Isolation and Identification of Aza-Arenes of Tobacco Smoke*

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INTRODUCTION

Nitrogen analogues of polynuclear aromatic hydrocarbons (termed aza-arenes) are important, biologically active compounds (1) and are found in numerous sources of the environment, such as petroleum distillate (2), coal tar, air pollution (3), automobile exhaust (4), and tobacco smoke (5, 6). Consequently, their analysis in environmental samples, as well as in tobacco smoke, has recently received considerable attention. In Schmeltz and Hoffmann's review of the nitrogen-containing compounds of tobacco and tobacco smoke (5), only about seven aza-arenes were stated as occurring in smoke, in addition to the quinolines and isoquinolines. Since then, several reports have appeared on the isolation, identification, and quantitation of aza-arenes in cigarette smoke condensate (CSC). Klus and Kuhn (7) reported a complicated seven-step procedure for isolating aza-arenes by a method involving several solvent partitioning steps, alumina chromatography, gel chromatography on Sephadex LH-20, ion exchange, and thin-layer chroma-

Figure 1. Aza-arenes isolation scheme.



3. HPLC

tography. They reported a large number of aza-arenes, containing up to three fused rings, and their alkyl derivatives. They also reported the presence of 3,4-benzacridine and a dimethyl derivative. Recently, Dong and co-workers (6) published their work on the isolation and quantitation of aza-arenes. They utilized a hydrochloric acid extraction, with subsequent alumina chromatography, to yield aza-arene containing fractions. We now report our work on the isolation and identification of aza-arenes in a basic fraction of cigarette smoke condensate. Our methodology involves a reduced number of steps to produce a concentrated, relatively pure aza-arene isolate, suitable for identification purposes. Our procedure successfully isolates the full range of aza-arenes from quinoline to azapyrene, including numerous alkyl derivatives.

EXPERIMENTAL

Preparation and Fractionation of Cigarette Smoke Condensate

All solvents were Burdick and Jackson** "distilled-in-glass" solvents. Cigarette smoke condensate was prepared from commercially available cigarettes as previously described (8). Fractionation of the condensate to obtain the bases was performed as reported (8) (Fig. 1). Briefly, condensate (1 kg) was partitioned between diethyl ether (E) and 1 N aqueous NaOH to separate acids. The diethyl ether layer was then extracted with 1 N HCl to isolate the bases. The HCl solution was adjusted to pH 12 with NaOH pellets (cooling with ice) and extracted with diethyl ether. The diethyl ether solution was dried over anhydrous Na₂SO₄ and evaporated to yield 56.3 g of a free bases fraction (5.63%) of original cigarette smoke condensate).

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Silicic Acid Chromatography

The free bases were redissolved in about 300 ml of diethyl ether. Silicic acid (125 g, Mallinckrodt, 100 mesh, washed with MeOH, dried at 150 °C for 16 h) was added to the diethyl ether solution. The diethyl ether solvent was evaporated from the silicic acid (SA) slurry on a rotary evaporator, leaving the bases deposited on the SA. The SA-sample mixture was placed on top of a column of silicic acid (1000 g) packed in petroleum ether [PE (Burdick and Jackson, 30-60°C grade)] and eluted with the following solvents to give fractions F-A to F-E (Fig. 1): 8 l benzene/ether (B/E) (1:1), 6 l B/E (1:3), 6 l diethyl ether, 6 l acetone, and 3 1 MeOH. The fractions were evaporated on a rotary evaporator and the yields in grams and percentage composition of cigarette smoke condensate were: F-A, 6.49 g, 0.65%; F-B, 5.8 g, 0.58%; F-C, 11.4 g, 1.1%; F-D, 16.9 g, 1.7%; F-E, 10.1 g, 1.0%.

