Aza-Arenes in Tobacco Smoke [1,2]

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Abstract

Quinoline is a hepatocarcinogen in rats and has recently been found to be mutagenic in Salmonella typhimurium. **Its presence and that of related compounds in tobacco and tobacco smoke were established. This report describes an analytical method for the qualitative and quantitative determination of quinolines and other basic aza-arenes in tobacco smoke. The method consists of solvent partitioning, column chromatography, and gas chromatography-mass spectrometry. High-pressure liquid chromatography (HPLC) and spectroscopic techniques were used to ascertain the identity of isomeric aza-arenes. Since quinoline was found to be the most abundant aza-arene in the smoke, ^{14}C-quinoline was synthesized for use as an internal standard in quantitative determinations. The mainstream smoke of a U.S.-blended, 85-mm cigarette without filter tip contains 1.67 μg of quinoline, 0.12 μg of isoquinoline, and a total of 0.70 μg of methylquinolines. Four isomers of benzoquinolines, azafluoranthenes, and azapyrenes were also quantitated. Sidestream smoke was found to contain about 11 times more quinoline than mainstream smoke. The analytical procedure was applied to various tobacco products as well as to unburned tobacco. Studies using cigarettes made entirely from cellulose showed that tobacco leaf proteins are major precursors for quinoline in the smoke. Non-selective reduction of quinoline levels in smoke can be effected by filtration; selective reduction can be achieved by use of tobacco of low protein content.**

Introduction

Aza-arenes in tobacco smoke are products of thermal decomposition of proteins and other nitrogenous leaf materials. In 1960, Van Duuren *et al.* demonstrated the presence of dibenzacridines in cigarette smoke (1). Candeli *et al.* confirmed these findings in 1963 (2). Quinolines, isoquinolines, and benzoquinolines were also identified in the smoke, although not quantitated (3). Ishiguro and Sugawara have recently determined the quinoline levels in the smoke of the lamina and midrib of flue-cured tobacco leaves and found them to be 2.1 and 1.2 μg/cig., respectively (4). However, comprehensive quantitative data on aza-arenes in tobacco smoke are lacking, although several aza-arenes, e.g., alkyl derivatives of benzacridines and dibenzacridines, are established carinogens in experimental animals, and others are suspected carcinogens (5). Recently, quinoline was found to induce hepatomas in rats (6). Quinoline, 5- and 8-hydroxyquinolines, all methylquinolines and some benzoquinolines were found to be mutagenic in *Salmonella typhimurium* (7-9). These findings suggest that quinoline and, possibly, certain aza-arenes, play a role in tobacco carcinogenesis. Therefore, we have now begun a more comprehensive survey of aza-arenes in the basic portion of tobacco smoke. Neutral aza-arenes such as carbazoles and indoles, which have been studied earlier (10-13), are not included in this study.

[1]Presented in part at the 31st Tobacco Chemists' Research Conference, Greensboro, North Carolina, Oct. 3-5, 1977.

[2]This is No. LV of Chemical Studies on Tobacco Smoke.

Experimental

Reagents

Reference aza-arenes were obtained from K & K, Aldrich Chemical Co., and Pfaltz and Bauer, Inc. All solvents used were spectrograde. Alumina (activity II-III) was obtained from ICN Pharmaceuticals, Inc. For the sysnthesis of quinoline-3-^{14}C, 50 μCi (31 mCi/mmol) glycerol -2-^{14}C, was obtained from New England Nuclear, Inc. Cigarettes and other tobacco products were purchased on the open market in Westchester County, New York, during 1976/77. Quinoline and methylquinoline reference compounds were purified by preparative liquid chromatography, as described previously (14).

Apparatus

All GC analyses were performed on a 10', 1/8" o.d. stainless steel column, packed with 3% Dexsil 300 GC on Chromosorb AW/HP (80/100 mesh) obtained from Applied Science Laboratories. This column was used on a Hewlett-Packard 5710A GC with FID detector. GC-MS analyses were conducted on a Hewlett-Packard Model 5982 dual source combined GC-MS system interfaced with a Hewlett-Packard 5933A data system. Mass spectra were recorded by electron impact at 70 eV.

