The Determination of Benzo[a]Pyrene and Benz[a]Anthracene in Mainstream and Sidestream Smoke of the Kentucky Reference Cigarette 1R4F and a Cigarette Which Heats but Does Not Burn Tobacco: A Comparison*

by

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SUMMARY

A quantitative method for the determination of benzo[a]anthracene (B[a]A) and benzo[a]pyrene (B[a]P) in mainstream and sidestream cigarette smoke by high performance liquid chromatography (HPLC) has been developed. Mainstream and sidestream particulate matter is collected on Cambridge filter pads. The polycyclic aromatic hydrocarbons (PAH) are extracted with cyclohexane and subjected to normal-phase chromatography to isolate target fractions which are concentrated and then diluted with acetonitrile or acetonitrile/water prior to reverse-phase analytical chromatography.

The Kentucky Reference cigarette 1R4F and a cigarette which heats but does not burn tobacco (New Cigarette) developed at R. J. Reynolds Tobacco Company were analyzed and compared with respect to B[a]P and B[a]A in both mainstream and sidestream smoke.

ZUSAMMENFASSUNG

Es wurde eine Methode zur quantitativen Bestimmung von Benz[a]anthracen (B[a]A) und Benzo[a]pyren (B[a]P) im Haupt- und Nebenstromrauch von Zigaretten durch Hochleistungsflüssigkeits-Chromatographie (HPLC) entwickelt. Die Partikelphase des Haupt- und Nebenstromrauchs wurde mit Cambridge-Filtern gesammelt. Die polyzyklischen aromatischen Kohlenwasserstoffe (PAH) wurden mit Cyclohexan extrahiert und einer konventionellen Säulenchromatographie unterwor-

fen. So wurden bestimmte Fraktionen isoliert, die konzentriert und dann in Acetonitril oder einem Acetonitril/Wassergemisch gelöst wurden; anschließend wurde eine analytische Umkehrphasen-Chromatographie durchgeführt.

Die Kentucky-Referenz-Cigarette 1R4F und eine Cigarette, die den Tabak zwar erhitzt aber nicht verbrennt (Neue Cigarette, sie wurde bei R. J. Reynolds entwickelt), wurden analysiert und in ihrem Gehalt an B[a]P und B[a]A im Haupt- und Nebenstromrauch miteinander verglichen.

RESUME

On a développé une méthode quantitative pour déterminer au moyen de chromatographie liquide à haute pression les substances benz[a]anthracène (B[a]A) et benzo[a]pyrène (B[a]P) contenues dans le courant principal et secondaire de la fumée de cigarette. La phase particulaire de la fumée du courant principal et secondaire a été collectée sur filtres Cambridge. Les hydrocarbures aromatiques polynucléaires ont été extraits à l'aide de cyclohexane et isolés sur chromatographie en phase normale. Avant l'analyse par chromatographie en phase inverse les fractions ont été concentrées et puis diluées dans de l'acétonitrile ou un mélange d'acétonitrile et d'eau. L'analyse du contenu en B[a]P et (B[a]A) dans la fumée du courant principal et secondaire a été réalisée d'une part sur la cigarette référence de Kentucky 1R4F et d'autre part sur une cigarette qui chauffe mais ne brûle pas le tabac (cigarette nouvelle, développée chez R. J. Reynolds Tobacco Company).

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INTRODUCTION

Many methods applying various analytical techniques have been developed for the determination of polycyclic aromatic hydrocarbons (PAH) in cigarette smoke condensate. These techniques include high performance liquid chromatography (HPLC) (1-6), gas chromatography (7-14), thin-layer chromatography (15, 16), paper chromatography (17) and spectrophotometry (15, 18-21). Many of these procedures require either a large sample or an extensive sample preparation procedure and are directed exclusively at the determination of benzo[a]pyrene (B[a]P) in mainstream cigarette smoke. Frequently, B[a]P has been used as an indicator for the entire compound class.

Little work has been done to quantify PAH's other than B[a]P in tobacco smoke (either mainstream or sidestream) by HPLC. At this time, HPLC coupled with fluorescence detection appears to be the method of choice for these non-volatile compounds (1-4, 6). To follow the distribution and quantity of PAH's in smoke condensate, two PAH's with different structures, B[a]A and B[a]P, were monitored. This provides a more complete picture with respect to the overall class.