Gel Chromatography

The gel chromatographic system consisted of four (1.25 cm × 109 cm) glass Cheminert liquid chromatography columns (Laboratory Data Control, Riviera Beach, Florida) connected in series and packed with Bio-Beads S-X12 (a neutral, porous, styrene-divinyl-benzene co-polymer, molecular exclusion 400; Bio-Rad Laboratories, Richmond, California) in benzene. Total length of the wet gel bed was approximately 400 cm. Fraction F-A residue was dissolved in 10 ml of benzene and aliquots were placed on the first gel column with a 1.0 ml loop injection valve. Three 1 ml aliquots of F-A were individually chromatographed through the gel columns. Benzene was pumped (Chromatronix CMP-3 pump) at a flow rate of 120 ml/h and 50 8 ml fractions were collected. Eluted gel fractions (GF) from all of the runs with the same number were combined. After Fraction 50, 500 ml of the column eluant was collected to elute a colored material and was evaporated to give combined GF 50-115. Column eluant was monitored at 280 nm with a Chromatronix Model 230 detector. The system gave reproducible elution volumes for standards.

Gas Chromatography (GC)

The gel fractions were analyzed by gas chromatography on a 1.83 m \times 0.32 cm outside diameter nickel column, packed with 6% OV-17 on 100/120 mesh Chromosorb G/HP. A Hewlett-Packard 5750 gas chromatograph, with FID, was employed under the following conditions: temperature program, 90 to 250 °C at 2°/min; injector, 290 °C; detector, 350 °C; helium flow, 20 ml/min. The gel fractions were also analyzed with a Hewlett-Packard 5830 gas chromatograph equipped with a nitrogen-phosphorus (NP) selective detector under the same chromatographic conditions as above.

Identification of Compounds

Each individual gel fraction from GF-40 to GF-49 and the combined GF 50-115 were submitted to preparative gas chromatography on a Hewlett-Packard 5720A gas chromatograph, equipped with a thermal conductivity detector and a 1.83 m × 0.32 cm nickel column packed with 6% OV-17 on 100/120 mesh Chromosorb G/HP (injector, 290 °C; detector, 300 °C; helium flow, 20 ml/min; temperature program, 90-250 °C at 2°/min). Separated components (GC cuts) were collected in melting point capillaries cooled by dry ice, and were rinsed into UV cuvettes with cyclohexane. UV spectra were recorded on a Beckman Acta C III spectrophotometer. Each gel fraction was also submitted to GC - mass spectrometry (MS) analysis on a Hewlett-Packard 5700A gas chromatograph - 5930A mass spectrometer system. Aza-arene standards were commercially obtained. 4-Azapyrene was purchased from K & K Labs, ICN Pharmaceutical, Inc., Plainview, New York. 3-Phenylindole was kindly supplied by D. Hoffmann, American Health Foundation, Valhalla, New York.

High-Pressure Liquid Chromatography

A Varian 8500 liquid chromatograph, equipped with a $25 \text{ cm} \times 4.9 \text{ mm}$ Whatman Partisil 10-micron ODS-2 column, was used in the analyses of the collected GC cuts. The solvent was programmed from $50^{\circ}/_{0}$ MeOH /

Compound	Percentage distribution									
	Petroleum ether	Benzene / petroleum ether (1:3)	Benzene / petroleum ether (1:1)	Benzene	Benzene / diethyl ether (1:1)	Diethyl ether				
Naphthalene	_	100	_							
Anthracene	_	100	 .	— , ¹	_	-				
Benzo[a]pyrene	_	100		_	_	- -				
Quinoline		-		_	79	21				
6-Methylquinoline	-	_		_	83	17				
Acridine	-	_	_	·	100					
Benz[c]acridine		. —	-	69	31					
Phenanthridine	-	_	- .	_	100	· _				
1-Azapyrene	-	_	-	-	100	_				

Table 1. Silicic acid chromatography of heteroatom-PAH.

 H_2O to 90% MeOH / H_2O at 4% min. Elutions were monitored at 254 nm. Separated components were collected and UV spectra obtained.