For HPLC analyses, a 30cm x 4mm i.d. column of μ-Bondapak/C_{18} (Waters Assoc.) was used on an ALC/GPC model 202 liquid chromatograph (Waters Assoc.) with Model M-6000 pumps, a Model 660 solvent programmer, and a 254-nm differential UV detector. Spectrographic data were obtained with a Cary 118 UV-VIS spectrophotometer and an Aminco Bowman Spectrofluorimeter. The β-radiation of the ^{14}C-labelled internal standard was counted with a Nuclear Chicago Isocap 300 Liquid Scintillation Counter.

Synthesis of Quinoline-3-^{14}C

Quinoline-^{14}C was synthesized via the Skraup reaction (15) (see Figure 1). Glycerol-2-^{14}C (148 μg, 50 μCi) was mixed with 14 μl of "cold" glycerol, 5 μl conc. H_2SO_4 and 1 mg $FeSO_4$ in a 1.0-ml, tightly capped reacti-vial (Applied Science Laboratories). The mixture was heated in an oil bath of 150°C for 3 hours and was then subjected to solvent partitioning and liquid chromatography on alumina. The overall yield, based on ^{14}C-activity was 31%. The purity of the product was checked by GC-MS and TLC with a Packard radio-scanner. The retention time, R_f-value, and mass spectrum corresponded with those of reference quinoline. About 4 mg of quinoline-3-^{14}C (15.7 μCi/mmole) were synthesized.

Smoke Analyses

Mainstream Smoke. For the collection of mainstream

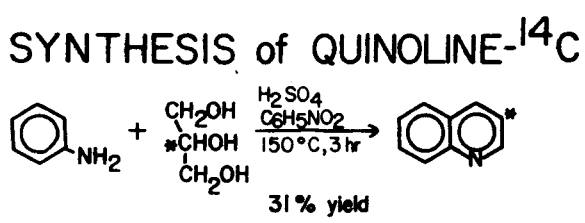

Figure 1. Synthesis of Quinoline-3- ^{14}C.

smoke, 120 cigarettes or little cigars were humidified overnight (60% rh 22°C) and were then smoked on a Borgwaldt automatic 30-port smoking machine (16) under standard conditions (1 puff/minute, 2-second puff duration, 35-ml puff volume, 23-mm butt length) (11). Smoke was led through a sampling system consisting of a trap immersed in dry ice/ethylene glycol monoethyl ether, a gas wash bottle containing 100 ml of *N* HCl and a Cambridge filter (44 mm). Mainstream cigar smoke (30 cigars per sample) was collected similarly under standard cigar smoking conditions (1 puff of 1.5-sec. duration, every 40 seconds, 20-ml volume, 33-mm butt length) (17).

Sidestream Smoke. Cigarettes (20-40 per sample) were smoked individually in a sidestream-apparatus (18) using the same smoking conditions described for mainstream smoke. A single-port Borgwaldt smoking machine was used for withdrawing 10-11 puffs of mainstream smoke during the collection of sidestream. Air was drawn through the apparatus at a rate of 25 ml/sec. Cigar sidestream smoke (4-6 cigars per analysis) was collected in a similar fashion.

Analytical Procedure. The analysis of aza-arenes in tobacco smoke is summarized in Figure 2. The contents of the cold trap and the material on the Cambridge filters were washed

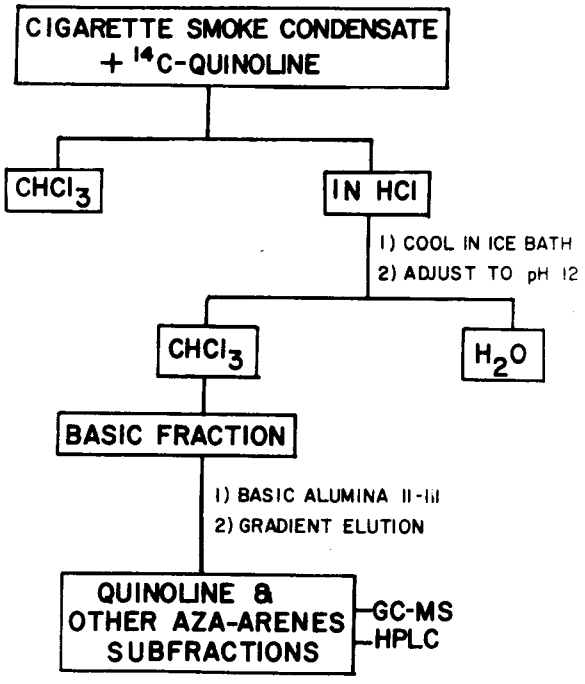

Figure 2. Analytical scheme for the determination of quinolines and aza-arenes in tobacco smoke.

