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Analysis of 23 polycyclic aromatic hydrocarbons in smokeless tobacco by gas chromatography-mass spectrometry

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Abstract

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 8 different polycyclic aromatic hydrocarbons (PAH) 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 PAH in smokeless tobacco after a simple two-step extraction and purification procedure. The method produced coefficients of variation under 10% for most PAH. The limits of quantitation for different PAH varied between 0.3 ng/g tobacco 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 include 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 PAH in conventional moist snuff averaged 11.6 ($\pm$ 3.7) μ g/g dry weight, 20% of this amount being comprised by carcinogenic PAH. The levels of PAH in new spit-free tobacco products were much lower than those in moist snuff, the sum of all detected PAH averaging 1.3 ($\pm$ 0.28) μ g/g dry weight. Our findings render PAH one of the most prevalent groups of carcinogens in smokeless tobacco, along with tobacco-specific nitrosamines. Urgent measures are required from the U.S. tobacco industry to modify manufacturing processes so that the levels of these toxicants and carcinogens in the U.S. moist snuff are greatly reduced.

Keywords

PAH; moist snuff; smokeless tobacco; cancer

INTRODUCTION

Investigation of toxic and carcinogenic compounds present in smokeless tobacco is a critical step towards 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 U.S. (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

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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). Since moist snuff is characterized by high moisture and salt content, its use generates excess saliva and, therefore, usually requires spitting, creating a rather unsanitary image. In recent years, the tobacco industry has been also 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 (TSNA)¹, volatile *N*-nitrosamines, volatile aldehydes, polycyclic aromatic hydrocarbons (PAH), certain lactones, urethane, metals, polonium-210 and uranium-235 and -238 (6,10,11). Among these, TSNA are commonly acknowledged as the most important group from the quantitative point of view (12,13). Over the last 2 decades, TSNA 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 PAH – 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 7 other PAH are present in U.S. smokeless tobacco, some of them at unexpectedly high levels (21). These findings inspired further investigation of the presence of PAH in smokeless tobacco products.

In this study, we developed a gas chromatography-mass spectrometry (GC-MS) method to analyze 23 PAH in smokeless tobacco. We expanded the list of PAH 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 PAH that, according to IARC, are present in cigarette smoke. The method allows analysis of all of these PAH in a single GC-MS run after a simple two-step extraction and purification procedure. PAH analyzed here include naphthalene (NP), acenaphthylene (ANP), acenaphthene (ANE), fluorine (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 (DBaA), 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.

¹Abbreviations: acenaphthene (ANE), acenaphthylene (ANP), anthracene (ANT), 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; chrysene (CHR), DBaA, dibenz[*a,h*]anthracene; fluorine (FLR), fluoranthene (FLT), GC-MS, gas chromatography-mass spectrometry; IcdP, indeno[1,2,3-*cd*]pyrene; methylchrysene (MC), naphthalene (NP), phenanthrene (PHE), pyrene (PY), TSNA, tobacco-specific nitrosamines.

MATERIALS AND METHODS

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). Triumph Snus, Grand Prix Snus, and Tourney Snus were purchased in Columbus, Ohio. Marlboro Snus was purchased in Indianapolis, Indiana, and Camel Snus and Nordic Ice were procured in Minneapolis.

Chemicals

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

Caution

Many PAH are strong toxicants and carcinogens, and should be handled with extreme care, in a well-ventilated hood and with personal protective equipment.

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). PAH were extracted by shaking 300 mg tobacco with 1 mL cyclohexane at room temperature for 3 h. The tobacco particles were removed by centrifugation and 500 µL of the extract was mixed with the ¹³C-labeled internal standard mix. The mixture was loaded on 50-mg BondElut Silica cartridges (Varian) pre-equilibrated with 1 mL cyclohexane. The cartridge was washed with 2 mL cyclohexane, and the eluants from both the load and wash were combined and concentrated under a gentle stream of N₂ to a final volume of 200 µL. The concentrated samples were transferred to amber glass microinsert vials. Three µL of the sample were analyzed by GC-MS.

