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Extraction from Moist Snuff with Artificial Saliva of Benzo[*a*]pyrene and Other Polycyclic Aromatic Hydrocarbons *

by

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SUMMARY

The present study evaluated *in vitro* extractability of various polycyclic aromatic hydrocarbons (PAHs) from moist snuff, when the extracting agent was water or artificial saliva. The extraction was performed on nine brands of moist snuff samples that are commercially available and were purchased from the market in January 2018. The moist snuff brands were selected to represent brands with different tobacco cut size descriptors and flavors. For the measurement of PAHs, two different analytical methods were used, an HPLC (High Performance Liquid Chromatography) method for measuring only benzo[*a*]pyrene (BaP) and a GC/MS/MS (Gas Chromatography Tandem Mass Spectrometry) method for measuring 21 PAHs (including BaP). These methods were modifications of preexistent methods reported in the literature. The results for BaP indicated that by extracting 500 mg of freeze-dried moist snuff with 6 portions of 20 mL water (120 mL), or with 4 portions of 20 mL artificial saliva, followed by two portions of 20 mL water, the BaP remains close to 100% in the solid material and it is not detected in the extracting solution. PAHs with a molecular weight similar or heavier than BaP also showed no extractability. Lighter PAHs such as fluorene, phenanthrene, anthracene, and 5-methylanthracene showed a relatively good extractability. An intermediate group including fluoranthene, pyrene, and benz[*a*]anthracene showed some extractability in the conditions of this *in vitro* experiment. This study is not a substitute for clinical studies regarding PAH uptake in human users of moist snuff. However, the results indicate very limited bioavailability of BaP and heavier PAHs from moist snuff. Higher, but variable bioavailability was indicated for lighter PAHs. Important implications of these findings are that: 1) measurably different BaP content of two moist snuff products is unlikely to result in any meaningfully different consumer exposure to BaP; and 2) biomarkers for one PAH cannot necessarily be used as a reliable indicator of exposure to another PAH, particularly if the molecular weights of the precursor PAHs differ since their bioavailabilities can be very different. [Beitr. Tabakforsch. Int. 28 (2019) 214–223]

KEY WORDS:

Moist snuff; polycyclic aromatic hydrocarbons; PAHs; benzo[*a*]pyrene; BaP; extractability

ZUSAMMENFASSUNG

In der vorliegenden Studie wird die Extrahierbarkeit von verschiedenen polyzyklischen aromatischen Kohlenwasserstoffen (PAK) aus Moist Snuff bei Einsatz von Wasser oder künstlichem Speichel als Extraktionsmittel *in vitro* untersucht. Die Extraktion wurde an Proben neun handelsüblicher Moist-Snuff-Marken durchgeführt, die im Januar 2018 im Handel erworben wurden. Die Moist-Snuff-Marken wurden so ausgewählt, dass sie unterschiedliche

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Tabakschnittgrößendeskriptoren und -aromen repräsentierten. Zur Messung der PAK wurden zwei verschiedene Analysemethoden angewandt: Die HPLC-Methode (Hochleistungsflüssigkeitschromatographie) wurde ausschließlich zur Messung von Benzo[*a*]pyren verwendet und die GC/MS/MS-Methode (Gaschromatographie mit Tandem-Massenspektrometrie) wurde zur Messung von 21 PAK (einschließlich BaP) eingesetzt. Diese Methoden waren Variationen bereits existierender, aus der Literatur bekannter Methoden. Die Ergebnisse für BaP ergaben, dass bei der Extraktion von 500 mg gefriergetrocknetem Moist Snuff mit sechs Portionen Wasser zu je 20 mL (120 mL) bzw. mit vier Portionen künstlichem Speichel zu je 20 mL, gefolgt von zwei Portionen Wasser zu je 20 mL, das BaP zu beinahe 100% im Feststoff bleibt und nicht in die Extraktionslösung übergeht. Bei PAK mit einem Molekulargewicht, das gleich oder höher als bei BaP war, konnte ebenfalls keine Extrahierbarkeit beobachtet werden. Leichtere PAK wie Fluoren, Phenanthren, Anthracen und 5-Methylanthracen zeigten eine relativ gute Extrahierbarkeit. Eine Zwischengruppe mit Fluoranthen, Pyren und Benz[*a*]-anthracen zeigte eine gewisse Extrahierbarkeit unter den Bedingungen dieses *In-Vitro*-Experiments. Diese Studie ersetzt nicht klinische Studien mit menschlichen Konsumenten zur Aufnahme von PAK aus Moist Snuff. Die Ergebnisse weisen jedoch auf eine sehr begrenzte Bioverfügbarkeit von BaP und schwereren PAK aus Moist Snuff hin. Eine höhere, jedoch variable Bioverfügbarkeit ergab sich bei leichteren PAK. Aus diesen Ergebnissen lassen sich folgende wichtige Implikationen ableiten: 1) Es ist unwahrscheinlich, dass ein messbar unterschiedlicher Gehalt an BaP bei zwei verschiedenen Moist-Snuff-Produkten zu einem bedeutsamen Unterschied bei der Exposition der Konsumenten gegenüber BaP führen wird; und 2) Biomarker für einen PAK können nicht notwendigerweise als verlässlicher Indikator für die Exposition gegenüber einem anderen PAK verwendet werden, insbesondere dann nicht, wenn die Molekulargewichte der Ausgangsstoffe sich unterscheiden, da ihre Bioverfügbarkeiten sehr unterschiedlich sein können. [Beitr. Tabakforsch. Int. 28 (2019) 214–223]