with $CHCl_3$. The washings were then extracted with HCl (3 x 150 ml). Prior to partitioning between $CHCl_3$ and HCl, approximately 20 μg of quinoline-^{14}C (10^5dpm) was added to the $CHCl_3$ layer. The HCl solution from the gas wash bottle was used in the first partioning. The acidic extracts were combined and filtered. The filtrate was cooled in an ice bath and adjusted to pH 12 with a saturated solution of NaOH. This basified solution was then three times extracted with equal volumes of $CHCl_3$. The combined $CHCl_3$ solutions were dried (Na_2SO_4), filtered, and carefully concentrated to 5 ml.

The resulting brown concentrate containing the basic portion of smoke condensate was mixed with 5 g of basic alumina and placed on top of a packed alumina column (1.5 cm x 40 cm, 150 g of basic alumina II-III). The column was eluted in sequence with 500 ml each of n-hexane and n-hexane containing 10,15,20,25,30,50, and 75% $CCHCl_3$. These were followed by 500 ml each of $CHCl_3$ and MeOH. All aza-arenes eluted before nicotine, which appeared with hexane and 30% $CHCl_3$. Eluants of the column were sampled in 15 ml-portions in an automatic fraction collector. The ^{14}C-activity of these fractions was monitored by liquid scintillation counting, and the radioactive fractions were combined to give the "quinoline" fraction. 8-Methylquinoline and benzo(h)quinoline eluted first, followed by 2-methylquinoline, quinoline, acridine, benzo(f)quinoline, phenanthridine, other methylquinolines, azapyrenes, azafluoranthenes, and isoquinolines. Aza-arene subfractions were collected and concentrated to 0.5 ml.

A 10-μl aliquot of each subfraction was examined by GC-MS. The GC column temperature was programmed from 90-300°C at 8°C/min, and ions specific for aza-arenes were monitored during these runs. This technique of mass fragmentography allows sub-ng detection of trace components in complex mixtures. Mass spectral background subtractions were often necessary to obtain spectra for trace aza-arenes that were not always well resolved from interferences. Synthetic mixtures of aza-arene standards of known concentration were injected routinely to generate retention data and mass spectral data.

Peak areas resulting from regular GC-FID runs were used for quantitation, usually after peak identity and purity had been ascertained by mass spectrometry. The integrated specific ion currents of some aza-arenes of low concentrations were also used for quantitation after proper calibration.

HPLC. GC-MS data were supplemented by HPLC analyses on reversed phase columns. The details of these chromatographic separations were described earlier (19). Major peak effluents with retention times of aza-arenes of interest were collected and subsequently examined by UV absorption or fluorescence spectrometry. The identities of isomeric aza-arenes, which cannot be distinguished by MS-analysis, were thus ascertained.

Results and Discussion

Aza-arenes in Cigarette Smoke

The quantitative data for aza-arenes in the mainstream and sidestream smoke of U.S.-blended, 85-mm non-filter cigarettes are presented in Table I. Quinoline was identified by UV absorption, mass spectroscopy, and chromatographic retention times and was quantitated by isotope dilution technique. Its abundance relative to other aza-arenes is apparent. Its concentration of 1.67 μg/cig. (average of 4 determinations using the isotope dilution method with recoveries of 60-70% and a deviation coefficient of 6.8%) is

an order of magnitude higher than that of methylquinolines and isoquinoline, and two orders of magnitude higher than that of any 3- or 4-ring aza-arene in the mainstream smoke. It is, however, lower than that of pyridine at 30 μg/cig. (20) and ammonia at 130 μg/cig. (21), and about the same as that of naphthalene at 2.7 μg/cig. (22). However, it is 70 times higher than that of benzo (a) pyrene (25 ng/cig.) in the same cigarette (23). The role of quinoline in tobacco carcinogenesis is currently under investigation.

The levels of methylquinolines isolated from the smoke are reported as the averages of two determinations. Some methylquinolines are not separated by GC and are therefore reported as a group. Levels of methylquinolines total 0.70 μg/cig.

