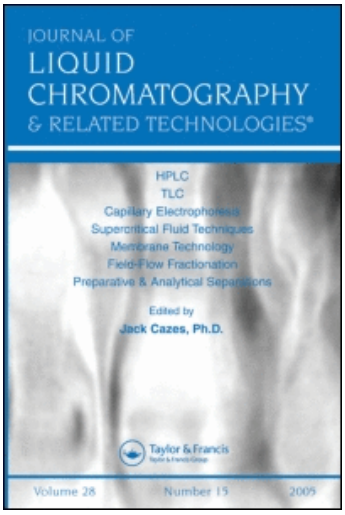

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DETERMINATION OF BENZO(a)PYRENE IN TOTAL PARTICULATE MATTER OF VIRGINIA AND BLACK TOBACCO SMOKE BY HPLC WITH FLUORIMETRIC DETECTION

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ABSTRACT

A method for the determination of the benzo(a)pyrene by RP-HPLC with fluorimetric detection in total particulate matter of Virginia and blak tobacco smoke is developed. The total particulate matter was collected on glass fibre filters, which were ultrasonically extracted with cyclohexane. A fraction of the resulting extract was cleaned up on Sep-Pak Vac Si cartridges and the PAHs were eluted with mehylene chloride. The eluate was evaporated to drynees and the residue was dissolved in methanol. The mean recoveries in the extraction and clean-up steps were 100.1% and 49.2%, respectively, with relative standard deviations of 3.8% and 2.4%, respectively, in the concentration range 20-140 ng. The quantification is achieved by means of external standard method.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have been the subject of intensive analytical investigation in studies relating to tobacco smoke (1, 2), air (3-5), water (6, 7) and food (8, 9) because of the carcinogenic activity of some members of this family. Benzo(a)pyrene (B(a)p), a carcinogenic polycyclic aromatic hydrocarbon, has received the most attention. In recent years, a number of methods for the quantitative analysis of B(a)p have been published, which employed improved techniques such as GC and HPLC and laborious procedures (10, 11). The determination of B(a)p in cigarette smoke is a major analytical problem, because the particulate matrix of cigarette smoke contains thousands of PAHs or their derivatives (12) and the absolute deliveries of B(a)p are not large, usually 6-30 ng B(a)p/cigarette (cig.)(13).

The isolation of the B(a)p fraction from the extract of total particulate matter of cigarette smoke must involve a solid phase extraction using a cartridge (12, 14) or HPLC system with a semipreparative column (12, 13). The first method is much cheaper, simpler and faster, but the second once is more efficient in terms of recovery and precision. Reversed phase high performance liquid chromatography (RP-HPLC) coupled with a fluorescence detector offers

good selectivity and sensitivity for the determination of PAHs, due to the specific columns used (5, 15).

In this paper we report the development and optimization of a fast, cheap, simple and precise method for the determination of B(a)p in total particulate matter of Virginia and black tobacco smoke by HPLC with fluorimetric detection.

EXPERIMENTAL

Apparatus and material

The chromatographic system consisted of the following components: a high pressure gradient Milton Roy CM 4000 pump, a Rheodyne 7125 injection valve with a 20 μl loop, a Perkin-Elmer LS 30 luminescence spectrometer and a Milton Roy CI 4100 integrator. A P-Selecta Precisterm bath was used to maintain the column temperature at 22°C. The column was an Ultraspher 5 μm particle size (125 mmx 4 mm I.D.) from Merck. A P-Selecta ultrasonic bath was used to extract PAHs from the filters. For the clean-up of the extract a Sep-Pak Vac Si, 500 mg (6 ml) cartridge from Millipore were used. A Visiprep vacuum manifold system from

Supelco was also used. Solvents used to prepare the mobile phase and organic extracts from the tobacco smoke particulate samples were filtered through 0.45 μm pore size nylon Lida membrane filters.

Reagents

Stock solutions of the PAHs with concentrations in the range $(10-1.0)x10^{-4}M$ were prepared by dissolving the solids (from Sigma) in methanol; more dilute solutions were prepared by diluting with this solvent. HPLC purity acetonitrile, methanol and methylene chloride from Carlo Erba were used. Water was purified with a Milli-Q system from Millipore. Other chemicals used were of analytical reagent grade.

Cigarettes

Both commercial Virginia and black tobacco cigarette brands used in this study were purchased in a Madrid tobacco nist's. The cigarettes are 85 mm in length, 8 mm in diameter, and filtered.

