Analysis of 23 Polycyclic Aromatic Hydrocarbons in Smokeless Tobacco by Gas Chromatography−Mass Spectrometry

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Smokeless tobacco contains 28 known carcinogens and causes precancerous oral lesions and oral and pancreatic cancer. A recent study conducted by our research team identified eight different polycyclic aromatic hydrocarbons (PAHs) in U.S. moist snuff, encouraging further investigations of this group of toxicants and carcinogens in smokeless tobacco products. In this study, we developed a gas chromatography−mass spectrometry method that allows simultaneous analysis of 23 various PAHs in smokeless tobacco after a simple two-step extraction and purification procedure. The method produced coefficients of variation under 10% for most PAHs. The limits of quantitation for different PAHs varied between 0.3 and 11 ng/g tobacco, starting with a 300 mg sample. The recovery of the stable isotope-labeled internal standards averaged 87%. The method was applied to analysis of 23 moist snuff samples that included various flavors of the most popular U.S. moist snuff brands, as well as 17 samples representing the currently marketed brands of spit-free tobacco pouches, a relatively new type of smokeless tobacco. The sum of all detected PAHs in conventional moist snuff averaged 11.6 (±3.7) μg/g dry weight; 20% of this amount was comprised of carcinogenic PAHs. The levels of PAHs in new spit-free tobacco products were much lower than those in moist snuff; the sum of all detected PAHs averaged 1.3 (±0.28) μg/g dry weight. Our findings render PAHs one of the most prevalent groups of carcinogens in smokeless tobacco. Urgent measures are required from the U.S. tobacco industry to modify manufacturing processes so that the levels of these toxicants and carcinogens in U.S. moist snuff are greatly reduced.

Introduction

Investigation of toxic and carcinogenic compounds present in smokeless tobacco is a critical step toward the reduction of harmful health effects associated with its use. The "smokeless" category includes a variety of tobacco products intended for oral or nasal use, including chewing tobacco and moist snuff for oral use and dry snuff for nasal use. Among these, oral moist snuff is the most popular type of smokeless tobacco in the United States (*1*). This is, to a certain degree, a result of considerable investments made by the tobacco industry into its marketing. Even though overall smokeless tobacco use has declined substantially between 1986 and 2003 (*2*), accompanied by an 11% decrease in overall smokeless tobacco sales volume (*3*), the use of moist snuff increased more than 80-fold over the same period (*2*). In 2005, moist snuff accounted for more than 80% of total sales of smokeless tobacco (*1*). Because moist snuff is characterized by high moisture and salt contents, its use generates excess saliva and, therefore, usually requires spitting, creating a rather unsanitary image. In recent years, the tobacco industry has also been promoting spit-free smokeless tobacco designated for oral use. These new products are sold as small pouches of flavored tobacco with low moisture content and are marketed to cigarette smokers to be used in situations where smoking is not allowed (*4*).

Oral smokeless tobacco use can lead to precancerous oral lesions and oral and pancreatic cancer (*5*−*7*) and is associated with an increased risk of esophageal cancer (*8*). These carcinogenic effects are believed to be caused by various carcinogens present in smokeless tobacco (*9, 10*). The International Agency for Research on Cancer (IARC) lists 28 carcinogens present in smokeless tobacco (*6*). This list includes tobacco-specific *N*-nitrosamines (TSNAs)[1], volatile *N*-nitrosamines, volatile aldehydes, polycyclic aromatic hydrocarbons (PAHs), certain lactones, urethane, metals, polonium-210, and uranium-235 and -238 (*6, 10, 11*). Among these, TSNAs are commonly acknowledged as the most important group from the quantitative point of view (*12, 13*). Over the last 2 decades, TSNAs have become the main focus of studies dealing with chemical analysis of various smokeless tobacco products, their levels in a particular product being used as an indicator of its overall carcinogenic potency (*14*−*19*). At the same time, there was a common assumption that PAHs—ubiquitous environmental carcinogens formed during the incomplete combustion of organic matter—are present in smokeless tobacco only in trace amounts. This assumption was based on the fact that the use of smokeless tobacco does not involve burning. It was also supported by the low amounts of benzo[*a*]pyrene (BaP)—a representative carcinogenic PAH—quantified in some brands of U.S. moist snuff (*20*). However, our recent report demonstrated that, in addition to BaP, at least seven other PAHs are present in U.S. smokeless

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[1] Abbreviations: ANE, acenaphthene; ANP, acenaphthylene; ANT, anthracene; BaA, benz[*a*]anthracene; BbF, benzo[*b*]fluoranthene; BjF, benzo[*j*]fluoranthene; BkF, benzo[*k*]fluoranthene; BaP, benzo[*a*]pyrene; BeP, benzo[*e*]pyrene; BghiPy, benzo[*g*,*h*,*i*]perylene; CHR, chrysene; DBahA, dibenz[*a*,*h*]anthracene; FLR, fluorene; FLT, fluoranthene; GC-MS, gas chromatography−mass spectrometry; IcdP, indeno[1,2,3-*cd*]pyrene; MC, methylchrysene; NP, naphthalene; PHE, phenanthrene; PY, pyrene; TSNA, tobacco-specific nitrosamines.