RESUME

La présente étude évalue l'extractibilité *in vitro* de divers hydrocarbures aromatiques polycycliques (HAP) présents dans le tabac à priser humide, avec pour agent extracteur, de l'eau ou de la salive artificielle. L'extraction fut réalisée sur des échantillons prélevés sur neuf marques de tabac à priser humide disponibles dans le commerce et commercialisées sur le marché en janvier 2018. Les marques de tabac à priser humide furent sélectionnées dans le souci d'inclure des marques présentant différents descripteurs de coupes et d'arômes du tabac. En vue de mesurer les HAP, deux méthodes analytiques distinctes furent utilisées, une HPLC (chromatographie en phase liquide à haute performance) pour la mesure du seul benzo[*a*]pyrène (BaP) et une GC/MS/MS (chromatographie en phase gazeuse couplée à une spectrométrie de masse en tandem) pour la mesure des 21 HAP (y compris le BaP). Ces méthodes furent des adaptations d'approches préexistantes relayées dans la littérature. Les résultats pour le BaP indiquèrent qu'en extrayant 500 mg de tabac à priser humide lyophilisé à l'aide de 6 portions de 20 mL d'eau (120 mL) ou de 4 portions de 20 mL de salive artificielle, suivies de deux portions de 20 mL d'eau, la teneur en BaP demeurait proche des 100% dans la matière solide, sans détectable transfert du BaP dans la solution d'extrait. Pour les HAP dont le poids moléculaire était similaire ou supérieur au BaP, aucune extractibilité ne fut observée. Les HAP plus légers tels que le fluorène, le phenanthrène, l'anthracène et le 5-méthylanthracène affichèrent une extractibilité relativement bonne. Un groupe intermédiaire incluant le fluoranthène, le pyrène et le benz[*a*]anthracène présentèrent un certain degré d'extractibilité dans les conditions de cet expérimentation *in vitro*. Cette étude ne se substitute point aux études cliniques consacrées à l'absorption des HAP chez les humains consommant du tabac à priser humide. Toutefois, les résultats indiquèrent une biodisponibilité très limitée du BaP et des HAP plus lourds présents dans le tabac à priser humide. Une biodisponibilité plus élevée, quoique variable, fut observée dans le cas des HAP plus légers. Les implications importantes de ces observations sont multiples: 1) La teneur en BaP distincte et quantifiée de deux produits de tabac à priser humide n'est pas susceptible de donner des degrés d'exposition au BaP différents et significatifs pour les consommateurs et 2) les biomarqueurs pour un HAP ne peuvent pas nécessairement servir d'indicateurs fiables d'exposition à un autre HAP, tout particulièrement si les poids moléculaires des HAP précurseurs diffèrent puisque leurs biodisponibilités peuvent être très différentes. [Beitr. Tabakforsch. Int. 28 (2019) 214–223]