GC-MS Analysis—Separation and analysis of PAH was 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, the oven temperature being programmed as follows: 15 °C/min ramp from 80 °C 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, scan width was set at *m/z* 0.100, and scan time was 0.010 sec. The instrument was operated in selected ion monitoring mode (SIM) (Table 1). The filament was turned off for the first 6 min. To assure maximum selectivity and sensitivity, the PAH were divided into 11 SIM

groups. The PAH 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 ng, 100 ng, 250 ng, and 500 ng of each PAH. A non-spiked 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 5 measurements obtained from 5 aliquots of a cyclohexane extract of Copenhagen Snuff (a representative moist snuff product). The limit of quantitation 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 3:1 signal to noise ratio for the corresponding quantitation ion.

Other Analyses

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 PAH 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 clean-up 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 ¹³C-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 co-eluted with BjF, and 4MC co-eluted with 6MC (inserts A and B in Figure 1). The latter two compounds also overlapped with the 5MC peak.

Characteristics of the method

Accuracy, precision, and limits of quantitation for all PAH are summarized in Table 3. There was a minor contribution of stable isotope-labeled PAH to the ion chromatograms of corresponding unlabeled PAH. This contribution was consistent over the range of labeled PAH concentrations from 0.05 ng/μL to 1 ng/μL, and varied from 0.03% of ¹³C₆-ANT area observed in the ANT ion chromatogram to 6.7% of ¹³C₄-BaP peak area observed in the BaP ion chromatogram. These values were subtracted from peak areas of corresponding PAH in all subsequent calculations. The difference between the PAH content measured in non-spiked 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 PAH). Analysis of 5 non-spiked aliquots of Copenhagen Snuff extract produced coefficients of variation under 10% for most PAH (Table 3). The limits of

quantitation varied between 0.3 ng/g and 10.9 ng/g tobacco, and the limits of detection varied between 0.1 ng/g 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 PAH in various smokeless tobacco products marketed and test-marketed in the U.S. 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 PAH were very similar across various brands of conventional moist snuff. Average amounts of detected PAH in these products ranged from 7.5 ng/g dry weight for DBahA to 4700 ng/g tobacco for PHE. Total MC in Table 4 represent the calculated sum of 1 MC, 3MC, 4MC, and 6MC. 5MC was not detected in any of the tested products. We also consistently observed an additional peak which, based on its spectrum and retention time (24.16 min, Figure 2), could be 2-methylchrysene (34). However, we did not have a corresponding standard to confirm its identity. The sum of all detected PAH (total PAH) in 23 samples of conventional moist snuff averaged 11639 ($\pm$ 3702) ng/g dry weight. Hawken Long Cut Wintergreen was relatively low in total PAH content – only 1253 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 PAH averaging 1281 ($\pm$ 275.7) 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; FLT content in the same products was 5.6 ng/g and 59.7 ng/g dry weight, respectively, and BaP content was below the limit of quantitation 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: 1726 ($\pm$ 392.3) ng/g dry weight in moist snuff and 1112 ($\pm$ 207.0) ng/g dry weight in the spit-free products, accounting for 15% and 87%, respectively, of average total PAH in these products. Average relative contributions of other individual PAH to the sum of 22 PAH (excluding NP) are illustrated in Figure 3.

DISCUSSION

A recent study conducted by our research team identified 8 PAH in moist snuff, encouraging further investigations of this group of toxicants and carcinogens in smokeless tobacco products. In this study, we used gas chromatography-mass spectrometry to analyze 23 different PAH in some U.S. moist snuff brands, as well as in some new spit-free smokeless tobacco products currently marketed and test-marketed in the U.S. This is the first study to report the presence of naphthalene, acenaphthene, fluorene, benz[*a*]anthracene, chrysene, 1-methylchrysene, 3-methylchrysene, 4-methylchrysene, 6-methylchrysene, benzo[*j*]fluoranthene, benzo[*e*]pyrene, indeno[1,2,3-*cd*]pyrene, benzo[*g,h,i*]perylene, and dibenz[*a,h*]anthracene in U.S. moist snuff. Our findings render PAH 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 PAH 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% were comprised of ANP, ANE, MC isomers, BbF, BjF, BkF, BaP, BeP,