The relatively lower concentration of isoquinoline (0.12 μg/cig.) is surprising, although it is quite consistent with model pyrolysis studies of N-compounds (24). Four isomers of benzoquinoline were identified and found at levels of 10-40 ng/cig. Azafluoranthenes and azapyrenes were the only 4-ring aza-arenes found in the smoke. Benzacridine and dibenzacridine were not detectable in our analyses and would likely require further enrichment steps. Levels of quinoline in sidestream smoke amount to 18 μg/cig. , and all metylquinolines in sidestream smoke add up to 8.0 μg/cig. Thus, the concentration ratio of sidestream (SS) to mainstream (MS) for quinoline is about 11 for these cigarettes. The SS/MS ratio for nicotine is 2.7; whereas the SS/MS ratios for other volatile bases of pyrolytic origin are substantially higher: NH_3 is 106; pyridine is 20.3; and 3-vinylpyridine is 43 (25).

Quinoline Levels in the Smoke of Other Tobacco Products

Since quinoline is by far the most abundant of the aza-arenes (except for pyridines) and its level may be indicative of higher aza-arenes, we determined its content in the smoke of various other commercial cigarettes and cigars. Results are summarized in Table II. Mainstream smoke concentrations range from 0.6-4.0 μg/cig. A cellulose acetate filter tip removes 30-50% of quinoline with little selective filtration (selectivity ~0.9). The sidestream smoke quinoline levels vary from 18-187 μg/cig. Cigars have a higher SS/MS ratio (30-90, c.f. quinoline ~ 11 in cigarettes), probably reflecting the weight of tobacco consumed per cigar, smoldering characteristics, and porosity of wrappers. These factors are currently under investigation. The fractions from the alumina column that contained the ^{14}C-activity (from ^{14}C-quinoline, the internal standard) were combined to give the "quinoline fraction", which contains quinoline and also methylquinolines, dimethyl-, and ethylquinolines, 3-n-butylpyridine, 3-vinylpyridine, and dimethylpyridines. Typical GC traces of the quinoline fraction in MS and SS cigarette smoke are shown in Figures 3 and 4. These profiles are easily reproducible from run to run. The use of a nitrogen-selective GC detector (Hewlett-Packard) showed little difference from that of a flame ionization detector, indicating that the major compounds in this fraction contain nitrogen. The UV absorbance HPLC detector, however, is quite selective for aza-arenes because of their high molar absorptivity coefficient at 254 nm. A representative HPLC reversed phase chromatogram of cigarette MS smoke is shown in Figure 5.

Precursors for Quinoline in Tobacco Smoke

Unburned tobacco from a popular U.S. cigarette (Table I) contained no detectable level of quinoline (< 0.02 μg/g tobacco). This result indicated that quinoline in MS (1.67 μg cig.) and in SS (18 μg cig.) is primarily formed during the burning of the cigarette. Several pyrolysis studies have suggested nicotine (24) and amino acids, especially tryptophan as precursors for quinoline (26). In model studies we therefore spiked the 62-mm column of an 85-mm cellulose cigarette (free of nicotine, 0.54% nitrogen; analysis by Gailbraith Laboratories, Inc.) with the suspected precursors of quinoline by syringe technique (27). Nicotine was added as tartrate in an aqueous solution and F-1 tobacco protein (28,29) in a 2% NaCl solution.

Table III summarizes the result of the mainstream smoke analysis of the spiked cigarettes. The 62-mm column of the nicotine-spiked cellulose cigarette contained about 4.0%

Table I. Quinolines and Aza-arenes in Mainstream (MS) and Sidestream (SS) of Cigarette Smoke. (85-mm U.S. Blended Nonfilter Cigarette)

Aza-arenes	Names	Mol Wt	Isolated Quantities (μg/cig) MS	SS	
	quinoline	129	1.67[a]	18	
	2-methylquinoline	143	0.23[b]	1.6	
	3-,6-,7-methylquinolines	143	0.11[b]	0.70	2.7	8.00
	4-,5-methylquinolines	143	0.24[b]	2.2	
	8-methylquinoline	143	0.12[b]	1.4	
	C_2-quinolines	157	0.48	2.0	
	C_3-quinolines	171	0.09	1.0	
	isoquinoline	129	0.12	ND	
	methylisoquinolines	143	0.21	ND	
	C_2-isoquinolines	157	0.07	ND	
	Benzo [h] quinoline	179	0.01	0.1	
	acridine	179	0.04	0.1	
	phenanthridine	179	0.01	0.2	
	Benzo (f) quinoline	179	0.01		
	4-Azafluorenes	167	0.07[c]	ND	
	1-Azafluoranthenes	203	0.005[c]	ND	
	4-aza-pyrenes	203	0.005[c]	ND	

a. Average of four determination by isotope dilution method. The values are 1.66, 1.83, 1.63, 1.56 μg/cig. Analytical Recovery is between 60-70%.
b. Average of 2 determinations is isolated amounts.
c. Values include other isomers.
ND Not determined.