EXPERIMENTAL

A. Apparatus for Preparative Liquid Chromatography and Reagents

1. Isolation/preparation of the Cambridge pad extract of the cigarette smoke condensate for B[a]P and B[a]A was performed on a Waters Associates liquid chromatograph equipped with a single Model 6000A pump. Detection was accomplished by means of a Waters Model 420 fluorescence detector equipped with a G4T5 mercury lamp, 254 nm excitation band-pass filter and 375 nm emission long-pass filter. Sample extracts were injected via a Model U6K injector equipped with a 1000 μ l sample loop. All detector outputs were monitored via a Model 730 module and data were acquired on-line by a computer (Hewlett-Packard RTE 1000 and Beckman Computer Automated Laboratory System).
2. A Waters Radial-Pak μ Bondapak-NH₂, 8 mm $\times$ 10 cm, 10 μ m particle size column was used at ambient temperature in a Waters Z-module.
3. B[a]P and B[a]A (Aldrich) and all solvents (Burdick and Jackson) were obtained and used as received. Distilled water was further purified by passage through a Barnstead Nanopure System.

B. Smoke Collection and Cigarettes

Mainstream and sidestream cigarette smoke particulate matter was collected on Cambridge filter pads by pro-

cedures described by PILLSBURY et al. (22) and JOHNSON (23), respectively. Reference cigarettes 1R4F were obtained from the Tobacco and Heath Research Institute, Lexington, Kentucky. The New Cigarette was obtained in-house and has been described elsewhere (24).

C. Apparatus for Analytical Liquid Chromatography

1. The analytical determination of B[a]P and B[a]A was done on a separate Waters liquid chromatograph consisting of two Model 6000A pumps, a Perkin-Elmer 650-10S fluorescence detector and a WISP 710B autosampler with an injection volume of 200 μ l.
2. The analytical column was a Vydac 201TP54, 4.6 mm $\times$ 250 mm, 5 μ m particle size, polymeric octadecylsilane on silica (The Separations Group).

D. Analytical Procedure: Normal-Phase Chromatography

The normal-phase isolation/preparation of B[a]A was similar to that for B[a]P described elsewhere (1). B[a]A has one less aromatic ring than B[a]P and therefore elutes in an earlier fraction that contained mostly isomers of the same ring number. Attempts to isolate both B[a]A and B[a]P in a single fraction of 1R4F condensate were not successful.

E. Analytical Procedure: Reverse-Phase Chromatography

The conditions and procedures for the determination of B[a]P have been described previously (1). In order to determine the optimum excitation and emission wavelength settings for the detection of B[a]A, fluorescence spectra of B[a]A in acetonitrile (ACN) were obtained. Data in Table 1 show the wavelength maxima obtained with the more prominent wavelengths in bold type. Combinations of these wavelength sets were evaluated on the detector at a constant standard concentration so that the most sensitive (highest response) set of wavelengths could be determined. The excitation wavelength of 287 nm and emission wavelength of 409 nm were the most responsive and probably the most selective under sample chromatographic conditions (see below).

Table 1.
Excitation and emission wavelengths for B[a]A.

	Wavelength (nm) ^a
Excitation	287 , 328, 340, 358
Emission	385, 409 , 432, 464

^a - more prominent wavelengths in bold type

Table 2.
Gradient conditions for the separation of B[a]A from chrysene
(flow rate at 1.5 ml/min).

Time (min.)	% Acetonitrile	% Water
0	45	55
20	45	55
50	95	5
60	95	5
64	45	55
84	45	55

Since other 4-ring compounds are present in tobacco smoke (5, 8, 10, 11, 17), e.g., chrysene, the analytical column was evaluated with respect to the separation of the B[a]A-chrysene pair. Isocratic conditions were of little value for separating this pair. Data in Table 2 show the linear gradient that was most effective in separating the B[a]A-chrysene pair. The gradient included a column cleaning segment (95 % acetonitrile for 10 minutes) to help prevent excessive buildup of strongly retained sample components. Note that there are two twenty-minute segments for column equilibrium prior to the next injection. These longer equilibrium conditions helped to minimize the "degassing" which occurred at these mixtures of acetonitrile and water. Figure 1 illustrates the resolution obtained between B[a]A and chrysene with the described gradient. B[a]A had a significantly greater response factor than chrysene at these wavelengths which showed the selectivity that can be attained by fluorescence detection. The standards used in Figure 1 were dissolved in 1:1, acetonitrile/water. Attempts to chromatograph these compounds in 100 percent acetonitrile resulted in a

Figure 1.
B[a]A - Chrysene Separation. Conditions: Vydac 201TP54 (4.6 mm x 250 mm, 5 μ m); acetonitrile/water, 45:55 initially for 20 minutes to 95:5 over 30 minutes with linear gradient at 1.5 ml/min.; injection volume, 200 μ l; fluorescence detection, 287 nm excitation, 409 nm emission; B[a]A, 8.3 ng/ml, chrysene, 276 ng/ml in 1:1, acetonitrile/water.

