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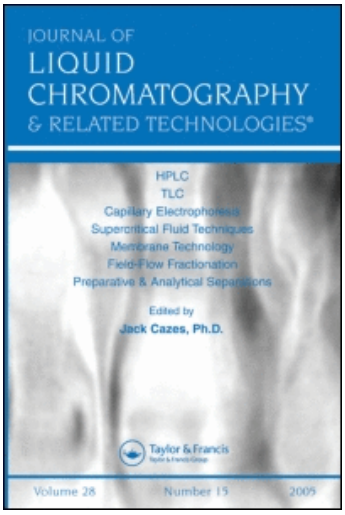

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Analysis of Benz[C]acridines in Cigarette Smoke by High-Performance Liquid Chromatography

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ANALYSIS OF BENZ[C]ACRIDINES IN CIGARETTE SMOKE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method has been developed for the analysis of mutagenic and carcinogenic benz[c]acridines (BAcs) in cigarette smoke condensates on filter tips. The BAcs were extracted from cigarette filter tips with chlorobenzene in a Soxhlet apparatus, purified by liquid-liquid partition and column chromatography, and then analysed by high-performance liquid chromatography (HPLC) using fluorescence detection. Recoveries of BAcs spiked at

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0.5 ng/tip were 81.2-90.4%, and the limit of detection was found to be 5 pg. Typically, smoked cigarette filter tips were found to contain the known carcinogens benz[c]acridine and 9-methylbenz[c]acridine. The identification of peaks in each sample chromatogram was confirmed by comparing the retention times and the fluorescence spectra with its standard respectively.

INTRODUCTION

Because of their mutagenic and carcinogenic characteristics the identification and quantification of aza-arenes have recently become important research subjects (1).

Aza-arenes are formed as trace pollutants during incomplete combustion or pyrolysis of nitrogen-containing substances that are found in cigarette smoke (2-4), urban suspended particulate matter (5-7), automobile exhaust (8,9), and industrial stack effluents (10). They are also present in coal tar (11,12), crude oil (13,14), and high boiling petroleum distillates (15,16).

In studies of mutagenicity and carcinogenicity of several aza-arenes, BAcs are known to show strong carcinogenicity and constitute a health hazard for man (17). Several techniques have been described for the analysis of BAcs in environmental samples. However, few reports on the analysis of BAcs in cigarette smoke have been amassed in comparison with other environmental samples (2-4). The determination of BAcs in cigarette smoke samples has proven to be a difficult task because of both their very low concentrations and interference from other compounds.

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The analytical techniques utilized require not only extreme sensitivity but also high selectivity.

BAcs in mixed samples have been analysed by means of thin-layer chromatography (TLC) (18,19), capillary-column gas chromatography (GC)(3,4,9,19-21), and high-performance liquid chromatography (HPLC) (19,22,23). However, due to the low levels of BAcs in the samples being investigated, positive identification and quantification was found to be difficult when using GC. HPLC, using fluorescence detection, was then chosen for the determination because its application to BAcs had been widely accepted, as being a more rapid and sensitive technique than the other possible methodologies.

In the present paper, a method is described for the isolation and determination of BAcs in cigarette smoke condensate, which includes Soxhlet extraction, pre-separation by liquid-liquid partition and column chromatography, and then separation and determination by reversed-phase HPLC with fluorescence detection.