IcdP, BghiPy, and DBahA. Overall, of the 22 PAH detected in moist snuff, 9 are classified by IARC as group 1, 2A, or 2B carcinogens (Table 5), their sum averaging 2.36 $\mu\text{g/g}$ dry weight, or 20% of total PAH. This is the first study to demonstrate that the sum of carcinogenic PAH in U.S. moist snuff reaches microgram per g levels. The relatively high levels of FLT and PY in moist snuff are also disturbing, as these PAH act as co-carcinogens when applied to mouse skin along with, or prior to, BaP treatment (26–28). Co-application 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). Co-carcinogenicity with BaP has been also reported for BeP and BghiPy (26,27). Some of the PAH found in smokeless tobacco, however, were reported to inhibit carcinogenicity of other PAH. Thus, NP and BaA inhibit carcinogenicity of BaP (30,31), while BeP inhibits carcinogenicity of DBahA (32).

Comparison to the levels of PAH 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 PAH in the smoke of full flavor varieties of the U.S. cigarettes Marlboro, Camel, and Newport were 1.15 $\mu\text{g/cigarette}$, 1.29 $\mu\text{g/cigarette}$, and 1.15 $\mu\text{g/cigarette}$, respectively (23). The reported PAH 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 B_jF (which could not be separated from BbF, but accounted for only about 1% of the sum) in one gram of product averaged 5.41 (± 1.66) μg . Thus, an average single portion of traditional moist snuff (1–2 g) can contain about 5 times higher amounts of the sum of these 14 PAH 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).

Among moist snuff products analyzed here, Hawken Long Cut Wintergreen is drastically different from the rest of the products, the sum of all PAH in this product being only about 11% of the average total PAH 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 PAH. 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 U.S.(33)

Of the 5 analyzed MC isomers, 5MC 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 4MC and 6MC, because these two peaks co-eluted (Figure 2). The identity of 2MC could not be confirmed because of the lack of the standard compound. The average contributions of 1MC and 3MC to the total amount of MC isomers identified in moist snuff were 40% and 42%, respectively, while the sum of 4MC and 6MC accounted for 18% of the total amount. These relative contributions are different from those observed in cigarette smoke, where MC are formed as a result of tobacco pyrolysis (34). Among the detected methylchrysenes, 3MC is a strong tumor initiator and shows some carcinogenic activity in animal studies (34). The amount of 3MC 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 PAH in spit-free tobacco pouches were very low. Similarly to Hawken, the sum of all PAH in these products was only about 11% of the average total PAH 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 PAH, including carcinogenic NP, BaA, CHR, IcdP, and DBahA, in many new spit-free tobacco samples. The sum of carcinogenic PAH in these products averaged 1.18 $\mu\text{g/g}$ dry weight, which is somewhat similar to moist snuff.

However, this amount was mainly due to high naphthalene 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 PAH in spitless tobacco was about 10 % of that in moist snuff (0.066 $\mu\text{g/g}$ dry weight vs. 0.64 $\mu\text{g/g}$ dry weight, respectively). Moreover, NP content was the major contributor to the sum of all PAH detected in newer brands, accounting for more than 80% of total PAH; the sum of all other PAH in this category was about 2% of that in moist snuff (0.25 $\mu\text{g/g}$ dry weight vs. 10.1 $\mu\text{g/g}$ dry weight, respectively).

The differences in PAH levels, as well as the differing patterns in the relative contribution of individual PAH 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 PAH. 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 PAH in an urban location in U.K. 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. Since there is no direct contact with the wood smoke, the major source of contamination of these products with PAH 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.

In summary, our findings demonstrate that PAH 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 PAH, along with smoking. The low amounts of PAH 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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Figure 1.

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

Figure 2.

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

Figure 3.

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

Table 1

Ions and ion ratios for the analyzed polycyclic aromatic hydrocarbons and corresponding internal standards.