1. INTRODUCTION

Moist snuff is typically made using a tobacco blend that includes fire cured tobacco. Since fire cured tobacco is made by exposing the tobacco to smoke obtained from low smoldering fires of hardwood, it contains several polycyclic aromatic hydrocarbons (PAHs). As a result, moist snuff contains several PAHs (1–3), including benzo[*a*]pyrene (BaP) which is listed as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) (4). BaP is also listed as one of the nine harmful and potentially harmful constituents (HPHC) of smokeless tobacco products on which Food & Drug Administration (FDA) requires reporting (5). The distribution of PAHs within the moist snuff matrix is not known. The transfer of BaP and other PAHs from moist snuff to a human user, is likely to involve an extraction process from the snuff matrix into saliva. In order to better understand this process, an *in vitro* study was performed to evaluate the extraction of BaP from several brands of moist snuff into water or into artificial saliva. Following the study on BaP, the extraction of other PAHs was also evaluated. Results of the present study indicate very limited bioavailability of BaP and of other PAHs from moist snuff. Since other pathways of PAHs adsorption by the moist snuff users are possible (ingestion, contact with oral mucosa), present results are not intended to be a substitute for clinical studies regarding PAHs transfer to human. However, the findings raise important implications in that context.

For the evaluation of the extraction of BaP and of other PAHs from moist snuff to water or artificial saliva, two methods for measuring these compounds were utilized. One method was a modification of a procedure for BaP analysis by LC (Liquid Chromatography) with fluorescence detection (6). The other method was a modification of a GC/MS/MS technique utilized for the analysis of PAHs (3, 7) in smokeless tobacco products.

An initial attempt was made to measure BaP in the extract of a sample of moist snuff with water. However, the results indicated that the BaP level in the water extract was below the detection limit of the analytical method, indicating that the extraction was extremely weak. For this reason, the study was performed by measuring the levels of PAHs in the solid moist snuff material before and after the extraction with water or with artificial saliva.

2. MATERIALS AND METHOD

2.1. Materials

The EPA 610 Polynuclear Aromatic Hydrocarbons Mix solution of standards was obtained from Supelco (Bellefonte, PA, USA). In addition, other PAHs were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Seven deuterated PAHs were obtained from Isotech Lab. Inc., (Champaign, IL, USA). Other chemicals and solvents were also obtained from Sigma-Aldrich. Artificial saliva Cat. No. 1700-0305 was purchased from Pickering Laboratories (Mountain View, CA, USA). This artificial saliva had an adjusted pH but did not contain enzymes. It should be indicated that human saliva does not contain cytochromes P450 known to oxidize BaP and the absence in the artificial saliva of enzymes (such as α-amylase present in human saliva) was not considered important for the purpose of the study. Bond Elut-CH, 500 mg, 3 mL SPE cartridges were purchased from Agilent (Wilmington, DE, USA). Autovial PVDF filters with 0.45 mm pore size were purchased from Whatman (Florham Park, NJ, USA). Nine moist snuff samples that are commercially available were purchased from the market in January 2018, and kept in a freezer at −20 °C during the experiments.

2.2. Instrumentation

Two different methods were used in the study, an HPLC method for measuring only BaP and a GC/MS/MS method for measuring 21 PAHs (including BaP). The HPLC analysis with fluorescence detection was performed on a 1260-1290 HPLC system from Agilent. The HPLC was equipped with a Zorbax Eclipse XDB-C18 column, 4.6 × 250 mm 5 µm, also from Agilent. For assuring lack of interferences in HPLC analysis of BaP, during the validation step of the analytical procedure, a PAH C18 column, 2 × 250 mm 5 µm from Waters (Milford, MA, USA) was also used. The GC/MS/MS analysis was performed on a 7890B/7000C GC/MS/MS system from Agilent equipped with a 60 m × 0.25 mm, 0.25 µm film ZB-PAH column from Phenomenex (Torrance, CA, USA). A freeze-dryer FreeZone 4.5 from Labconco (Kansas City, MO, USA) was used for freeze-drying the moist snuff samples. The moisture of samples was also measured using a HE53 Moisture Analyzer (Mettler Toledo GmbH, Greifensee, Switzerland).