RESULTS AND DISCUSSION

Several standard polynuclear aromatic hydrocarbons (PAH) and aza-arenes were chromatographed on silicic acid with solvents of various polarities (Table 1). The results indicated that the more polar nitrogen compounds required benzene/ether solvent mixtures to elute them from the column, while the PAH could be eluted with benzene / petroleum ether mixtures. Nicotine and other alkaloids began to elute from silicic acid with 75% benzene / ether and were mainly eluted with acetone and MeOH. Thus, the elution procedure allowed the more abundant alkaloids to be separated from the less abundant aza-arenes.

Since gel chromatography with Bio-Beads S-X12 in benzene was successful in isolating, in a relatively pure form, the PAH from silicic acid fractions of cigarette smoke condensate neutrals (9), it was expected that similar purification could be achieved for the aza-arenes. The elution characteristics of a number of PAH and their aza-arene analogues (Table 2) confirmed that azaarenes were also retained by an adsorption-type mechanism by the Bio-Beads polystyrene gels, using benzene as solvent. In comparison, aliphatic compounds were eluted early from the gels and were cleanly separated from the aromatic compounds. The results also showed that the aza-arenes eluted slightly earlier than the PAH, presumably due to their slightly lower ring π -electron density. A full discussion of the elution properties of heteroatom-PAH from Bio-Beads has been given elsewhere (10). Alkylated aza-arenes eluted before their parent compounds and elution was in order of increasing ring number. These two properties of the gels allowed the identification of individual components in complex mixtures by partial separations and selective enrichments.

The retention properties of silicic acid and Bio-Beads gels were successfully utilized to develop the isolation scheme for the aza-arenes shown in Fig. 1. A free bases fraction, representing 5.63% of cigarette smoke condensate, was isolated from cigarette smoke condensate and chromatographed on silicic acid. Elution with 50% benzene / ether yielded an aza-arene-containing fraction (F-A), representing 0.65% of cigarette smoke condensate. Preliminary experiments had shown that essentially no aza-arene was eluted from the silicic acid column prior to the 50% benzene / ether solvent system. The need for gel chromatography is evident from the comparison of gas chromatograms of F-A and a gel fraction of the aza-arene isolate (Fig. 2). Quinoline and isoquinoline can barely be detected among the multitudes of peaks in the gas chromatogram of the unrefined F-A fraction. However, after gel chromatography, the gas chromatogram of one of the gel fractions from F-A shows the dramatic enrichment and purification achieved.

The characteristics of the gel chromatographic step for F-A are shown in Fig. 3. Also shown are the elutions of several standard aza-arenes. As can be seen, most of the weight of F-A (about $63^{0}/_{0}$) eluted from the gels before the beginning (GF-39) of the elution of the aza-arenes. Eighty percent of the weight of F-A eluted before the elution of the 2- and 3-ring aza-arenes (GF-40). After GF-49, it was noticed that weakly fluorescent material (observed with portable long-wave UV light source) continued to be eluted from the gels. It required an additional 500 ml of benzene to com-

Compound	Gel fraction number										
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	42	43	44	45	46	47	48	49			
Naphthalene	0.4	25.7	53.7	19.3	0.9						
Quinoline		9.4	47.4	37.8	5.3	0.1					
2-Methylnaphthalene	3.8	44.6	40.8	9.2	1.5		5				
4-Methylquinoline	3.1	52.6	43.0	1.4							
Anthracene	0.2	11.2	46.9	34.4	6.7	0.5					
Phenanthrene	0.4	1.1	22.2	45.0	27.3	4.7					
Acridine	1.5	26.4	48.5	19.2	4.1	0.3					
Phenanthridine	÷	6.3	37.4	36.9	· 17.1	2.3					
Benzo[f]quinoline		1.4	24.7	46.4	22.7	4.2					
Pyrene			0.1	4.3	26.9	42.1	21.7	4.9			
1-Azapyrene			1.7	11.7	34.1	31.5	11.7	7.3			
	Gel fraction number										
	25	26	27	28	29	30	- A				
C ₃₁ -hydrocarbon	1.2	21.4	36.7	16.3	9.6	2.3					

Table 2. Gel chromatography of PAH and heteroatom-PAH in Bio-Beads S-X12 in benzene.