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MATERIALS AND METHODS

A. Reagents and Materials

The following twelve BAcs were synthesized according to the method previously described (24): benz[c]acridine, 7-methylbenz[c]acridine, 8-methylbenz[c]acridine, 9-methylbenz[c]acridine, 10-methylbenz[c]acridine, 11-methylbenz[c]acridine, 5,7-dimethylbenz[c]acridine, 7,9-dimethylbenz[c]acridine, 7,10-dimethylbenz[c]acridine, 7,11-

dimethylbenz[c]acridine, 7,9,10-trimethylbenz[c]acridine, 7,9,11-trimethylbenz[c]acridine. Acetonitrile (HPLC grade) was purchased from Nakarai Tesque, Inc (Kyoto, Japan). The SP Sepadex C25 was obtained from Pharmacia LKB (Uppsula, Sweden) and activated with 200 mL 0.1N hydrochloric acid, washed with 250 mL methanol-water (7:3), and then washed with 200 mL methanol before use. Silicagel (kiselgel 60, 230-400 mesh) was acquired from Merck Company (Darmstadt, Germany). All other solvents were analytical or reagent grade. Stock solutions of BAcs were prepared at 1 μg/mL in methanol.

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B. Apparatus and chromatographic conditions

The liquid chromatograph consisted of a JASCO Model BIP-1 pump (Japan Spectroscopic, Tokyo,Japan), a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector equipped with a 20 μL loop, a JASCO Model 8600-CO column oven, A JASCO Model FR-550A spectrofluorometer, and a Shimadzu chromatopac CR-3A digital integrator (Shimadzu Corp., Kyoto, Japan). The detector was fitted with a Xenon source lamp and operated at an excitation wavelength of 290 nm and emission wavelength of 400 nm.

The column was Cosmosil 5C18-AR (5μm particle size, 4.6 x 250 mm, Nacalai Tesque Inc.). The mobile phase was acetonitrile-water (75:25). Separations were carried out at a flow rate of 1.0mL/min and a column temperature of 40°C.

C. Sample Preparation

Smoked filters from different brands of filter-tipped cigarettes were carefully separated from the tobacco. 100 filters

were collectively Soxhlet-extracted for 8 hours using 250 mL of chlorobenzene. The extracted chlorobenzene layer was back extracted 2 times with 20 mL of 50% sulfuric acid. The acidic layers were combined, cooled in an ice bath, and neutralized to pH 12 with a saturated sodium hydroxide solution. This solution was then back-extracted 3 times with 50mL portions of chloroform. The combined chloroform portions were washed with 25 mL of 5% sulfuric acid and 100 ml of water. The chloroform layer was then evaporated to near dryness under reduced pressure with a rotary evaporator and transfered in methanol to the cation-exchange column (5g of SP-Sephadex C25 in a 2 x 35 cm glass column). The flask was rinsed with 5 mL of methanol which was added to the column. The column was eluted with 200 mL of methanol which was discarded. The BAcs were then recovered by elution of 100 mL of a buffer solution (mixture of 30 mL of 5N ammonium chloride, 10 mL of 5N aqueous ammonia, 10 mL of water and 50 mL of methanol). This collected fraction was diluted with 100 mL of water and extraction twice with 100 mL portions of hexane. The combined hexane layers were washed with 100 mL of water and then 50% aqueous methanol and finally evaporated to dryness. Following evaporation, the residue was transferred in hexane to a column of silica gel (20g of kiselgel 60 in a 2 x 50 cm glass column). The column was eluted with 200 mL of hexane, which was discarded. The BAcs were then recovered by elution with 100 mL of benzene. The column eluate was evaporated to near dryness for HPLC analysis. One µL of the sample solution was injected into the HPLC system. The resulting peaks were identified by retention times and fluorescence spectral analysis.