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Table 1. Ions and Ion Ratios for the Analyzed PAHs and Corresponding Internal Standards

analyte	abbreviation	SIM group (duration, min)	quantitation ion (*m/z*)	confirmation ion (*m/z*)	ion ratio	internal standard	quantitation ion (*m/z*)	confirmation ion (*m/z*)	ion ratio	response ratio[a]
naphthalene	NP	1 (10.00)	128.2	127.2	6.27	$^{13}C_6$-NP	134.2	133.2	5.35	0.88
acenaphthylene	ANP	2 (2.25)	152.1	151.1	4.54	$^{13}C_6$-ANP	158.2	157.1	3.64	0.86
acenaphthene	ANE	2 (2.25)	154.2	152.2	1.65	$^{13}C_6$-ANE	160.2	158.2	1.45	0.90
fluorene	FLR	3 (1.25)	166.2	165.2	0.97	$^{13}C_6$-FLR	172.2	171.2	0.89	0.93
phenanthrene	PHE	4 (2.00)	178.2	176.2	4.59	$^{13}C_6$-PHE	184.2	182.2	4.52	0.95
anthracene	ANT	4 (2.00)	178.2	176.2	4.66	$^{13}C_6$-ANT	184.2	182.2	4.50	0.92
fluoranthene	FLT	5 (3.50)	202.3	200.3	4.32	$^{13}C_6$-FLT	208.3	206.3	4.67	1.03
pyrene	PY	5 (3.50)	202.3	200.3	4.51	$^{13}C_6$-PY	205.2	203.3	2.59	1.14
benz[*a*]anthracene	BaA	6 (4.00)	228.3	226.3	3.47	$^{13}C_6$-BaA	234.3	232.3	3.36	0.96
chrysene	CHR	6 (4.00)	228.3	226.3	3.12	$^{13}C_6$-CHR	234.3	232.3	3.04	0.84
1-methylchrysene	1MC	7 (4.00)	242.3	239.3	2.97	$^{13}C_6$-CHR	234.3	232.3	3.12	1.67
3-methylchrysene	3MC	7 (4.00)	242.3	239.3	2.98	$^{13}C_6$-CHR	234.3	232.3	3.12	0.98
4-methylchrysene + 6-methylchrysene	4MC, 6MC	7 (4.00)	242.3	239.3	2.35	$^{13}C_6$-CHR	234.3	232.3	3.12	1.22
5-methylchrysene	5MC	7 (4.00)	242.3	239.3	2.20	$^{13}C_6$-CHR	234.3	232.3	3.12	1.32
benzo[*b*]fluoranthene + benzo[*j*]fluoranthene	BbF, BjF	8 (2.50)	252.3	250.3	3.39	$^{13}C_6$-BbF	258.3	256.3	3.54	0.97
benzo[*k*]fluoranthene	BkF	8 (2.50)	252.3	250.3	3.68	$^{13}C_6$-BkF	258.3	256.3	3.72	1.01
benzo[*e*]pyrene	BeP	9 (3.50)	252.3	250.3	2.89	$^{13}C_4$-BeP	256.3	254.3	3.06	0.92
benzo[*a*]pyrene	BaP	9 (3.50)	252.3	250.3	3.86	$^{13}C_4$-BaP	256.3	254.3	3.06	1.01
indeno[1,2,3-*cd*]pyrene	IcdP	10 (7.00)	276.3	274.3	3.84	$^{13}C_6$-IcdP	282.3	280.4	5.01	1.08
dibenz[*a*,*h*]anthracene	DBahA	10 (7.00)	278.3	276.3	3.81	$^{13}C_6$-DBahA	284.3	282.4	3.41	1.01
benzo[*g*,*h*,*i*]perylene	BghiPy	10 (7.00)	276.3	274.3	3.68	$^{13}C_6$-IcdP	282.3	280.4	5.01	0.91

[a] Analyte to internal standard peak area ratio in the range of analyte concentrations from 0.01 to 10 ng/μL.

tobacco, some of them at unexpectedly high levels (*21*). These findings inspired further investigation of the presence of PAHs in smokeless tobacco products.

In this study, we developed a gas chromatography−mass spectrometry (GC-MS) method to analyze 23 PAHs in smokeless tobacco. We expanded the list of PAHs analyzed in smokeless tobacco to include the priority environmental PAH pollutants identified by the U.S. Environmental Protection Agency (EPA), as well as those carcinogenic PAHs that, according to IARC, are present in cigarette smoke. The method allows analysis of all of these PAHs in a single GC-MS run after a simple two-step extraction and purification procedure. The PAHs analyzed here include naphthalene (NP), acenaphthylene (ANP), acenaphthene (ANE), fluorene (FLR), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PY), benz[*a*]anthracene (BaA), chrysene (CHR), methylchrysene (MC) isomers, benzo[*b*]fluoranthene (BbF), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), benzo[*e*]pyrene (BeP), BaP, indeno[1,2,3-*cd*]pyrene (IcdP), dibenz[*a*,*h*]anthracene (DBahA), and benzo[*g*,*h*,*i*]perylene (BghiPy). The method was applied to analysis of 23 moist snuff samples that include various flavors of the most popular U.S. moist snuff brands, as well as 17 samples of spit-free tobacco pouches representing the currently marketed brands of this relatively new type of smokeless tobacco.

Materials and Methods

Caution: *Many PAHs are strong toxicants and carcinogens and should be handled with extreme care in a well-ventilated hood and with personal protective equipment.*

Tobacco Samples. Products collected for analysis represent conventional moist snuff and new smokeless spit-free tobacco products. Conventional moist snuff was obtained from retailers in Minneapolis, MN, between July, 2007, and July, 2009. New spit-free varieties of smokeless tobacco were purchased in retail stores between August, 2008, and July, 2009. Some of these products were supplied by Dr. Biener (University of Massachusetts, Boston, MA). Triumph Snus, Grand Prix Snus, and Tourney Snus were purchased in Columbus, OH. Marlboro Snus was purchased in Indianapolis, IN, and Camel Snus and Nordic Ice were procured in Minneapolis.

Chemicals. ^{13}C-labeled PAH mix was purchased from Cambridge Isotope Laboratories (Andover, MA). 3-MC and 4-MC were purchased from the NCI Chemical Carcinogen Reference Standard Repository. 1-MC and 6-MC were kindly provided by Dr. Amin at the Penn State College of Medicine. Other unlabeled PAH standards were obtained from Cambridge Isotope Laboratories and Sigma-Aldrich (Milwaukee, WI). All other chemicals and solvents were purchased from Sigma-Aldrich Chemical Co. or Fisher Scientific (Fairlawn, NJ).