Analyte	Abbreviation	SIM group (duration, min)	Quantitation ion (<i>m/z</i>)	Confirmation ion (<i>m/z</i>)	Ion ratio	Internal standard	Quantitation ion (<i>m/z</i>)	Confirmation ion (<i>m/z</i>)	Ion ratio	Response ratio ^a
Naphthalene	NP	1 (10.00)	128.2	127.2	6.27	¹³ C ₆ -Naphthalene	134.2	133.2	5.35	0.88
Acenaphthylene	ANP	2 (2.25)	152.1	151.1	4.54	¹³ C ₆ -Acenaphthylene	158.2	157.1	3.64	0.86
Acenaphthene	ANE	2 (2.25)	154.2	152.2	1.65	¹³ C ₆ -Acenaphthene	160.2	158.2	1.45	0.90
Fluorene	FLR	3 (1.25)	166.2	165.2	0.97	¹³ C ₆ -Fluorene	172.2	171.2	0.89	0.93
Phenanthrene	PHE	4 (2.00)	178.2	176.2	4.59	¹³ C ₆ -Phenanthrene	184.2	182.2	4.52	0.95
Anthracene	ANT	4 (2.00)	178.2	176.2	4.66	¹³ C ₆ -Anthracene	184.2	182.2	4.50	0.92
Fluoranthene	FLT	5 (3.50)	202.3	200.3	4.32	¹³ C ₆ -Fluoranthene	208.3	206.3	4.67	1.03
Pyrene	PY	5 (3.50)	202.3	200.3	4.51	¹³ C ₆ -Pyrene	205.2	203.3	2.59	1.14
Benz[<i>a</i>]anthracene	BaA	6 (4.00)	228.3	226.3	3.47	¹³ C ₆ -Benz[<i>a</i>]anthracene	234.3	232.3	3.36	0.96
Chrysene	CHR	6 (4.00)	228.3	226.3	3.12	¹³ C ₆ -Chrysene	234.3	232.3	3.04	0.84
1-Methylchrysene	1MC	7 (4.00)	242.3	239.3	2.97	¹³ C ₆ -Chrysene	234.3	232.3	3.12	1.67
3-Methylchrysene	3MC	7 (4.00)	242.3	239.3	2.98	¹³ C ₆ -Chrysene	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 -Chrysene	234.3	232.3	3.12	1.22
5-Methylchrysene	5MC	7 (4.00)	242.3	239.3	2.20	¹³ C ₆ -Chrysene	234.3	232.3	3.12	1.32
Benzo[<i>b</i>]fluoranthene + Benzo[<i>j</i>]fluoranthene	BbF, BjF	8 (2.50)	252.3	250.3	3.39	¹³ C ₆ -Benzo[<i>b</i>]fluoranthene	258.3	256.3	3.54	0.97
Benzo[<i>k</i>]fluoranthene	BkF	8 (2.50)	252.3	250.3	3.68	¹³ C ₆ -Benzo[<i>k</i>]fluoranthene	258.3	256.3	3.72	1.01
Benzo[<i>e</i>]pyrene	BeP	9 (3.50)	252.3	250.3	2.89	¹³ C ₄ -Benzo[<i>a</i>]pyrene	256.3	254.3	3.06	0.92
Benzo[<i>a</i>]pyrene	BaP	9 (3.50)	252.3	250.3	3.86	¹³ C ₄ -Benzo[<i>a</i>]pyrene	256.3	254.3	3.06	1.01
Indeno[1,2,3- <i>cd</i>]pyrene	IcdP	10 (7.00)	276.3	274.3	3.84	¹³ C ₆ -Indeno[1,2,3- <i>cd</i>]pyrene	282.3	280.4	5.01	1.08
Dibenzo[<i>a,h</i>]anthracene	DBaA	10 (7.00)	278.3	276.3	3.81	¹³ C ₆ -Dibenzo[<i>a,h</i>]anthracene	284.3	282.4	3.41	1.01
Benzo[<i>ghi</i>]perylene	BghiPy	10 (7.00)	276.3	274.3	3.68	¹³ C ₆ -Indeno[1,2,3- <i>cd</i>]pyrene	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.