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RESULTS AND DISCUSSION

Extraction and preseparation

Soxhlet extraction by organic solvents was used to isolated BAcs from the filter tips of cigarettes. 1 ng/tip of each of six BAcs (benz[c]acridine, 7-methylbenz[c]acridine, 9-methylbenz[c]acridine, 7,11-dimethybenz[c]acridne, 7,10-dimethylbenz[c]acridine, and 7,9,10-trimethylbenz[c]acridine) was added to nonsmoked filter tips. The filter tips were Soxlet extracted for 8 hours, and the extracts evaporated and analyzed directly without further treatment. Of the seven solvents initially investigated for extraction (acetone, methanol, ethyl acetate, hexane, chloroform, benzene and chlorobenzene), the highest overall yield of BAcs was obtained with chlorobenzene, as shown in Table 1. Because arenes occur much more abundantly in nature than aza-arenes, the extracts of cigarette smoke condensates may contain many impurities related to arenes. In order to isolate the weakly-polar BAc fraction of the extract, the liquid-liquid partition and cation-exchange column chromatography steps were added for pre-separation as described in the previous paper (19). Furthermore, silicagel column chromatography was used to separate the more abundant nicotine and other alkaloids from the less abundant BAcs (3). After the extract was deposited on the top of the silica gel column, the column was briefly eluted successively with hexane and then benzene. The benzene fraction was concentrated, dried, diluted with methanol, and analyzed by HPLC.

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TABLE 1

Recovery (%) of Soxhlet extraction with different solvents.

Benz[c]acridines	Solvent						
	CH_3OH	CH_3COCH_3	C_6H_{14}	$CHCl_3$	C_6H_6	C_6H_5Cl	$CH_3COOC_2H_5$
benz[c]acridine	16.9	16.9	36.3	20.5	74.3	93.3	30.0
7-methylbenz[c]acridine	14.2	16.0	42.3	23.3	71.4	91.4	24.6
9-methylbenz[c]acridine	18.0	14.3	42.5	18.0	71.8	93.5	37.1
7,10-dimethylbenz[c]acridine	39.6	27.1	68.7	45.3	74.0	90.2	54.9
7,11-dimethylbenz[c]acridine	24.5	24.0	51.0	28.0	71.4	94.6	49.5
7,9,10-trimethylbenz[c]acridine	22.3	21.1	46.3	31.2	67.1	94.7	41.8

TABLE 2
Retention times and fluorescence spectral data of BAcs.

Benz[c]acridines	Retention time (min)	Fluorescence spectra[1)]	
		Ex.λ max (nm)	Em.λ max (nm)
benz[c]acridine	11.0	286	399
7-methylbenz[c]acridine	13.2	289	399
8-methylbenz[c]acridine	13.8	279	401
9-methylbenz[c]acridine	14.7	278	396
10-methylbenz[c]acridine	14.2	286	402
11-methylbenz[c]acridine	20.1	279	394
5,7-dimethylbenz[c]acridine	17.2	291	396
7,9-dimethylbenz[c]acridine	17.2	291	396
7,10-dimethylbenz[c]acridine	17.2	289	402
7,11-dimethylbenz[c]acridine	24.6	281	395
7,9,10-trimethylbenz[c]acridine	20.8	291	400
7,9,11-trimethylbenz[c]acridine	33.5	294	393

1) The fluorescence spectra of BAcs were measured in 75% (v/v) acetonitrile in water.

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HPLC of BAcs

A reversed-phase ODS column was used for the separation of the BAcs. The separation conditions were optimized by adjusting the strength of the mobile phase. The best isocratic elution was found to be 75 % acetonitrile in water. However, 5,7-dimethylbenz[c]acridine, 7,9-dimethylbenz[c]acridine, 7,10-dimethylbenz[c]acridine essentially coeluted using the reversed-phase ODS column as described in a previous paper (25). Table 2 shows the retention times and fluorescence spectral data of BAcs. Figure 1 shows the separation of standard BAcs under the HPLC conditions described above with an excitation wavelength of