69





pletely remove this material from the gel column. All of this material was pooled and designated GF 50-115. Each gel fraction from GF-40 to GF-49 and combined GF 50-115 was submitted to analysis by analytical gas. chromatography (including correlation with GC retention times of standards), preparative gas chromatography with subsequent UV analyses of GC cuts, and GC-MS. In addition, high-pressure liquid chromatography (HPLC) was used to separate and characterize components of the preparative GC cuts.

The results of our identification studies are shown in Figures 4-9. The gas chromatogram of GF-40 is given in Fig. 4. As expected, many alkyl derivatives were found in the early gel fractions. The only parent compounds found in this fraction were the 2- and 3-vinylpyridines and 2- and 3-phenylpyridines. GF-40 contained major amounts of methyl-, dimethyl-, trimethyl-, and tetramethylpyridines, vinylpyridines, quinolines, isoquinolines, phenylpyridines, 4-azafluorenes, benzoquinolines and benzoisoquinolines. Standards were available for the 2-, 4-, 6-, 7-, and 8-methylquinolines. The elutions of the other 2 isomers were assumed to be identical with those found by Dong and co-workers (6). Three different peaks were found to correspond to compounds with a molecular weight of 182. Their UV spectra were very similar to that of harman; however, none of them co-chromatographed with standard harman. Harman is a methylazacarbazole (1-methyl-9H-[3,4-b]indole) and has been reported to be present in smoke (11). Acetone was required to elute standard norharman and harman from the silicic acid column and thus these compounds would be expected to be found in silicic acid fraction F-E. In fact, norharman and harman were found in large quantities in F-E. The presence of alkylazacarbazoles in the less polar 50% benzene / ether fraction from the silicic acid chromatography strongly indicated that these were N-alkylated derivatives. Since there are four possible isomers of azacarbazole: 1-, 2-, 3-, and 4-azacarbazole (also designated α -, β -, γ -, δ -carbolines), it appeared that the three different m/e 182 peaks were different N-methylazacarbazoles. The UV spectra of 1- and 2-azacarbazole are very similar (12), but literature UV spectra for the other two isomers could not be found to compare with the isolated compounds. Several other compounds were found with even molecular weights, i.e. 130 and 168. Analysis of GF-40 with a nitrogen-phosphorus selective detector indicated that these compounds did not contain nitrogen and they were probably present because of incomplete solventsolvent extractions.

The gas chromatogram of GF-42 (Fig. 5) showed that in this gel fraction the multialkyl derivatives were greatly reduced and the parent aza-arenes were beginning to appear. Among these compounds were quinoline, isoquinoline, and 4-azafluorene. The nitrogen-phosphorus detector indicated that the compound (peak) with m/e 132, eluting just before quinoline, did not contain nitrogen. The possible presence of diazanaphthalenes, such as quinoxaline and quinazoline, which have been reported to be present in smoke (5, 7), was considered. Although standards and literature UV spectra were available for most of the isomers of diazanaphthalene, none was found in the analyzed gel fractions of F-A. Their presence in other silicic acid fractions (F-B to F-E) is being investigated.

Major quantities of the benzoquinolines (BQ) and benzoisoquinolines (BIQ) eluted in GF-45 (Fig. 6). All eight possible isomers of BQ/BIQ were found, with four isomers (B[g]Q, B[f]IQ, B[g]IQ, B[h]IQ) being reported present in cigarette smoke condensate for the first time. Quinoline, isoquinoline, and their alkyl derivatives were also concentrated in GF-45. HPLC was a great asset in the identification of individual components. The material in the GC peak labeled "B[c]Q (phenanthridine) + B[f]Q" was collected by preparative GC and submitted to HPLC by reverse-phase chromatography. Separated components (Fig. 7) were collected and their UV spectra were obtained, which in conjunction with GC-MS data, proved that the peak contained tetramethylquinoline, B[c]Q, and B[f]Q.