Table 2

Average recovery of stable isotope-labeled polycyclic aromatic hydrocarbons used as internal standards

Internal standard	Average recovery, %
¹³ C ₆ -Naphthalene	76.9
¹³ C ₆ -Acenaphthylene	75.6
¹³ C ₆ -Acenaphthene	74.7
¹³ C ₆ -Fluorene	80.2
¹³ C ₆ -Phenanthrene	83.9
¹³ C ₆ -Anthracene	80.4
¹³ C ₆ -Fluoranthene	90.5
¹³ C ₆ -Pyrene	93.0
¹³ C ₆ -Benz[<i>a</i>]anthracene	92.4
¹³ C ₆ -Chrysene	91.0
¹³ C ₆ -Benzo[<i>b</i>]fluoranthene	96.7
¹³ C ₆ -Benzo[<i>k</i>]fluoranthene	96.0
¹³ C ₄ -Benzo[<i>a</i>]pyrene	98.4
¹³ C ₆ -Indeno[1,2,3- <i>cd</i>]pyrene	94.2
¹³ C ₆ -Dibenz[<i>a,h</i>]anthracene	88.5

Table 3

Characteristics of the method

Analyte	Accuracy	Precision	Limit of quantitation, ng/g tobacco
	%	% variation (N = 5)	
Naphthalene	109	9.93	4.8
Acenaphthylene	112	8.29	4.0
Acenaphthene	105	7.89	10.9
Fluorene	118	7.98	1.6
Phenanthrene	124	7.69	2.0
Anthracene	118	9.51	2.6
Fluoranthene	103	8.25	0.3
Pyrene	115	9.89	0.3
Benz[<i>a</i>]anthracene	109	8.03	0.9
Chrysene	108	8.15	1.0
1-Methylchrysene	109	8.05	2.0
3-Methylchrysene	110	7.02	2.0
4-Methylchrysene + 6-Methylchrysene	112	9.31	2.6
5-Methylchrysene	104	nd ^a	nd
Benzo[<i>b</i>]fluoranthene + Benzo[<i>j</i>]fluoranthene	115	6.32	1.8
Benzo[<i>k</i>]fluoranthene	118	5.72	1.6
Benzo[<i>e</i>]pyrene	113	7.70	2.1
Benzo[<i>a</i>]pyrene	109	4.47	1.6
Indeno[1,2,3- <i>cd</i>]pyrene	113	7.20	2.3
Dibenz[<i>a,h</i>]anthracene	112	10.83	3.9
Benzo[<i>g,h,i</i>]perylene	113	12.31	2.4

^a nd, not determined (not present in tobacco).

Table 4

Levels of polycyclic aromatic hydrocarbons in smokeless tobacco marketed in the U.S.

Sample content	Moisture, %	PAH ^d , ng / g dry weight																	Total PAH
		NP	ANP	ANE	FLR	PHE	ANT	FLT	Py	BaA	CHR	Total MC	BbF+BjF	BkF	BeP	BaP	Fluor	B[a]h[a]P	
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
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
Copenhagen Snuff	54.7	1860	85	112	709	4960	784	1650	1420	220	269	95	278	26	102	60	27	26	LOQ ^d
Copenhagen Long Cut	54.6	2020	96	120	878	6030	1050	1790	1490	222	293	95	186	28	70	68	31	27	LOQ
Kodiak Straight	56.2	2210	114	165	1110	6340	1070	2010	1880	346	462	217	189	32	111	102	40	34	10
Kodiak Wintergreen	55.1	2100	174	200	1440	8660	1440	2540	2250	328	477	139	139	37	89	86	49	41	11
Grizzly Snuff	52.7	1330	140	83	840	4230	835	975	1090	154	153	69	61	16	35	40	13	14	LOQ
Grizzly Pouches Straight	27.1	1240	107	53	482	2860	535	999	1060	178	177	86	73	20	40	46	16	16	LOQ
Grizzly Pouches Mint	60.7	1990	113	46	782	3840	701	785	853	112	109	48	41	12	28	27	8.9	12	LOQ
Kayak Long Cut Straight	53.2	2120	56	22	609	4170	651	989	1070	139	130	64	48	11	31	30	10	13	LOQ
Kayak Long Cut Mint	51.2	2270	37	26	496	3800	567	961	1090	149	148	71	56	14	34	30	11	13	LOQ
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
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
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
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
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
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
Timber Wolf Apple	49.7	1590	122	128	897	5140	981	1820	1560	245	247	106	115	22	53	74	20	17	8.8