Table 3.
Linear correlation of chromatographic system for B[a]P.

1R4F Range		New Cigarette Range	
Standard (ng/ml)	Height (mV)	Standard (ng/ml)	Height (mV)
1.4	70.7	0.042	42.8
2.8	135.8	0.084	63.4
4.2	213.4	0.210	141.0
5.6	289.2	0.420	238.3
8.4	436.3	0.840	447.3
14.0	714.2	1.050	544.2

$R^2 = 0.9996$

$R^2 = 0.9991$

Table 4.
Linear correlation of chromatographic system for B[a]A.

1R4F Range		New Cigarette Range	
Standard (ng/ml)	Height (mV)	Standard (ng/ml)	Height (mV)
1.30	43.7	0.052	24.2
2.60	88.9	0.104	35.6
5.20	176.0	0.260	85.7
6.50	222.2	0.416	130.0
9.75	324.8	0.780	247.6
13.00	448.2	1.040	373.3

$R^2 = 0.9992$

$R^2 = 0.9908$

single peak. Resolution can only be achieved when the samples are dissolved in a solvent similar in composition to the mobile phase.

Linearity of detector response in the appropriate ranges for the two cigarettes are given in Tables 3 and 4 for B[a]P and B[a]A, respectively.

F. Sample Preparation

For the determination of B[a]A and B[a]P in mainstream smoke condensate of 1R4F, five cigarettes were smoked; for the New Cigarette, twenty cigarettes were smoked. The wet total particulate matter (WTPM) is recorded and the Cambridge pad, in both cases, was extracted for one hour by wrist-action shaking of the pad in 10 ml of cyclohexane.

For sidestream smoke condensate, two Kentucky Reference 1R4F cigarettes and six New Cigarettes were used. The 1R4F pad was extracted with a total of 50 ml of cyclohexane, 10 ml being used to rinse the hood, and similarly, the New Cigarette Cambridge pad was extracted with a total of 20 ml cyclohexane. These samples also were shaken one hour. Standards in cyclohexane

were injected (1 ml) onto the normal-phase, NH_2 column with hexane as the mobile phase at a flow of 2 ml/min. Only when there was a steady baseline and at least two identical chromatograms of the standard were attained, was the sample extract injected. The sample fraction corresponding to 2 minutes prior to the standard peak elution threshold time until 2 minutes following complete elution of the standard was collected in a 25-ml collection tube. The collected B[a]A fraction in hexane (usually ca. 14 ml) was heated at 50 °C under nitrogen until the hexane was completely evaporated. Prior to the following analytical determination, 1 ml of 1:1, acetonitrile/water was used to dissolve the residue.

A variety of blank determinations also were done as described above. For mainstream and sidestream smoke, the same number of unlit cigarettes were subjected to the same conditions as the lit cigarette samples, simultaneously, while the sample cigarettes were being smoked. Also, in the case of sidestream and mainstream smoke from 1R4F, a backup Cambridge pad was used to determine if the initial pad became overloaded. The cyclohexane solvent also was analyzed for B[a]A and B[a]P.

Table 5.
Precision for the determination of B[a]P in 1R4F reference cigarette and the New Cigarette.