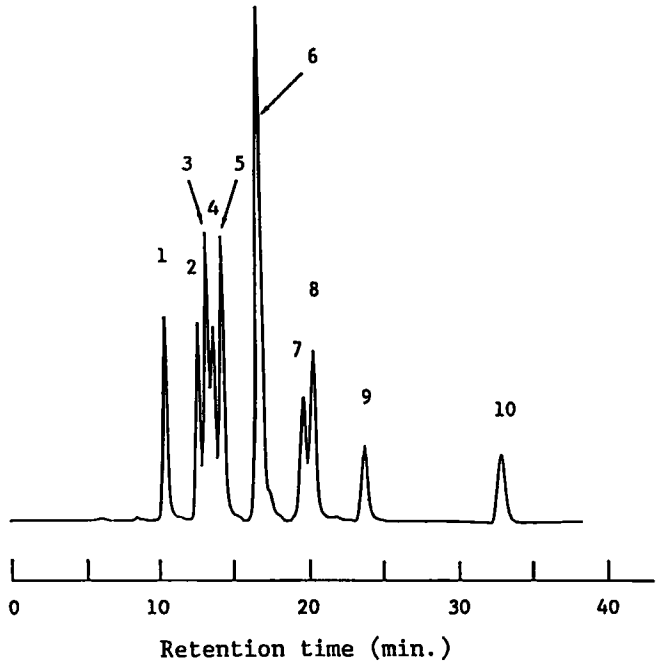

Figure 1. Chromatogram of BAcs standards. Peaks: 1=benz[c]acridine, 2=7-methylbenz[c]acridine, 3=8-methylbenz[c]acridine, 4=10methylbenz[c]acridine, 5=9-methylbenz[c]acridine, 6=5,7-dimethylbenz[c]acridine, 7,9-dimethylbenz[c]acridine and 7,10-dimethylbenz[c]acridine, 7=11-methylbenz[c]acridine, 8=7,9,10-trimethylbenz[c]acridine, 9=7,11-dimethylbenz[c]acridine, 10=7,9,11-trimethylbenz[c]acridine.

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TABLE 3

Recovery and coefficient of variation (c.v.) of BAcs from spiked cigarette filter tips.

Benz[c]acridines	Spiked concentration ng/tip	Number of analysis	Recovery (%)	c.v. (%)
benz[c]acridine	0.5	5	85.2	2.86
7-methylbenz[c]acridine	0.5	5	81.7	2.57
9-methylbenz[c]acridine	0.5	5	86.5	2.54
7,10-dimethylbenz[c]acridine	0.5	5	81.2	2.61
7,11-dimethylbenz[c]acridine	0.5	5	90.4	2.01
7,9,10-trimethylbenz[c]acridine	0.5	5	81.6	3.55

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290 nm and emission wavelength of 400 nm (These wavelengths were chosen as a best compromise to detect all components of interest).

Linearity, reproducibility, and recovery

A linearity study (ratio of concentration to peak height) of BAcs showed linearity of response over the concentration range of 10 - 100 pg with a coefficient of correlation (r) of >0.99 for each BAc. The limit of detection for an individual BAc was approximately 5 pg at a signal to noise ratio of 2. The reproducibility and actual recovery of the proposed method was determined by the analysis of replicate nonsmoked filter tips spiked with known amounts of standard BAcs. The results are presented in Table 3. The average recoveries of BAcs were 81.2% or greater (n=5) with a coefficient of variation (c.v %) at ±3.55%

or better for BAc levels of 0.5 ng/tip. These results indicate the proposed procedure to be quite efficient and capable of measuring BAcs in cigarette filter tips.

Resulting BAcs in cigarette filter tips

The proposed method was then applied to the analysis of BAcs in smoked cigarette filter tips. Severson et al. have reported that cigarette filters not only reduced the amount of cigarette smoke condensates but also selectively removed arenes. The data obtained has shown that filters can effectively decrease the arenes in the smoke to 52-70% contents (26). The ability to analyze BAcs from cigarette filter tips would be valuable in determining environmental pollution sources and health hazards to man. A typical chromatogram of the BAc fraction isolated from cigarette smoke condensate onto the filter is shown in Figure 2. The peaks were identified by comparing their retention times and spectral pattern with standard BAcs. The chromatogram shows that the combination of a pre-separation step for isolating the BAcs and then HPLC with fluorescence detection for their measurement was very satisfactory, especially when the small amount of BAcs present was considered. Numbered peaks in Figure 2 correspond to the standard BAcs, and individual components were characterized with the fluorescence spectra by means of stop-flow and scanning techniques. As shown in Figure 3, the two spectra of the peaks obtained are very similar to the standard BAcs. The concentrations of the isolated BAcs (benz[c]acridine and 9-dimethylbenz[c]acridine) in cigarete filter tips were at the levels of 0.37ng/tip and 0.11ng/tip, respectively. Since no other