2.3. HPLC method for BaP analysis

The moisture content of the moist snuff samples was initially measured using the HE53 Moisture Analyzer. After that, about 4–5 g of moist snuff was freeze-dried from each brand. The weight of the samples was measured before and after freeze-drying, and the same moisture contents as using the Moisture Analyzer were obtained. For the HPLC analysis 100 ± 0.01 mg of dried moist snuff was precisely weighed in 4-mL screw cap vials. To each vial 1.5 mL methanol was added as an extracting solvent. The moist snuff was extracted for 30 min at 78 °C with occasional agitation. After cooling at room temperature, the solution from each extract was filtered through a 0.45 mm pore size PVDF filter and placed in 2-mL screw cap HPLC autosampler vials. The HPLC separation used a Zorbax Eclipse XDB-C18, 4.6 × 250 mm, 5 µm particle column. The separation was obtained using gradient starting with 15% water and 85% acetonitrile for 0.5 min, then to 100% acetonitrile at 8 min (linear), holding at 100% acetonitrile for 5 min. At 13 min the eluant was returned to initial composition over 0.5 min and held for column equilibration for another 1.5 min (total run time 15 min). The detection was done using fluorescence with excitation at 378 nm and emission at 405 nm. The injection volume was 20 µL. Photomultiplier gain for the Agilent 1290 FLD was set at 10. A typical chromatogram of a moist snuff extract is shown in Figure 1.

For quantitation, a calibration curve was generated using a set of six standard solutions with BaP concentrations between 2.17 ng/mL and 69.31 ng/mL in methanol. The calibration was linear with an equation used for measuring the unknown concentrations of the form:

$$\textit{Conc.}\ (\text{ng/mL}) = 0.19109 \times \text{Peak area} + 0.30952 \qquad [1]$$

The R^2 value for the linear calibration was 0.99954.

A summary validation for the analytical procedure was performed. In order to assure the method selectivity, an additional column (Waters PAH C18, 2 × 250 mm 5 µm) was connected in series with the Zorbax Eclipse XDB-C18 column, and the conditions for the chromatographic separation were modified starting with 20% water and 80% acetonitrile for 0.5 min, then to 100% acetonitrile at 16 min (linear), holding at 100% acetonitrile for 3 min. At 19 min the eluant was returned to initial composition over 0.5 min and held for column equilibration for another 2.5 min (total run time 22 min). No differences were detected in the BaP peak areas for several samples of extracted moist snuff. Also, the separate injection of a benzo[*e*]pyrene (BeP) sample showed that this compound does not interfere with BaP measurement. Precision of the procedure was also demonstrated to be very good, with relative standard deviations (RSD%) for standards lower than 3% and for samples run in triplicate lower than 5%. The linearity of calibration is also very good as the correlation coefficient R^2 of the calibration very close to unit. A determination of LOD (level of detection) was not performed, all the analyzed

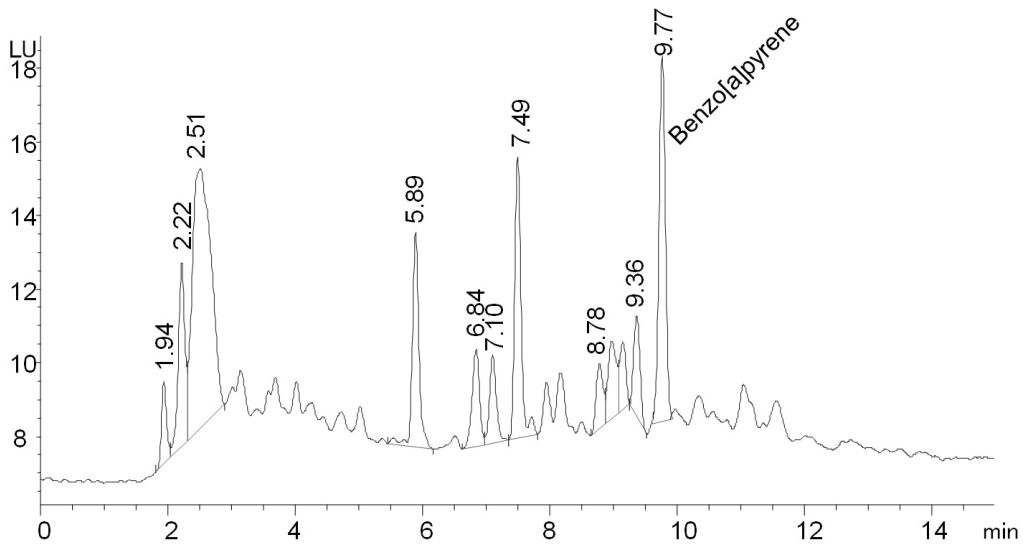

Figure 1. Chromatogram of a moist snuff extract showing the peak of BaP.

samples containing a BaP level were within the limits of calibration standards. Extraction efficiency was also verified. This was done by two procedures. One was adding 10 ng of BaP in solution on a moist snuff sample and calculate the recovery. This recovery was between 98% and 106% (for three replicates). The other was extending the extraction time of the samples from 30 min to 1 h and 2 h. No difference in the level of the detected BaP was noticed (within 5% RSD) for three different moist snuff samples.