PAH Analysis. Sample Preparation. Tobacco samples were extracted and purified by a slight modification of a method originally developed by Ding et al. (*22*) for PAH analysis in cigarette smoke and later adapted by our group for smokeless tobacco analysis as described elsewhere (*21*). PAHs were extracted by shaking 300 mg of tobacco with 1 mL of cyclohexane at room temperature for 3 h. The tobacco particles were removed by centrifugation, and 500 μL of the extract was mixed with the ^{13}C-labeled internal standard mix. The mixture was loaded on 50 mg BondElut Silica cartridges (Varian) pre-equilibrated with 1 mL of cyclohexane. The cartridge was washed with 2 mL of cyclohexane, and the eluants from both the load and the wash were combined and concentrated under a gentle stream of N_2 to a final volume of 200 μL. The concentrated samples were transferred to amber glass microinsert vials. Three microliters of the sample was analyzed by GC-MS.

GC-MS Analysis. Separation and analysis of PAHs were based on the method originally developed by Ding et al. (*23*). The analysis was performed on a Thermo Scientific system composed of a TSQ Quantum GC tandem mass spectrometer, coupled with a Trace GC Ultra gas chromatograph and a Tri Plus autosampler. The GC was equipped with a 60 m (0.25 mm internal diameter, 0.25 μm film thickness) DB-5MS fused silica capillary column (J&W Scientific, Folsom, CA) and a 2 m × 0.53 mm deactivated fused silica guard column. The constant flow rate was 1.2 mL/min He, and the splitless injection port temperature was set at 250 °C. The total GC run time was 46.5 min, and the oven temperature was programmed as follows: 15 °C/min ramp from 80 to 275 °C, then 5 min at 275 °C, then 5 °C/min to 285 °C, then 10 min at 285 °C, then 20 °C/min to 315 °C, and 15 min at 315 °C. The MS was operated in the positive EI mode. The ion source temperature was set at 300 °C, the emission current was 50 μA, the scan width was set at *m/z* 0.100, and the scan time was 0.010 s. The instrument was operated in selected ion monitoring mode (SIM) (Table 1). The filament was turned off for the first 6 min. To ensure maximum selectivity and sensitivity, the PAHs were divided into 11 SIM groups. The PAHs

Table 2. Average Recovery of Stable Isotope-Labeled PAHs Used as Internal Standards

internal standard	average recovery (%)
$^{13}C_6$-NP	76.9
$^{13}C_6$-ANP	75.6
$^{13}C_6$-ANE	74.7
$^{13}C_6$-FLR	80.2
$^{13}C_6$-PHE	83.9
$^{13}C_6$-ANT	80.4
$^{13}C_6$-FLT	90.5
$^{13}C_6$-PY	93.0
$^{13}C_6$-BaA	92.4
$^{13}C_6$-CHR	91.0
$^{13}C_6$-BbF	96.7
$^{13}C_6$-BkF	96.0
$^{13}C_4$-BaP	98.4
$^{13}C_6$-IcdP	94.2
$^{13}C_6$-DBahA	88.5

reported here were analyzed in the first 10 SIM groups (Table 1), while the last group was intended to monitor dibenzo[*a*,*e*]pyrene and dibenzo[*a*,*i*]pyrene, which were not detected under these conditions.

Method Characteristics. The accuracy of the method was tested by spiking Hawken Long Cut Wintergreen (which, according to our preliminary experiments, is low in PAH content) with 5, 100, 250, and 500 ng of each PAH. A nonspiked sample of the same product was added to the set. Each sample was analyzed in duplicate. The results at each spiking level were expressed as % of added PAH, after the subtraction of the amount of each PAH measured in the nonspiked sample. The average of these determinations was then calculated for each PAH. The precision of the method for each PAH was determined by calculating the coefficient of variation of five measurements obtained from five aliquots of a cyclohexane extract of Copenhagen Snuff (a representative moist snuff product). The limit of quantitation (LOQ) was determined as the lowest amount of each PAH in the 300 mg tobacco sample that can be reliably quantified at 10:1 signal-to-noise ratio for the corresponding quantitation ion. The detection limit of each PAH was determined at a 3:1 signal-to-noise ratio for the corresponding quantitation ion.

Other Analyses. The moisture content was measured via the difference in weight of a tobacco sample before and after drying, as previously described (*21*).

Results

Modification of the Method. The method used in our previous study (*21*) produced satisfactory results; however, the recovery of the higher molecular weight PAH was somewhat inconsistent. We suggested that some of the PAHs were retained on the cartridge and removed only with the last part of the 2 mL cyclohexane volume. In an effort to achieve better efficiency of sample cleanup during solid-phase extraction and improve the PAH recovery from the cyclohexane tobacco extracts, we examined sample extraction on 50 mg BondElut Silica cartridges (Varian). This change in the sample purification protocol led to excellent recovery of all ^{13}C-labeled standards (Table 2).

A representative total ion GC-MS trace of a standard mix containing all 23 PAH and 15 stable isotope-labeled internal standards is illustrated in Figure 1. Selected ion chromatograms demonstrated excellent separation for most of the analytes. Two pairs of compounds were not well-resolved: BbF coeluted with BjF, and 4-MC coeluted with 6-MC (inserts A and B in Figure 1). The latter two compounds also overlapped with the 5-MC peak.

Characteristics of the Method. Accuracy, precision, and LOQs for all PAHs are summarized in Table 3. There was a minor contribution of stable isotope-labeled PAHs to the ion chromatograms of corresponding unlabeled PAHs. This contribution was consistent over the range of labeled PAH concentrations from 0.05 to 1 ng/μL and varied from 0.03% of $^{13}C_6$-ANT area observed in the ANT ion chromatogram to 6.7% of $^{13}C_4$-BaP peak area observed in the BaP ion chromatogram. These values were subtracted from peak areas of corresponding PAHs in all subsequent calculations. The difference between the PAH content measured in nonspiked and spiked Hawken samples was in good agreement with the amount of PAH added to each sample (Table 3), and the linearity of the method was satisfactory in the studied range of concentrations ($R^2 > 0.99$ for all PAHs). Analysis of five nonspiked aliquots of Copenhagen Snuff extract produced coefficients of variation under 10% for most PAHs (Table 3). The LOQs varied between 0.3 and 10.9 ng/g tobacco, and the limits of detection (LODs) varied between 0.1 and 3.8 ng/g tobacco, starting with a 300 mg sample (Table 3).