Procedures

Tobacco smoke particulate sampling and sample preparation

The Virginia and black tobacco smoke particulate was collected on glass fibre filters of 4 cm diameter using a smoking machine. The filters, containing the smoke particulate matter of five cigarettes, were treated with 10 ml of cyclohexane in the ultrasonic bath for thirty minutes. A 2 ml extract aliquot was injected into a cartridge, which had previously been conditioned with 15 ml of cyclohexane. The cartridge containing the extract aliquot was dried under a compressed air stream. The B(a)p was eluted with 2 ml of methylene chloride. This elution fraction was evaporated to small volume, transferred to 1.0 ml volumetric flask and then evaporated to dryness by means of the vacuum manifold system. The B(a)p residue was dissolved in 1 ml of methanol and determined by the chromatographic procedure by injecting 20 μl.

Chromatographic analysis

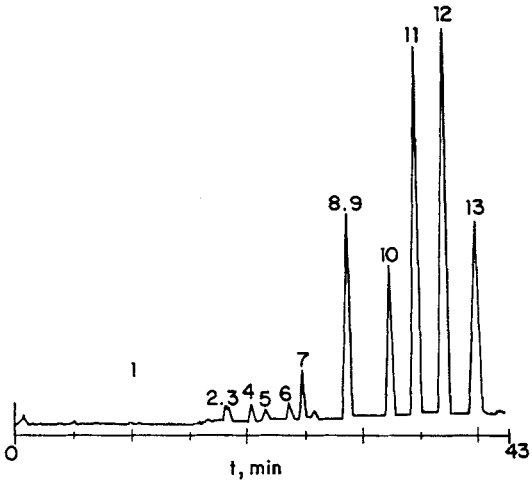
Once the column had been conditioned with the mobile phase, calibration graphs at five concentration levels were prepared from solutions containing B(a)p in the range

0.001-0.04 ng/μl. The mobile phase gradient was prepared by mixing water (A) and acetonitrile (B) and was degassed with helium. The gradient profile for the desorption of the analytes from the analytical column was: A-B (55-45) (0 min), which was held constant for 5 min, and subsequently changed linearly to 85% B in 25 min. The separations were carried out at 22°C, at a flow-rate of 1.0 ml/min. Detection was carried out fluorimetrically at the optimal excitation and emission wavelengths (295 and 405 nm) of B(a)p. The areas of the peaks were used to quantify B(a)p.

RESULTS AND DISCUSSION

Chromatographic and analytical characteristics

The chromatographic parameters were selected after a previous systematic study. Figure 1 shows the separation of B(a)p from the 12 PAHs using the optimized procedure which is specified in the experimental section. The B(a)p concentrations tested were in the range 0.001-0.04 ng/μl. Linearity was found with a regression coefficient of 0.999. The relative standard deviation (RSD, %) at concentration level of 0.02 ng/μl in the middle of the linear range studied was 5.3% (n=4) and the detection limit (DL = 3 S/N) was 24 ng/l.



PAH	c, ng/µl
1-Naphthalene	0.88
2-Acenaphthene	0.44
3-Fluorene	0.20
4-Phenanthrene	0.13
5-Anthracene	0.11
6-Fluoranthene	0.05
7-Pyrene	0.06
8-Benzo(e)anthracene	0.08
9-Chrysene	0.16
10-Benzo(e)pyrene	0.13
11-Benzo(a)pyrene	0.04
12-Dibenzo(ah)anthracene	0.10
13-Benzo(ghi)perylene	0.08

FIGURE 1. HPLC chromatogram of a standard mixture of 13 PAHs. Conditions: Ultraspher (125 x 4 mm, 5 µm) column; temperature, 22°C; acetonitrile/water programming from 55:45 (held for 5 min), to 85:15 at 30 min; flow rate, 1 ml/min; injection volume, 20 µl; fluorimetric detection, 295 nm excitation and 405 nm emission wavelengths.

Determination of B(a)p in tobacco smoke particulate

1. Optimization of extracting agent and recovery study

Three solvents (acetonitrile, methylene chloride and cyclohexane) were evaluated as media for extracting B(a)p from filters containing tobacco smoke particulate. Cyclohexane was chosen as the extractant for B(a)p from mainstream smoke condensate because acetonitrile and methylene chloride extract more interfering material.

The B(a)p recovery study was carried-out on twelve filters containing of B(a)p in the range 20-140 ng, applying the experimental procedure without the clean-up step. The results of the analysis are shown in Table I. The mean B(a)p recovery was 100.1% and the relative standard deviation was 3.8%. The B(a)p was not detected in the filter used as a blank.