2.4. GC/MS/MS method of PAH analysis

The GC/MS/MS analysis utilized for PAHs measurement was based on two methods previously published (3, 7) on which were made several modifications (8–13). The PAHs determined by this procedure included: naphthalene (NP), acenaphthylene (ANY), acenaphthene (ANE), fluorene (FLR), phenanthrene (PHE), anthracene (ANT), 5-methyl-anthracene (5-Me-ANT), fluoranthene (FLT), pyrene (PYR), benz[*a*]anthracene (BaA), chrysene (CHR), 5-methyl-chrysene (5-Me-CHR), 6,12-di-Me-chrysene (6,12-diME-CHR), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*e*]pyrene (BeP), benzo[*a*]pyrene (BaP), perylene (PER), indeno[1,2,3-*cd*]pyrene (IN1,2,3-cdPY), dibenzo[*a,h*]anthracene (DBahA), and benzo[*g,h,i*]perylene (BghiPE). Seven internal standards added to the samples were also measured, including: d8-naphthalene (d8-NP), d10-acenaph-thene (d10-ANE), d10-fluorene (d10-FLR), d10-pyrene (d10-PYR), d12-chrysene (d12-CHR), d14-dibenzo[*a,h*]anthra-cene (d14-DBahA), and d12-benzo[*g,h,i*]perylene (d12-BghiPE). The sample preparation for the GC/MS/MS analysis was as follows: 150 ± 0.01 mg of dried moist snuff or 300 ± 0.01 mg moist snuff (as is) was precisely weighed in 4-mL screw cap vials. To each vial 3.0 mL methanol was added as an extracting solvent and 45 µL I.S. (Internal standard). The moist snuff was extracted for 30 min at 78 °C with occasional agitation. After cooling at room temperature, the solution from each extract was filtered through a 0.45-mm pore size PVDF filter and placed into a test tube. From this solution 2 mL were further transferred into another test tube. To the 2 mL methanol sample solution, 3.71 mL water was added such that the ratio water/methanol was 65/35 (*v/v*). The whole content of the turbid solution in water/methanol was passed through a 500 mg, 3 mL SPE Bond Elut-CH cartridge under mild vacuum on a manifold. The cartridge was previously conditioned with 2 mL of methanol and then twice with 2 mL of water/methanol 65/35 (*v/v*). After the retention of PAHs on the SPE cartridge, this was rinsed three times with 2 mL water/methanol 65/35 (*v/v*) and dried for 20 min with air on the manifold. The sample was eluted with two portions of 0.75 mL cyclohexane. This solution was submitted for the GC/MS/MS analysis.

The GC separation for the GC/MS/MS analysis was performed on a 60 m × 0.25 mm, 0.25 mm film ZB-PAH column. The GC conditions are described in Table 1.

The detection of the analytes was performed in electron impact ionization positive (EI+) mode at 70 eV. The ion source temperature was set at 230 °C and both quadrupoles temperatures were set at 150 °C. A solvent delay of 10 min was employed. Helium quench gas was used at 4 mL/min with the N_2 collision gas turned off. The PAHs were monitored in "pseudo" MRM mode, in which both precursor and product ions are the same. Table 2 lists the PAHs along with their observed retention times and characteristic quantifying ions. The dwell time for all ions was 150 ms, and the resolution for both MS1 and MS2 was unity.

The quantitation of various PAHs utilized a set of 9 standards obtained by consecutive dilution to half concentrations starting with a stock solution. The stock solution contained:

1007 ng/mL NP	1268 ng/mL CHR
2014 ng/mL ANY	99.9 ng/mL 5-Me-CHR
1012 ng/mL ANE	100.1 ng/mL 6,12-diMe-CHR
1147.5 ng/mL FLR	200.2 ng/mL BbF
8569.6 ng/mL PHE	101.5 ng/mL BkF
1910.7 ng/mL ANT	101.5 ng/mL BaP
1168.2 ng/mL 5-Me-ANT	83.1 ng/mL PER
2497.2 ng/mL FLT	100.6 ng/mL IN1,2,3-cdPY
2298.6 ng/mL PYR	195.5 ng/mL DBahA
1515.3 ng/mL BaA	200 ng/mL BghiPE

Table 1. GC operating parameters.