Table II. Quinoline Levels in the Smoke of Commercial Cigarettes and Cigars

NO.	PRODUCT	WEIGHT G	MAINSTREAM G/CIG.	SIDESTREAM G/CIG.
1	CIGAR I	7.2	2.0	187
	CIGAR II	5.8	4.1	124
2	LITTLE CIGAR,F	1.2	0.66	25
3	CIGARETTE I (85 MM, NF)	1.0	1.67	18
4	CIGARETTE II (100 MM, F, 55 MM SMOKED)	1.1		
	WITH FILTER		0.62	---
	WITH FILTER REMOVED		1.2	---
5	FRENCH CIGARETTE (70 MM, 45 MM SMOKED	1.0		
	WITH FILTER		1.2	---
	WITH FILTER REMOVED		1.8	---

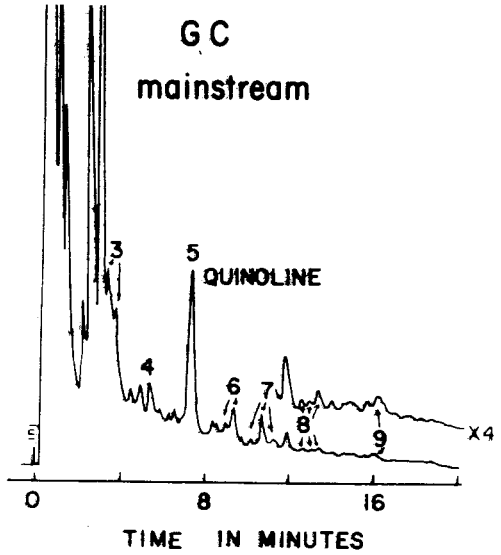

Figure 3. Representative gas chromatographic trace of the quinoline fraction from mainstream cigarette smoke. (Column = 10' x 1/8" o.d., stainless steel column filled with 3% Dexsil 300 on 80/100 mesh Chromosorb W (AW/HP). Flow rate = 50 ml He/min. Temperature = 90°C to 300°C at 8°C/min. Compounds identified = 1. C_2-pyridine; 2. 3-vinylpyridine; 3. C_3-pyridines; 4. 3-n-butylpyridine; 5. quinoline; 6. methylquinolines; 7. C_2-quinolines) 8. C_3-quinolines; 9. acridine).

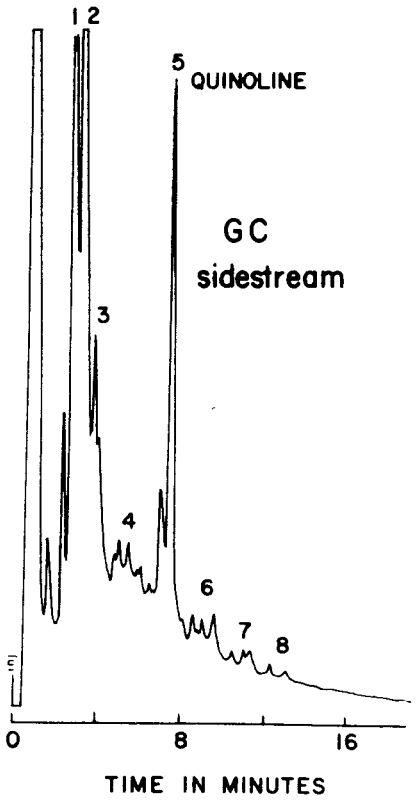

Figure 4. Representative gas chromatographic trace of the quinoline fraction from sidestream cigarette smoke. Chromatographic conditions and sequence of compounds as in Figure 3.

nicotine, compared to 2.0% nicotine in the popular 85-mm U.S. cigarette (Table I). Since the MS of the U.S. cigarette contains 1.67 μg of quinoline, one would expect about 0.35 μg of quinoline from the spiked cellulose cigarette, if the nicotine were to account for about 10% of the smoke quinoline. However, we found only 0.063 μg quinoline, suggesting that nicotine contributes merely a few percent to the quinoline in the smoke. This result is in good agreement with the finding in the smoke of a cigarette spiked with ^{14}C-labelled nicotine (30).