Sample content	Moisture, %	PAH ^a , ng / g dry weight																	Total PAH	
		NP	ANP	ANE	FLR	PHE	ANT	FLT	Py	BaA	CHR	Total MC	BbF+BjF	BkF	BbP	BaP	FluP	BghiPy		DB[a]A
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	25.86	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	865.9	LOQ	NQ	LOQ	13.5	LOQ	9.0	9.0	1.7	1.9	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	901.0
Marlboro Snus Mild	11.9	722.4	LOQ	NQ	LOQ	9.7	LOQ	6.9	7.6	1.1	1.3	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOQ	749.0
Marlboro Snus Spearmint	13.4	1065	LOQ	NQ	LOQ	10.0	LOQ	5.7	7.0	LOQ	1.4	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOD	1091
Marlboro Snus Peppermint	13.0	1225	8.1	NQ	LOQ	9.4	LOQ	5.6	6.0	1.1	1.5	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOQ	1257
Camel Snus Original	31.2	1108	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	1428
Camel Snus Spice	31.5	1084	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	1375
Camel Snus Frost	30.9	1073	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	1363
Camel Snus Mellow	33.6	1056	LOQ	NQ	LOQ	44.8	6.1	33.0	25.3	2.5	3.2	LOQ	LOQ	LOD	LOQ	LOQ	LOQ	LOQ	LOD	1173
Tourney Original	17.6	1061	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	1173
Tourney Spearmint	22.6	1129	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	1296
Tourney Wintergreen	20.1	992.6	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	1148
Grand Prix Original	25.1	1103	LOQ	129.1	6.1	42.4	LOQ	10.9	18.5	2.9	5.1	LOD	7.6	LOQ	LOQ	13.3	LOQ	LOQ	4.2	1344
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	1298
Grand Prix Wintergreen	21.3	932.2	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	1123
Triumph Original	54.0	1561	LOQ	LOQ	9.2	65.5	LOQ	53.1	48.5	5.3	11.7	LOD	45.1	LOQ	75.9	0.0	LOQ	LOQ	68.7	1944

Sample content	Moisture, %	PAH ^a , ng / g dry weight														Total PAH			
		NP	ANP	ANE	FLR	PHE	ANT	FLT	Py	BaA	CHR	Total MC	BbF+BjF	BkF	BeP		BaP	FltP	B[a]h[Py]
Triumph Mint	49.5	1513	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
Nordic Ice Snus	21.6	1314	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
Average for spit-free tobacco	21.5	1112	8.5	35.4	6.3	41.2	6.7	28.2	26.9	4.0	7.1	3.4	19.0	2.8	23.0	12.3	4.4	LOQ	16.6
STD	12.7	207.0	0.7	46.1	3.1	21.6	0.9	20.4	15.0	2.1	4.3	na ^e	14.0	1.4	26.2	4.9	0.4	na	21.8
																			275.7

Table 5

Carcinogenic polycyclic aromatic hydrocarbons analyzed in U.S. smokeless tobacco products.

PAH	Structure
Naphthalene

PAH	Structure
Benz[<i>a</i>] anthracene
Chrysene
Benzo[<i>b</i>]fluoranthene

PAH	Structure
Benzo[j]fluoranthene
Benzo[k]fluoranthene

PAH	Structure
Benzo[<i>a</i>]pyrene
Indeno [1,2,3- <i>cd</i>]pyrene
Dibenz[<i>a,h</i>]anthracene

^aInternational Agency for Research on Cancer (IARC) classification of carcinogens: group 1 – carcinogenic to humans; group 2A – probably carcinogenic to humans; group 2B – possibly carcinogenic to humans.