Analysis of Moist Snuff and Spit-Free Tobacco Pouches. The method was used to analyze PAHs in various smokeless tobacco products marketed and test-marketed in the United States. Twenty-three moist snuff samples and 17 spit-free tobacco products were included in the analysis. A typical GC-MS trace obtained upon analysis of conventional moist snuff is illustrated in Figure 2. The results are summarized in Table 4.

With the exception of Hawken Long Cut Wintergreen, the levels of individual PAHs were very similar across various brands of conventional moist snuff. Average amounts of detected PAHs in these products ranged from 7.5 ng/g dry weight for DBahA to 4700 ng/g tobacco for PHE. Total MCs in Table 4 represent the calculated sum of 1-MC, 3-MC, 4-MC, and 6-MC. 5-MC was not detected in any of the tested products. We also consistently observed an additional peak, which, on the basis of its spectrum and retention time (24.16 min, Figure 2), could be 2-MC (*34*). However, we did not have a corresponding standard to confirm its identity. The sum of all detected PAHs (total PAHs) in 23 samples of conventional moist snuff averaged 11600 (±3700) ng/g dry weight. Hawken Long Cut Wintergreen was relatively low in total PAH content—only 1250 ng/g dry weight.

The levels of PAH in the new spit-free tobacco products were much lower than those in moist snuff (Table 4), total PAHs averaging 1280 (±276) ng/g tobacco. The levels of individual PAH in these products were not as consistent across different brands as in moist snuff: PHE varied from 9.4 ng/g dry weight in Marlboro Snus Peppermint to 79.4 ng/g dry weight in Camel Snus Spice; the FLT content in the same products was 5.6 and 59.7 ng/g dry weight, respectively, and the BaP content was below the LOQ and 15.0 ng/g dry weight, respectively (Table 4). None of the tested new products had detectable levels of BghiPy, and only Nordic Ice Snus had quantifiable levels of MC isomers.

The only PAH that was present in both types of smokeless tobacco in comparable amounts was NP: 1730 (±392) ng/g dry weight in moist snuff and 1110 (±207) ng/g dry weight in the spit-free products, accounting for 15 and 87%, respectively, of the average total PAHs in these products. Average relative contributions of other individual PAHs to the sum of 22 PAHs (excluding NP) are illustrated in Figure 3.

Discussion

A recent study conducted by our research team identified eight PAHs in moist snuff, encouraging further investigations of this group of toxicants and carcinogens in smokeless tobacco products. In this study, we used GC-MS to analyze 23 different PAHs in some U.S. moist snuff brands, as well as in some new

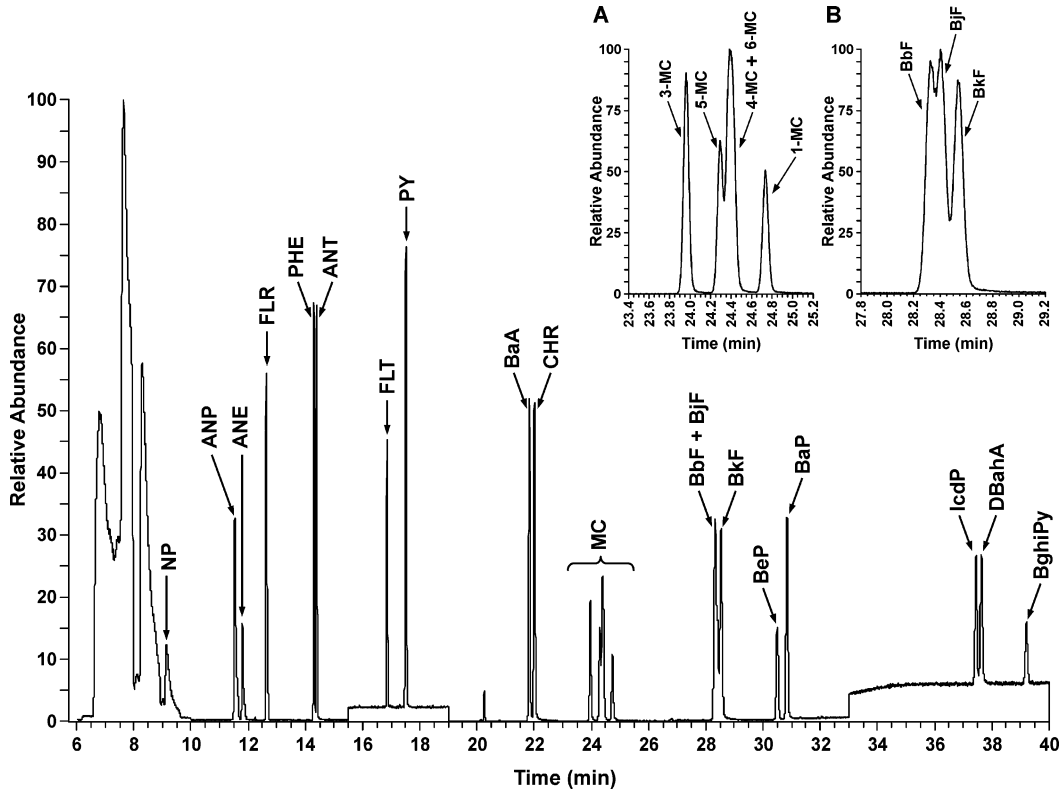


Figure 1. Typical total ion GC-MS chromatogram obtained upon analysis of a standard PAH mix containing 1 ng/μL each of 23 PAHs. All peaks were well-resolved with the exception of (A) MCs and (B) BbF and BjF. For PAH abbreviations, see Table 1.