When the 62-mm column of the cellulose cigarette was spiked with 8.5 mg of F-1 protein fraction, the column to be smoked contained about 1.4% protein, compared to about 15-20% in a regular tobacco cigarette. The finding of 0.07 μg of quinoline in the smoke of the cellulose cigarettes suggests that protein serves as a major precursor for quinoline. Therefore, it appears that quinolines and aza-arenes in the smoke could be reduced selectively by reducing the protein content of the tobacco used for cigarette manufacture.

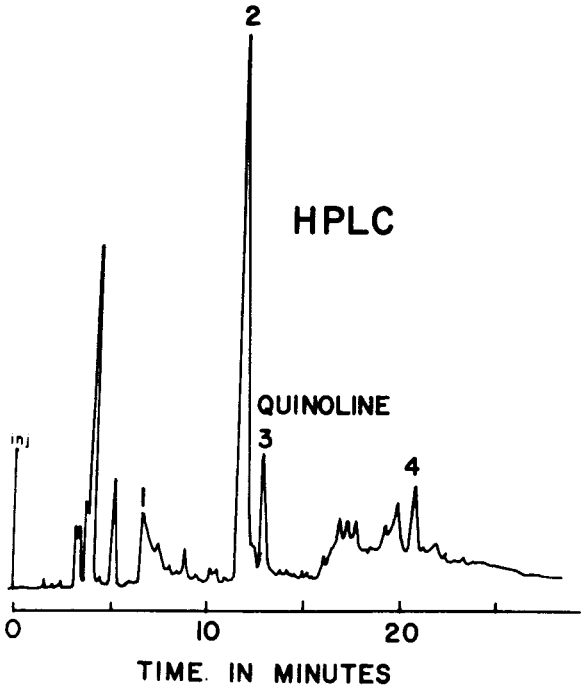

Figure 5. Representative HPLC trace of the quinoline fraction from the mainstream tobacco smoke. (Column = μ-Bondapak/C_{18}. Flow rate = 2.0 ml/min. Solvent program = 20% to 80% CH_3CN in H_2O at 2%/min. Compounds identified = 1. C_2-pyridine; 2. 3-vinylpyridine; 3. quinoline; 4. acridine.

Table III. Precursor Studies on the Formation of Quinoline in the Mainstream Smoke of Cellulose Cigarettes

PRECURSOR ADDED	MG ADDED PER CIG.	NG OF QUINOLINE ISOLATED PER CIG.
F-1-PROTEIN	8.5	70**
NICOTINE	26.5	63**
NONE (CELLULOSE ONLY) ***	--	40

*60% RECOVERY ASSUMED
**WITH CONTRIBUTION FROM CELLULOSE DEDUCTED
***CONTAINS 0.54% NITROGEN

Reducing the protein content of tobacco may be achieved through selective breeding or through special tobacco processing as proposed by Tso (28).

Tumorigenicity and Environmental Significance of Quinolines

Quinoline was found to be a rat liver carcinogen, a tumor co-initiator on mouse skin (9), and a mutagen in *Salmonella typhimurium*. We are currently testing quinoline and a number of methylquinolines in a full-term mouse skin assay for complete carcinogenicity and for cocarcinogenicity. The bioassays are important since quinolines are the most abundant basic aza-arenes in tobacco smoke, in addition to being important industrial chemicals used in the formulation of medicinals and dyes (31). Furthermore, quinoline is a common laboratory reagent, a major constituent of coal tar (32), shale oil (33), and coal liquefaction products (34). Due to its volatility and carcinogenicity, quinoline is of special concern in occupational health. Measurement of human exposure under occupational settings in conjunction with epidemiological studies should also be initiated.

This study of aza-arenes in tobacco smoke is part of our overall program to reduce tumor initiators, cocarcinogens, and promoters in tobacco smoke in order to establish criteria for the development of a less harmful cigarette (35,36).

Acknowledgment

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Note: Recently, we have had far better resolution of the smoke quinolines using a 10m x 0.35mm OV 101 glass capillary column.

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