1R4F Cigarette		
Chromatographic (Height) ^a	Overall (ng/cigarette)	
	Mainstream ^b	Sidestream ^c
218.1	9.66	161.6
215.2	9.51	145.6
210.2	10.62	136.8
206.8	8.66	138.2
204.6	8.98	149.7
205.6	7.78	155.8
Mean	9.20	148.0
Std. dev.	0.96	9.8
%RSD	10.43	6.6
New Cigarette		
Chromatographic (Height) ^a	Overall (ng/cigarette)	
	Mainstream ^e	Sidestream ^f
128.0	0.08	2.69
132.4	0.06	2.54
131.8	0.08	3.00
125.7	0.06	3.84
134.4	0.09	4.46
126.8	0.08	2.04
Mean	0.08	3.10
Std. dev.	0.01	0.90
%RSD	12.50	29.03

^a - 4.20 ng ml⁻¹ standard
^b - 5 cigarettes, 10 ml cyclohexane extract
^c - 2 cigarettes, total of 50 ml cyclohexane

^d - 0.21 ng ml⁻¹ standard
^e - 20 cigarettes, 10 ml cyclohexane extract
^f - 6 cigarettes, total of 20 ml cyclohexane

Table 6.
Precision for the determination of B[a]A in 1R4F reference cigarette and the New Cigarette.

1R4F Cigarette		
Chromatographic (Height) ^a	Overall (ng/cigarette)	
	Mainstream ^b	Sidestream ^c
185.8	9.6	249.6
188.5	12.3	198.5
184.6	10.6	186.0
184.4	10.8	184.0
181.4	8.6	170.5
181.8	11.4	326.0
Mean	10.5	219.1
Std. dev.	1.3	59.1
%RSD	12.3	27.0
New Cigarette		
Chromatographic (Height) ^d	Overall (ng/cigarette)	
	Mainstream ^e	Sidestream ^f
89.2	0.12	6.00
84.6	0.12	7.92
82.4	0.05	7.13
86.7	0.08	12.03
87.4	0.10	11.28
90.2	0.12	7.45
Mean	0.10	8.64
Std. dev.	0.02	2.43
%RSD	20.00	28.12

^a - 5.20 ng ml⁻¹ standard

^b - 5 cigarettes, 10 ml cyclohexane extract
^c - 2 cigarettes, total of 50 ml cyclohexane

^d - 0.26 ng ml⁻¹ standard

^e - 20 cigarettes, 10 ml cyclohexane extract
^f - 6 cigarettes, total of 20 ml cyclohexane

RESULTS AND DISCUSSIONS

Data in Tables 5 and 6 show both the instrumental and overall method precision for the determination of B[a]A and B[a]P in mainstream and sidestream smoke condensate of the Kentucky Reference 1R4F and the New Cigarette. As can be seen, the New Cigarette contained significantly less of these compounds than the 1R4F reference cigarette. Note that the % RSD for the New Cigarette was higher than that of 1R4F. This difference may be attributed to the small quantities involved and the higher sensitivity levels required to determine these small amounts.

Data in Table 7 show the percent recovery of B[a]P and B[a]A from the mainstream smoke condensate of the New Cigarette extracted from a Cambridge pad. In order to determine the reason for these low recoveries for B[a]P, standards in cyclohexane were taken through the preparative system and evaluated versus a similar standard run through the analytical system. Results showed that the standard in cyclohexane is only ninety percent recovered

Table 7.
Recovery/standard addition for B[a]P and B[a]A from the New Cigarette mainstream smoke.

B[a]P			
ng Added	ng Found	ng Recovered	% Recovery
0.00	1.54	—	—
1.50	2.90	1.36	90.66
3.00	4.23	2.69	89.66
4.50	5.08	3.54	78.66
6.00	6.51	4.97	82.83
Mean = 85.45			
Std. dev. = 5.71			
%RSD = 6.68			

B[a]A			
ng Added	ng Found	ng Recovered	% Recovery
0.00	2.50	—	—
1.25	3.98	1.48	118.40
2.50	5.08	2.58	103.20
3.75	6.22	3.72	99.20
5.00	7.66	5.16	103.20
Mean = 106.00			
Std. dev. = 8.48			
%RSD = 8.00			

from the preparative system. This result may explain the lower overall recovery for B[a]P. The γ -intercepts obtained from the data in Table 7 yield results practically identical to those obtained by external standard, 0.08 versus 0.08 ng/cigarettes for B[a]P and 0.12 versus 0.10 ng/cigarettes for B[a]A.