Table 3. Characteristics of the Method

analyte	accuracy (%)	precision, % variation ($N = 5$)	LOQ, ng/g tobacco
NP	109	9.93	4.8
ANP	112	8.29	4.0
ANE	105	7.89	10.9
FLR	118	7.98	1.6
PHE	124	7.69	2.0
ANT	118	9.51	2.6
FLT	103	8.25	0.3
PY	115	9.89	0.3
BaA	109	8.03	0.9
CHR	108	8.15	1.0
1-MC	109	8.05	2.0
3-MC	110	7.02	2.0
4-MC + 6-MC	112	9.31	2.6
5-MC	104	ND[a]	ND
BbF + BjF	115	6.32	1.8
BkF	118	5.72	1.6
BeP	113	7.70	2.1
BaP	109	4.47	1.6
IcdP	113	7.20	2.3
DBahA	112	10.83	3.9
BghiPy	113	12.31	2.4

[a] ND, not detected in tobacco.

spit-free smokeless tobacco products currently marketed and test-marketed in the United States. This is the first study to report the presence of NP, ANE, FLR, BaA, CHR, 1-MC, 3-MC, 4-MC, 6-MC, BjF, BeP, IcdP, BghiPy, and DBahA in U.S. moist snuff. Our findings render PAHs one of the most prevalent groups of carcinogens in smokeless tobacco.

The sum of NP, PHE, FLT, and PY accounted for about 78% of total PAHs in moist snuff. Of these, NP—a bicyclic aromatic hydrocarbon—is an IARC group 2B carcinogen (*24*) (Table 5), and FLT is a weak lung tumorigen in mice (*25*). The sum of FLR, ANT, and IARC group 2B carcinogens BaA and CHR in moist snuff accounted for an additional 18% of total PAH. The remaining 4% was comprised of ANP, ANE, MC isomers, BbF, BjF, BkF, BaP, BeP, IcdP, BghiPy, and DBahA. Overall, of the 22 PAH detected in moist snuff, nine are classified by IARC as group 1, 2A, or 2B carcinogens (Table 5), their sum averaging 2.36 μg/g dry weight or 20% of total PAHs. This is the first study to demonstrate that the sum of carcinogenic PAHs in U.S. moist snuff reaches μg/g levels. The relatively high levels of FLT and PY in moist snuff are also disturbing, as these PAHs act as cocarcinogens when applied to mouse skin along with, or prior to, BaP treatment (*26*−*28*). Coapplication of FLT or PY with BaP on mouse skin also results in a 56 and 66% increase, respectively, in the levels of BaP-derived DNA adducts (*29*). Cocarcinogenicity with BaP has been also reported for BeP and BghiPy (*26, 27*). Some of the PAHs found in smokeless tobacco, however, were reported to inhibit carcinogenicity of other PAHs. Thus, NP and BaA inhibit carcinogenicity of BaP (*30, 31*), while BeP inhibits carcinogenicity of DBahA (*32*).

Comparison to the levels of PAHs reported for cigarette smoke can be useful for a better understanding of the relative amounts of this group of chemicals found in U.S. moist snuff. In the report by Ding et al., the sums of 14 PAHs in the smoke of full flavor varieties of the U.S. cigarettes Marlboro, Camel, and Newport were 1.15, 1.29, and 1.15 μg/cigarette, respectively (*23*). The reported PAHs included NP, ANP, ANE, FLR, PHE, ANT, FLT, PY, BaA, CHR, BbF, BkF, BeP, and BaP. In our study, the sum of the same 14 PAH and BjF (which could not be separated from BbF but accounted for only about 1% of the sum) in 1 g of product averaged 5.41 ($\pm$1.66) μg. Thus, an average single portion of traditional moist snuff (1−2 g) can contain about five times higher amounts of the sum of these 14 PAHs than the smoke of a single cigarette. This is mostly due to the presence in moist snuff of relatively high levels of NP, PHE, FLT, and PY (Table 4). Of these, only NP is considered a group 2B carcinogen (Table 5).