The four-ring aza-arene compounds first appeared in GF-45 but major quantities eluted in GF-48 (Fig. 8). Three GC peaks in GF-45 and GF-48 had given GC-MS spectra with molecular ions of 203. The UV spectrum of the compound (peak) labeled "4-azapyrene" matched that of the standard compound. Although the standard was labeled "1-azapyrene", its spectrum matched that of benzo[lmn]phenanthridine (13), also called 4-azapyrene by the current numbering system. Another GC peak (compound) had an UV spectrum (λ_{max} 247, 272, 282, 286, 292, 306, 320) that closely matched that of 7-azafluoranthene (acenaphtheno[1,2-b]pyridine): literature values (13) were λ_{max} 240, 245, 270, 275, 281, 287, 292, 307, 317. The peak in the chromatograms of GF-45 and GF-48 labeled "N-Me-azacarbazole + azafluoranthene" had a complex UV spectrum. However, UV absorbance (14) indicated that 1-azafluoranthene, reported previously (6), was present.

UV spectra of 4-azapyrene and that of the next to last compound labeled "DiMe-4-azapyrene" in the chromatogram of GF-45 (Fig. 6) are compared in Fig. 9. The isolated compound had a m/e 231 and the shift in λ_{max} to longer wavelength confirms its identity as a dimethyl-4-azapyrene. The data in Tables 1 and 2 indicate that any 5- and 6-ring aza-arenes present should have been found in the fractions analyzed. Since none were found in these gel fractions, their concentrations must be so low in cigarette smoke condensate as to require fractionation of much larger quantities of cigarette smoke condensate.

The gas chromatogram of combined GF 50-115 is shown in Fig. 10. Since sp^3 -nitrogen compounds, such as indoles and carbazoles, are adsorbed by the Bio-Beads to a much greater extent than aza-arenes, it was no surprise to find 7-azaindole and its alkyl derivatives in this fraction. Although indoles and carbazoles are mainly found in the neutral fraction of smoke and are not readily extracted by HCl, the presence of the sp^2 -nitrogen caused these aza-derivatives to be extracted by HCl. Numerous alkylated derivatives of 7-azaindole were also found.



Figure 4. Gas chromatogram of Bio-Beads S-X12 gel fraction (GF) 40.



73



Figure 6. Gas chromatogram of gel fraction 45.

Quinolines (Q) + isoquinolines (IQ)



75



Figure 10. Gas chromatogram of combined gel fraction 50-115.

Detector response

Figure 11. Structures of some isomeric $(C_{11}H_8N_2)$ nitrogen aromatics found or postulated as present in cigarette smoke condensate.



Norharman (2-azacarbazole or β -carboline (Fig. 11)) was found to elute in GF 60-75. When the mass spectrum of combined GF 50-115 showed that the two largest peaks in the chromatogram corresponded to compounds with m/e 168 and 182, respectively, it was initially assumed that they were norharman and harman. which have been isolated from cigarette smoke condensate by Poindexter and co-workers (11). However, these two compounds did not have the norharman UV spectrum. Further, as mentioned, harman and norharman would not be expected to occur in the 50% benzene/ether fraction from silicic acid chromatography, but in a later fraction eluted by a more polar solvent. The compound indicated by the largest peak in Fig. 10 had an UV spectrum similar in shape to that of an azacarbazole isolated by Testa (15). However, our isolated compound had λ_{max} of 296, while that of Testa, about 274 nm. Testa indicated that his isolated azacarbazole was a-carboline. However, a-carboline has been reported to have a λ_{max} of 288 (16). We believe that our isolated compound was 5,6-benzo-7-azaindole (1H-pyrrolo[2,3b]-quinoline) and we are currently synthesizing this compound to verify its identity.

The possible presence of pyrroloquinolines (12) of the type shown in Fig. 11 was also considered. Compounds (peaks) in the latter half of the chromatogram of GF 50-115 (Fig. 10) gave UV spectra (λ_{max} 264) characteristic of pyrroloquinoline and its alkyl derivatives, which are indicated on the chromatogram as alkyl-m/e 168.