Figures 2 and 3 show preparative chromatograms of the cyclohexane extracts of mainstream and sidestream smoke condensate. Efforts were made to isolate both B[a]A and B[a]P from a single 1-ml injection of the 1R4F cyclohexane extract. However, it appears that too much material was collected from the normal-phase system as indicated by the number of interfering compounds in the reverse-phase chromatograms. Further studies might make it possible to isolate both B[a]A and B[a]P from a single injection of the New Cigarette condensate. However, this single step isolation of B[a]A and B[a]P was not done since a time-wavelength programmable fluorescence detector was not available. As can be seen from Figures 2 and 3, the material extracted from the New Cigarette smoke condensate under these conditions appeared to be less complicated than that of 1R4F. However, there were greater amounts of PAH's in the sidestream than mainstream smoke of both cigarettes. These conclusions are supported by the results of other workers (25). Fractions were collected from normal-phase sample injections based on the predetermined retention time of standards in cyclohexane and subjected to reverse-phase chromatography, Figures 4–7. As indicated by the μ -axis scale, larger amounts of B[a]A and B[a]P were observed in sidestream smoke compared to mainstream smoke. Smoke from the

Figure 2.
Normal-phase HPLC preparation of 1R4F and the New Cigarette mainstream smoke. Conditions: Waters μ Bondapak NH₂ (8 mm $\times$ 100 mm, 10 μ m); hexane eluent, 2 ml/min.; injection volume, 1 ml; fluorescence detection, 254 nm excitation, 375 nm emission. a=1R4F (10 ml cyclohexane, 5 cigarettes per pad); b=New Cigarette (10 ml cyclohexane, 20 cigarettes per pad).

Figure 3.
Normal-phase HPLC preparation of 1R4F and the New Cigarette sidestream smoke. Conditions: same as Figure 2. a=1R4F (50 ml cyclohexane, 2 cigarettes); b=New Cigarette (20 ml cyclohexane, 6 cigarettes).

New Cigarette contained less B[a]A and B[a]P than the 1R4F reference under these conditions. Results shown in Tables 5 and 6 indicate a hundred-fold reduction of B[a]P and B[a]A in mainstream smoke for the New Cigarette compared to 1R4F; a fifty-fold reduction of B[a]P and a twenty-five fold reduction of B[a]A in sidestream smoke. It is obvious from the chromatography (both preparative and analytical) that the sample matrices for these two cigarettes are different.

Since PAH's are ubiquitous and the amounts in cigarette smoke are low and, therefore, susceptible to contamination, a variety of blank determinations were done. PAH's in the cyclohexane solvent and mainstream unlit cigarette blanks were usually below detection limits (<0.01 ng/cigarette for both B[a]P and B[a]A). However, main-

Figure 4.

Reverse-phase HPLC analytical determination of B[a]P in 1R4F and the New Cigarette mainstream smoke. Conditions: Vydac 201TP54 (4.6 m × 250 mm, 5 μ m); acetonitrile/water, 90:10 at 1 ml/min; injection volume, 200 μ l; fluorescence detection, 378 nm excitation, 405 nm emission. a=1R4F; b=New Cigarette.

Figure 5.

Reverse-phase HPLC analytical determination of B[a]P in 1R4F and the New Cigarette sidestream smoke. Conditions: same as Figure 4. a=1R4F; b=New Cigarette.

Figure 6.

Reverse-phase HPLC analytical determination of B[a]A in 1R4F and the New Cigarette mainstream smoke. Conditions: same as Figure 1. a=1R4F; b=New Cigarette.

Figure 7.

Reverse-phase HPLC analytical determination of B[a]A in 1R4F and the New Cigarette sidestream smoke. Conditions: same as Figure 1. a=1R4F; b=New Cigarette.

stream and sidestream backup pad(s) – a pad in a Cambridge holder situated in-line behind the primary collection pad – from the smoking of 1R4F and the New Cigarette did contain detectable amounts of both B[a]P and B[a]A. The sidestream backup pad contained more than the mainstream and B[a]A was present in a greater amount than B[a]P. The presence of more B[a]A may be attributed to its higher volatility versus B[a]P and/or the large volume of air (which itself may contain B[a]A) passed through the system during sidestream smoke collection. These conclusions are supported by the significant amount of B[a]A (0.24 ng/cigarette) found in unlit cigarette blanks under sidestream smoking and collection conditions.

CONCLUSION

A method has been described for the determination of B[a]A in cigarette smoke which is both accurate and precise in the ranges to which it was applied. Both sidestream and mainstream smoke can be analyzed for B[a]A by this procedure and, with a slight modification of the technique, for B[a]P. The results for the New Cigarette show a substantial reduction of these compounds in smoke when compared to the Kentucky Reference cigarette 1R4F.

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