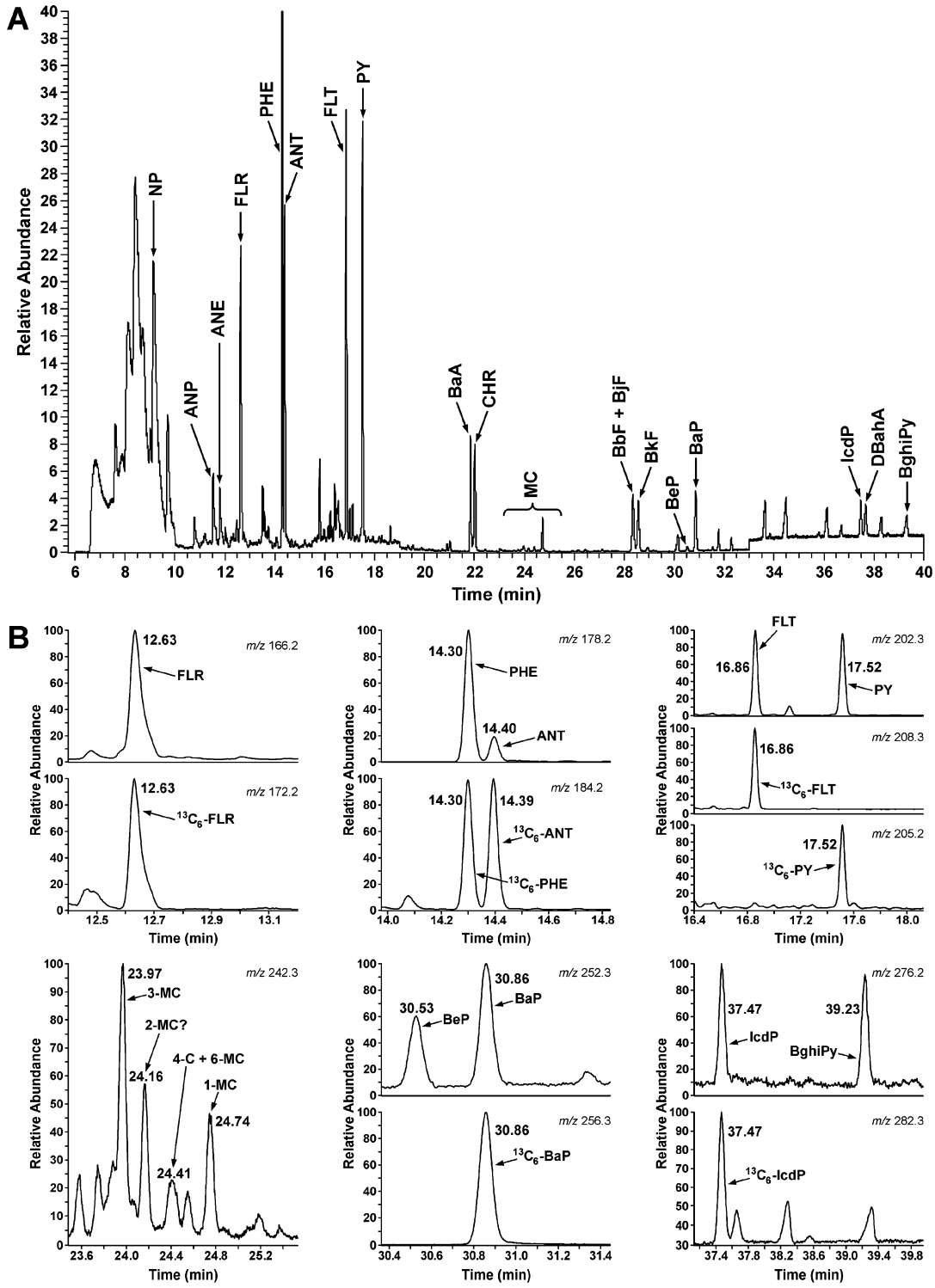


Figure 2. Typical GC-MS chromatogram obtained upon analysis of a moist snuff sample: A, total ion chromatogram; and B, some examples of individual ion chromatograms. For PAH abbreviations, see Table 1.

Among moist snuff products analyzed here, Hawken Long Cut Wintergreen is drastically different from the rest of the products; the sum of all PAHs in this product is only about 11% of the average total PAHs in the rest of moist snuff brands. Apparently, the tobacco processing method used in the manufacturing of Hawken does not lead to its contamination with PAHs. It is worth mentioning that Hawken brand has low prevalence of use, while high-PAH products such as Skoal, Copenhagen, and Grizzly are among the most popular smokeless tobacco brands in the United States (*33*).

Of the five analyzed MC isomers, 5-MC exhibits high carcinogenic activity similar to that of BaP (*34*). None of the products analyzed here had detectable levels of this isomer. We were not able to determine individual amounts of 4-MC and 6-MC, because these two peaks coeluted (Figure 2). The identity of 2-MC could not be confirmed because of the lack of the standard compound. The average contributions of 1-MC and 3-MC to the total amount of MC isomers identified in moist snuff were 40 and 42%, respectively, while the sum of 4-MC and 6-MC accounted for 18% of the total amount. These relative