2. Clean-up step recovery study

Acetonitrile, methylene chloride and cyclohexane were also tested for eluting the B(a)p. The methylene chloride

TABLE I.

Recovery of B(a)p standard in the extraction step.

B(a)p Amount added, ng	%Mean Recovery	n	%RSD
-	-	1	-
20	95.8	3	5.7
60	104.2	3	2.4
100	102.0	3	3.0
140	98.2	3	4.2
Average	100.1		3.8

n measure number

gave lowest interferences. Different elutant volumes were also tested , but volumes above 2 ml give higher chromatographic interferences.

The B(a)p recovery study was carried out on twelve filters containing B(a)p in the range 20-140 ng by applying the experimental procedure. Table II shows the results obtained. The mean B(a)p recovery was 49.2% and the relative standard deviation was 2.4%. B(a)p was not detected in the extract of the blank.

TABLE II.

Recovery of B(a)p standard in the clean-up step.

B(a)p Amount added, ng	%Mean Recovery	n	%RSD
-	-	1	-
20	48.4	3	4.2
60	50.5	3	3.7
100	48.0	3	1.0
140	49.8	3	5.1
Average	49.2		2.4

n measure number

3. B(a)p identification and quantification

Excitation and emission spectra and retention time were used to identify of B(a)p. Excitation and emission spectra corresponding to the chromatographic peaks of sample and standard solution were registered; the flow rate was stopped when the fraction of B(a)p was in detector's flow cell. The spectra are shown in Figures 2 and 3. A Perkin-Elmer LS 30 fluorimetric detector was used. This instrument can be used either as a spectrofluorimeter or as an LC detector. The comparison of spectra confirms the presence of B(a)p in the samples.

The proposed method was applied to determine the delivery of B(a)p in particulate matter of Virginia and black tobacco smoke.

Figures 4 and 5 compares the chromatograms of Virginia (Figure 4) and black (Figure 5) tobacco samples. Both profiles are remarkably similar in their general features. Quantitatively, chromatogram 5 (black tobacco) is more intense than chromatogram 4 (Virginia tobacco).

Table III compares the B(a)p deliveries of the Virginia and black tobacco when smoked. Of particular interest is the delivery of B(a)p from the black tobacco,

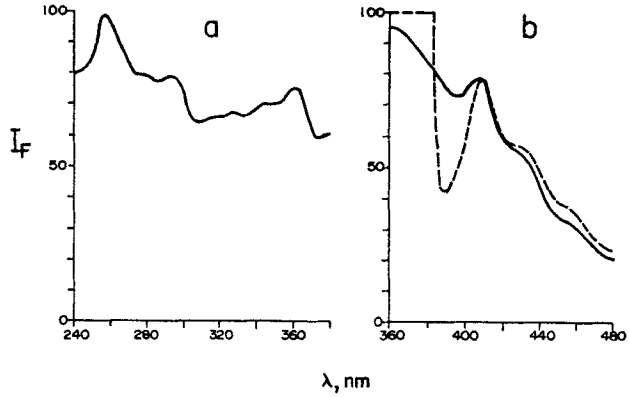


FIGURE 2. Excitation and emission spectra of B(a)p chromatographic peak of a sample; **a** = excitation (λ_{em} = 405 nm), **b** = emission (λ_{ex} = ——— 295, ---- 363 nm).

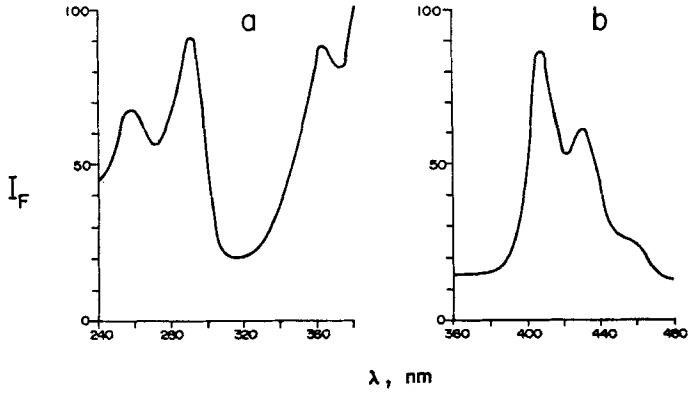


FIGURE 3. Excitation and emission espectra of B(a)p chromatographic peak of a standard solution; **a** = excitation (λ_{em} = 405 nm), **b** = emission (λ_{ex} = 295 nm).