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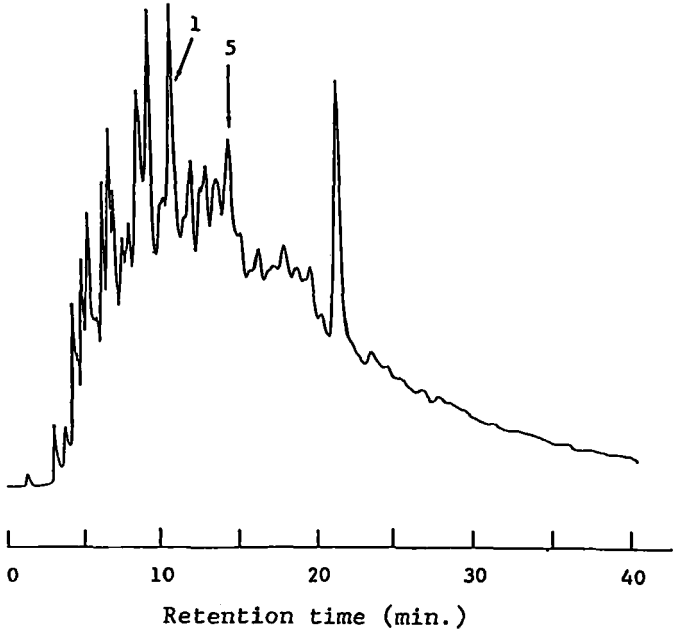

Figure 2. Chromatogram of BAcs fraction of the cigarette smoke condensate. Peak numbers refer to the compounds identified in Figure 1.

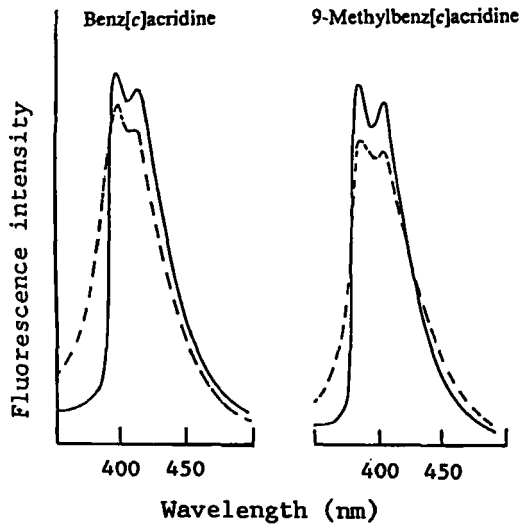

Figure 3. Fluorescence emission spectra of two species of standards (——) and identified BAc (-----) in cigarette smoke condensate. For each excitation, see Table 2.

BAcs were found at detection limits of 0.05ng/tip, their concentrations in cigarette filter tips must be very low in cigarette smoke as to require extraction of much larger quantities of cigarette filter tip.

CONCLUSIONS

The proposed procedure is useful for the identification and quantification of BAcs in cigarette smoke condensates. BAcs are efficiently extracted from cigarette filter tips by Soxhlet extraction with chlorobenzene. The use of liquid-liquid partion and column chromatography effectively was applied in the separation of BAcs from interfering aliphatic compounds and nicotine alkaloids. In addition, in combination with fluorescence detection, HPLC has been proven to be a poweful tool for the separation and determination of BAcs. Evidence of benz[c]acridine and 9-methylbenz[c]acridine in cigarette smoke condensates is presented.

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