SUMMARY

The nitrogen analogues of polynuclear aromatic hydrocarbons (aza-arenes) were isolated and identified in a basic fraction of cigarette smoke condensate. Silicic acid chromatography removed the predominant nicotine alkaloids, while gel chromatography on Bio-Beads S-X12 in benzene effectively separated the aza-arenes from interfering aliphatic compounds. In addition, the gel columns separated the aza-arenes by ring number and degree of alkylation on the basis of an adsorption-type mechanism. These gel characteristics facilitated the identifications of a large number of isomeric aza-arenes. Compounds identified included 2-vinylpyridine, 3-vinylpyridine and 2-phenylpyridine as well as quinoline, isoquinoline, 4-azafluorene, benzoquinolines, benzoisoquinolines, 1-azafluoranthene, 7-azafluoranthene, 4-azapyrene, 7-azaindole, pyrroloquinoline and their mono-, di- and trimethyl derivatives. All eight possible isomers of benzoquinoline and benzoisoquinoline were found, four of which are being reported for the first time. Evidence was also found for the probable presence of 5,6-benzo-7-azaindole.

ZUSAMMENFASSUNG

Die Stickstoffanalogen der polycyclischen aromatischen Kohlenwasserstoffe (Aza-arene) wurden in einer basischen Fraktion des Cigarettenrauchkondensates isoliert und identifiziert. Die überwiegend vorhandenen Nikotinalkaloide wurden mittels Chromatographie an Kieselsäure entfernt, während sich die Gel-Chromatographie an Bio-Beads S-X12 in Benzol als ein gutes Verfahren zur Abtrennung der Aza-arene von störenden aliphatischen Verbindungen erwies. Die Aza-arene wurden durch die Gel-Chromatographie darüber hinaus über einen adsorptionsähnlichen Mechanismus nach der Anzahl der Ringe und dem Grad der Alkylierung aufgetrennt. Auf der Basis dieser Gel-Charakteristika konnte eine große Zahl von isomeren Aza-arenen identifiziert werden. Es handelt sich u. a. um folgende Verbindungen: 2-Vinylpyridin, 3-Vinylpyridin und 2-Phenylpyridin sowie Chinolin, Isochinolin, 4-Azafluoren, Benzochinoline, Benzoisochinoline, 1-Azafluoranthen, 7-Azafluoranthen, 4-Azapyren, 7-Azaindol, Pyrrolochinolin und deren Monomethyl-, Dimethyl- und Trimethylderivate. Von Benzochinolin und Benzoisochinolin wurden alle acht möglichen Isomere gefunden, wobei über vier dieser Verbindungen zum erstenmal in der Literatur berichtet wird. Auch wurde wahrscheinlich gemacht, daß 5,6-Benzo-7azaindol im Cigarettenrauchkondensat vorkommt.

RÉSUMÉ

Les substances azotées analogues des hydrocarbures aromatiques polynucléaires (aza-arènes) ont été isolées et identifiées dans une fraction basique de condensats de fumée de cigarette. La chromatographie à l'acide silicique élimina les principaux alcaloïdes nicotiniques, tandis que la chromatographie sur gel Bio-Beads S-X12 dans du benzène, sépara effectivement les aza-arènes des composés aliphatiques interférants. De plus les colonnes de gel séparèrent les aza-arènes par nombre de cycles et degré d'alkylation selon un système à adsorption. Ces caractéristiques du gel facilitèrent l'identification d'un grand nombre d'aza-arènes isomères. Les composés identifiés étaient les suivants: 2-vinylpyridine, 3-vinylpyridine et 2-phénylpyridine, de même que quinoléine, isoquinoléine, 4-azafluorène, benzoquinoléines, benzoisoquinoléines, 1-azafluoranthène, 7-azafluoranthène, 4azapyrène, 7-azaindole, pyrroloquinoléine et leurs dérivés mono-, di- et triméthyle. Les huit isomères possibles de la benzoquinoléine et benzoisoquinoléine ont été décelés, pour quatre d'entre eux pour la première fois. On a également trouvé des indices de la présence du 5,6-benzo-7-azaindole.

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