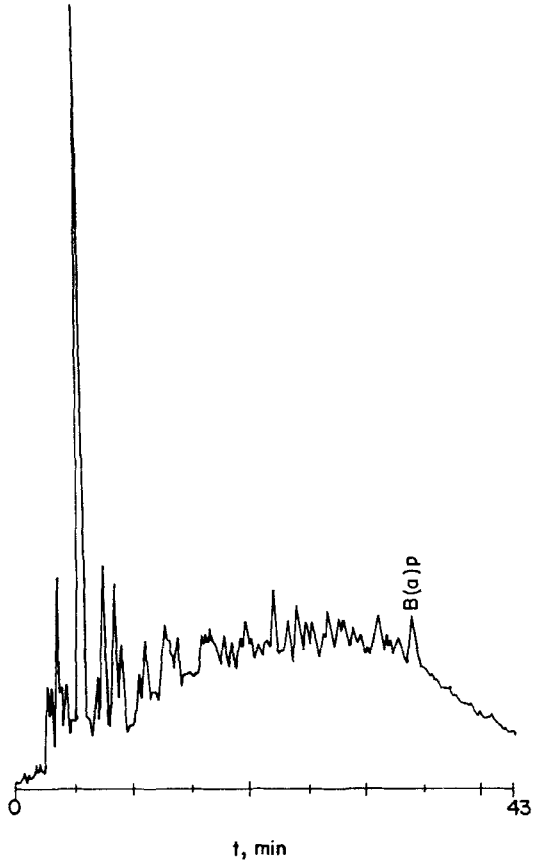


FIGURE 4. HPLC chromatogram of a Virginia tobacco sample. Conditions: see figure 1.

which is one and a half times that of the Virginia tobacco. The values reported exhibit a precision (expressed as relative standard deviation) of approximately 5.2-6.3%. The mean value obtained for the B(a)p delivery from a Virginia tobacco, 68.6 ng/5 cig. (5.2%, relative standard deviation, n=6), is in the range of that described for the commercial

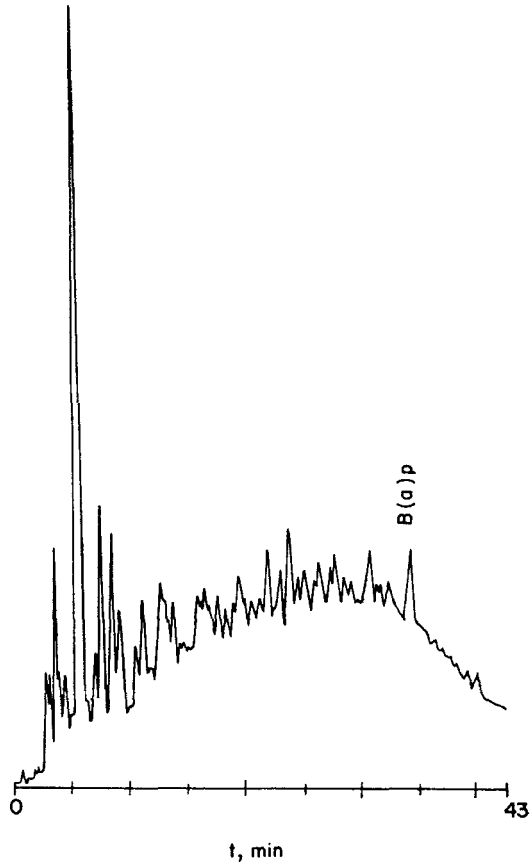


FIGURE 5. HPLC chromatogram of a black tobacco sample. Conditions: see figure 1.

tobacco cigarettes and from a black tobacco was 105.6 ng/5 cig. (6.3%, relative standard deviation, n=4). No references were found in the literature for the amount of B(a)p in black tobacco.

TABLE III.

Determination of B(a)p in Virginia and black tobacco.

Sample	Amount of B(a)p (ng/5 cigarettes)	
	Virginia tobacco	Black tobacco
1	73.9	100.7
2	68.6	101.3
3	68.6	105.4
4	69.4	115.1
5	62.7	-
6	68.4	-
$\bar{x}$	68.6	105.6
%RSD	5.2	6.3

CONCLUSIONS

The proposed method involves an extraction and a clean-up step for the determination of B(a)p in Virginia and black tobaco smoke. The optimized extraction and clean-up processes proved to be adequate for this type of samples the recoveries being 100.1% and 49.2%, respectively. The recovery in the clean-up step can be increased, but many interferences arise.

The B(a)p deliveries from the smokes of commercial cigarettes can be determined precisely, quickly and cheaply by applying our analytical procedure.

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