Table 4. Levels of PAHs in Smokeless Tobacco Marketed in the United States

		PAH[a] (ng/g dry weight)																		
sample content	moisture (%)	NP	ANP	ANE	FLR	PHE	ANT	FLT	PY	BaA	CHR	total MC	BbF + BjF	BkF	BeP	BaP	IcdP	BghiPy	DBahA	total PAH
moist snuff																				
Skoal Long Cut Straight	54.4	1990	132	126	783	4250	712	1180	1020	149	195	79	76	15	45	53	43	23	6.8	10900
Skoal Long Cut Mint	53.8	1940	143	163	945	5030	852	1460	1250	198	254	102	281	17	80	56	33	21	8.2	12800
Copenhagen Snuff	54.7	1860	85	112	709	4960	784	1650	1420	220	269	95	278	26	102	60	27	26	LOQ[b]	12700
Copenhagen Long Cut	54.6	2020	96	120	878	6030	1050	1790	1490	222	293	95	186	28	70	68	31	27	LOQ	14500
Kodiak Straight	56.2	2210	114	165	1110	6340	1070	2010	1880	346	462	217	189	32	111	102	40	34	10	16400
Kodiak Wintergreen	55.1	2100	174	200	1440	8660	1440	2540	2250	328	477	139	139	37	89	86	49	41	11	20200
Grizzly Snuff	52.7	1330	140	83	840	4230	835	975	1090	154	153	69	61	16	35	40	13	14	LOQ	10100
Grizzly Pouches Straight	27.1	1240	107	53	482	2860	535	999	1060	178	177	86	73	20	40	46	16	16	LOQ	7990
Grizzly Pouches Mint	60.7	1990	113	46	782	3840	701	785	853	112	109	48	41	12	28	27	8.9	12	LOQ	9500
Kayak Long Cut Straight	53.2	2120	56	22	609	4170	651	989	1070	139	130	64	48	11	31	30	10	13	LOQ	10200
Kayak Long Cut Mint	51.2	2270	37	26	496	3800	567	961	1090	149	148	71	56	14	34	30	11	13	LOQ	9780
Kayak Long Cut Wintergreen	52.5	NQ[c]	53	27	534	3610	607	870	910	120	114	57	41	11	27	26	7.9	11	LOQ	7020
Timber Wolf Fine Cut Natural	48.3	1530	138	156	1090	5970	1100	1940	1640	244	340	108	127	22	60	70	19	20	LOD	14600
Timber Wolf Long Cut Straight	53.6	2010	141	LOQ	1140	5700	1070	1900	1670	244	360	105	110	21	47	74	LOD[d]	LOD	6.7	14600
Timber Wolf Long Cut Wintergreen	53.8	886	153	136	1100	5600	1090	1760	1580	235	237	101	103	21	47	73	20	11	6.5	13100
Timber Wolf Fine Cut Wintergreen	54.0	1300	121	125	903	4220	850	1340	1160	169	170	78	77	16	34	57	14	8.2	LOQ	10600
Timber Wolf Long Cut Mint	53.9	1810	147	127	970	4990	1000	1610	1430	219	260	86	102	20	46	71	20	15	7.3	12900
Timber Wolf Apple	49.7	1590	122	128	897	5140	981	1820	1560	245	247	106	115	22	53	74	20	17	8.8	13100
Timber Wolf Peach	50.5	1530	96	120	841	4540	845	1520	1330	197	283	77	95	18	45	61	16	15	6.8	11600
Red Seal Natural	55.0	1860	111	153	924	5670	976	1800	1580	229	231	102	105	21	49	69	20	16	7.0	13900
Red Seal Long Cut Wintergreen	51.9	1610	89	117	781	4560	768	1350	1190	163	247	57	79	14	35	53	16	9.8	6.2	11100
Longhorn Long Cut Wintergreen	64.9	NQ	173	105	761	3890	935	1000	1140	188	175	96	70	17	40	45	12	15	LOQ	8670
Hawken Long Cut Wintergreen	25.9	1050	LOD	LOD	4.8	58	8.6	45	46	5.3	7.8	LOD	7.4	LOQ	LOQ	13	LOQ	LOQ	LOQ	1250
average for moist snuff	52.6	1730	111	105	827	4700	844	1400	1290	194	232	93	107	20	52	56	21	18	7.5	11600
STD	7.0	392	43	54	287	1570	278	537	429	71	110	35	70	6.6	24	22	12	8.3	1.9	3700
spit-free tobacco pouches																				
Marlboro Snus Rich	20.8	866	LOQ	NQ	LOQ	13.5	LOQ	9.0	9.0	1.7	1.9	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	901
Marlboro Snus Mild	11.9	722	LOQ	NQ	LOQ	9.7	LOQ	6.9	7.6	1.1	1.3	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOQ	749
Marlboro Snus Spearmint	13.4	1070	LOQ	NQ	LOQ	10.0	LOQ	5.7	7.0	LOQ	1.4	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOD	1090
Marlboro Snus Peppermint	13.0	1230	8.1	NQ	LOQ	9.4	LOQ	5.6	6.0	1.1	1.5	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOQ	1260
Camel Snus Original	31.2	1110	LOQ	LOQ	9.0	68.0	6.9	60.1	46.5	5.9	13.3	LOD	38.8	3.1	23.2	15.2	LOQ	LOQ	19.8	1430
Camel Snus Spice	31.5	1080	9.0	LOQ	11.8	79.4	8.1	59.7	45.4	5.5	9.1	LOD	30.2	3.1	LOQ	15.0	LOQ	LOQ	15.2	1380
Camel Snus Frost	30.9	1070	LOQ	LOQ	9.7	68.7	6.9	60.5	46.3	5.4	12.4	LOD	31.5	3.1	21.5	14.9	LOQ	LOQ	8.8	1360
Camel Snus Mellow	33.6	1060	LOQ	NQ	LOQ	44.8	6.1	33.0	25.3	2.5	3.2	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOD	1170
Tourney Original	17.6	1060	LOQ	17.7	5.2	36.2	LOQ	8.8	17.3	2.6	5.6	LOD	6.6	LOQ	LOQ	12.1	LOQ	LOQ	LOQ	1170
Tourney Spearmint	22.6	1130	LOQ	19.5	5.6	41.9	LOQ	23.7	28.2	5.5	9.9	LOD	9.8	3.4	LOQ	14.5	4.2	LOQ	LOQ	1300
Tourney Wintergreen	20.1	993	LOQ	20.7	5.9	34.9	LOQ	19.4	25.4	5.1	8.7	LOD	9.9	3.2	LOQ	14.0	4.0	LOQ	3.6	1150
Grand Prix Original	25.1	1100	LOQ	129	6.1	42.4	LOQ	10.9	18.5	2.9	5.1	LOD	7.6	LOQ	LOQ	13.3	LOQ	LOQ	4.2	1340
Grand Prix Spearmint	23.7	1100	LOQ	16.0	6.4	43.2	LOQ	37.0	35.4	7.1	11.1	LOD	11.9	3.6	3.2	15.0	4.4	LOQ	3.7	1300
Grand Prix Wintergreen	21.3	932	LOQ	LOQ	5.7	51.7	LOQ	36.4	37.5	7.6	11.5	LOD	12.6	4.4	2.9	15.6	LOD	LOQ	LOQ	1120
Triumph Original	54.0	1560	LOQ	LOQ	9.2	65.5	LOQ	53.1	48.5	5.3	11.7	LOD	45.1	LOQ	75.9	LOQ	LOQ	LOQ	68.7	1940
Triumph Mint	49.5	1510	LOQ	LOQ	7.4	44.4	LOQ	33.6	36.1	4.1	8.6	LOD	21.2	LOQ	32.4	5.9	LOQ	LOQ	8.6	1720
Nordic Ice Snus	21.6	1310	LOQ	NQ	2.6	36.7	5.7	15.6	17.5	3.2	3.7	3.4	2.9	LOQ	LOQ	LOQ	LOQ	LOQ	LOD	1410
average for spit-free tobacco	21.5	1110	8.5	35.4	6.3	41.2	6.7	28.2	26.9	4.0	7.1	NA[e]	19.0	2.8	23.0	12.3	4.4	LOQ	16.6	1280
STD	12.7	207	0.7	46.1	3.1	21.6	0.9	20.4	15.0	2.1	4.3	NA	14.0	1.4	26.2	4.9	0.4	NA	21.8	276

[a] For PAH abbreviations, see Table 1. [b] LOQ, detected but below the LOQ (see Table 2). [c] NQ, not quantified due to interfering peaks. [d] LOD, below the LOD (signal-to-noise ratio is less than 3 for quantitation ion). [e] NA, not applicable.

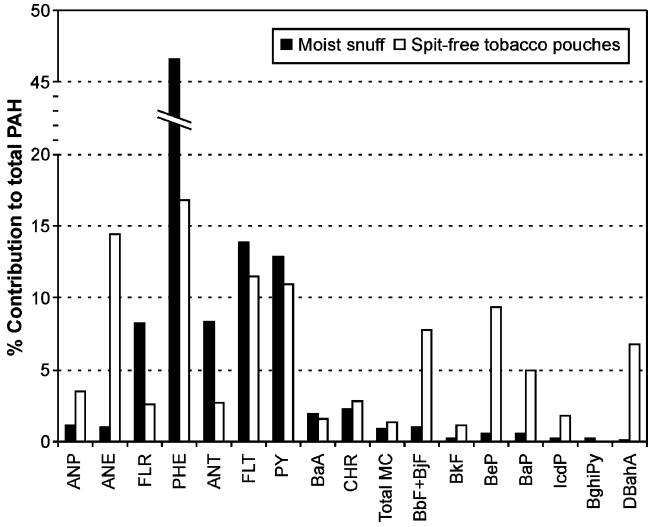


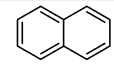
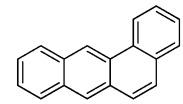
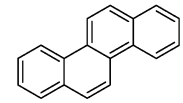
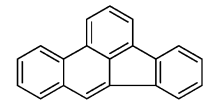
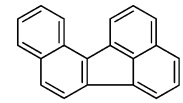
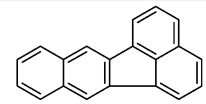
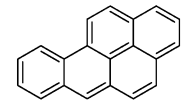
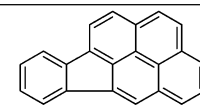
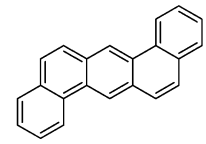
Figure 3. Comparison of average relative contributions of individual PAHs to total PAH content in moist snuff (solid bars) and spit-free tobacco pouches (open bars). NP, which accounted for 87% of total PAHs in spit-free tobacco products, is excluded from this chart. For PAH abbreviations, see Table 1.

contributions are different from those observed in cigarette smoke, where MCs are formed as a result of tobacco pyrolysis (*34*). Among the detected MCs, 3-MC is a strong tumor initiator and shows some carcinogenic activity in animal studies (*34*). The amount of 3-MC in moist snuff averaged 39.0 ng/g dry weight—comparable to an average 55.8 ng/g dry weight for BaP.

In agreement with our previous results (*21*), the levels of PAHs in spit-free tobacco pouches were very low. Similarly to Hawken, the sum of all PAHs in these products was only about 11% of the average total PAHs in moist snuff. In our previous study, we were not able to detect ANT, BbF, BkF, and BaP in most of the similar new smokeless tobacco products (*21*). In this study, probably due to the use of a more sensitive instrument and changes in the sample preparation procedure, we were able to detect these and other PAHs, including carcinogenic NP, BaA, CHR, IcdP, and DBahA, in many new spit-free tobacco samples. The sum of carcinogenic PAHs in these products averaged 1.18 μg/g dry weight, which is somewhat similar to moist snuff. However, this amount was mainly due to high NP content, which was quite uniform across the studied new brands and similar to that of moist snuff (Table 4). When NP was excluded from the calculations, the sum of the remaining carcinogenic PAHs in spitless tobacco was about 10% of that in moist snuff (0.066 vs 0.64 μg/g dry weight, respectively). Moreover, the NP content was the major contributor to the sum of all PAHs detected in newer brands, accounting for more than 80% of total PAHs; the sum of all other PAHs in this category was about 2% of that in moist snuff (0.25 vs 10.1 μg/g dry weight, respectively).

The differences in PAH levels, as well as the differing patterns in the relative contribution of individual PAHs to the total PAH content between conventional moist snuff and spit-free tobacco pouches, reflect the differences in the tobacco processing techniques used in the manufacturing of these two categories of smokeless tobacco. Tobacco used in moist snuff undergoes fire curing—the process that includes direct contact with the smoke of smoldering hardwoods, a rich source of PAHs. Moreover, the high chloride content in moist snuff (*21*) could possibly facilitate the absorption of PHE, FLT, and PY upon curing, leading to the high levels of these PAH in moist snuff. Thus, a study of atmospheric PAHs in an urban location in United Kingdom suggested that salt used to treat roads during the winter efficiently absorbs vehicular emissions of PHE, FLT, and PY (*35*). Tobacco used in the manufacturing of spit-free smokeless products is pasteurized. Because there is no direct contact with the wood smoke, the major source of contamination of these products with PAHs is probably their absorption from the air by tobacco leaves or processed tobacco. Spit-free smokeless tobacco is also low in salt content (*21*). As mentioned earlier, the only similarity between these two types of products was NP content, suggesting that the sources of NP contamination could be common for moist snuff and spit-free tobacco manufacturing.

Table 5. Carcinogenic PAHs Analyzed in U.S. Smokeless Tobacco Products

PAH	Structure	IARC group[a]
NP		2B
BaA		2B
CHR		2B
BbF		2B
BjF		2B
BkF		2B
BaP		1
IcdP		2B
DBahA		2A

[a] International Agency for Research on Cancer (IARC) classification of carcinogens: group 1, carcinogenic to humans; group 2A, probably carcinogenic to humans; and group 2B, possibly carcinogenic to humans.

In summary, our findings demonstrate that PAHs are one of the most prevalent groups of carcinogens in moist snuff and that the use of moist snuff can be considered an important source of human exposure to PAHs, along with smoking. The low amounts of PAHs in the brand Hawken and in various new spit-free smokeless brands represent direct and strong evidence that their amounts in moist snuff can be also brought to trace levels. Urgent measures are required from the U.S. tobacco industry to modify manufacturing processes so that the levels of these toxicants and carcinogens in U.S. moist snuff products are greatly reduced.

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