Parameter	Description	Parameter	Description
Initial oven temperature	100 °C	Purge flow	30 mL/min
Initial time	2.0 min	Inlet temperature	280 °C
Oven ramp rate	6 °C/min	Injection volume	4.0 mL
Final oven temperature	320 °C	Carrier gas	Helium
Final time	23.33 min	Flow mode	Constant flow
Total run time	62 min	Flow rate	1.1 mL/min
Inlet type	Split/splitless	Nominal initial pressure	22.71 psi
Inlet mode	Pulse splitless	Average flow velocity	27.675 cm/sec
Pulse pressure	30 psi	Outlet pressure	Vacuum
Pulse time	1.0 min	Total flow	34.1 mL/min
Purge flow time	1.2 min	Transfer line heater	290 °C

Table 2. MS/MS operating parameters.

Time segment (min)	Compounds	Internal Standard	Retention time (min)	Precursor > product ion	Gain	Collision Energy (EV)
10.0 – 15.0	d8-NP	-	12.716	136.0 > 136.0	1	10
	NP	d8-NP	12.804	128.0 > 128.0	1	10
15.0 – 21.0	ANY	d10-ANE	19.560	152.0 > 152.0	1	5
	d10-ANE	-	19.929	164.0 > 164.0	1	5
	ANE	d10-ANE	20.085	154.0 > 154.0	1	5
21.0 – 24.0	d10-FLR	-	21.963	176.0 > 176.0	1	5
	FLR	d10-FLR	22.108	166.0 > 166.0	1	5
24.0 – 28.0	PHE	d10-FLR	26.680	178.1 > 178.1	1	3
	ANT	d10-FLR	26.799	178.1 > 178.1	1	3
28.0 – 30.5	5-Me-ANT	d10-PYR	29.326	192.1 > 192.1	1	3
30.5 – 36.0	FLT	d10-PYR	31.873	202.1 > 202.1	1	3
	d10-PYR	-	33.052	212.1 > 212.1	1	3
	PYR	d10-PYR	33.136	202.1 > 202.1	1	3
36.0 – 39.5	BaA	d10-PYR	38.067	228.1 > 228.1	1	3
	d12-CHR	-	38.311	240.1 > 240.1	1	3
	CHR	d12-CHR	38.435	228.1 > 228.1	1	3
39.5 – 41.2	5-Me-CHR	d12-CHR	40.170	242.1 > 242.1	1	3
41.2 – 44.5	6,12-diMe-CHR	d12-CHR	41.950	256.2 > 256.2	2	2
	BbF	d12-CHR	43.266	252.2 > 252.2	2	2
	BkF	d12-CHR	43.395	252.2 > 252.2	2	2
44.5 – 50.0	BeP	d12-CHR	45.340	252.2 > 252.2	2	0.5
	BaP	d12-CHR	45.897	252.2 > 252.2	2	0.5
	PER	d12-CHR	46.743	252.2 > 252.2	2	0.5
50.0 – 56.0	IN1,2,3-cdPY	d14-DBahA	54.864	276.2 > 276.2	2	2
	d14-DBahA	-	54.364	292.2 > 292.2	2	2
	DBahA	d14-DBahA	54.742	278.2 > 278.2	2	2
56.0 – 62.0	d12- BghiPE	-	58.378	288.2 > 288.2	2	2
	BghiPE	d12- BghiPE	58.714	276.2 > 276.2	2	2

To each 1-mL standard solution was added 20 µL of I.S. (Internal standard) corresponding to 200 ng/mL of each I.S., and all concentrations were corrected to the change from 1000 µL to 1020 µL. The quantitation of BeP was done based on BaP calibration. The concentrations for individual compounds in the standards were selected to bracket the expected levels in the moist snuff extracts. All utilized calibration curves were quadratic. Although the deviation from linearity was small for all analytes (except for phenanthrene), a quadratic calibration was found to fit better the calibration points. All samples contained the PAHs in the calibration range.

Several aspects of the GC/MS/MS method were evaluated toward their validation. The first aspect was the selectivity of the method and lack of interferences. There was no interference regarding the standards, the method allowing excellent chromatographic separation between the pairs: PHE-ANT, FLT-PYR, BaA-CHR, or between BbF-BkF-BeP-BaP-PER, compounds that have the same product ion so that the MS did not offer selectivity. The only question remained the interference from the matrix of the moist snuff that contains numerous compounds, not all could be

eliminated by the SPE cleanup procedure. To confirm the absence of interferences, a limited number of runs was performed on moist snuff samples using the transition 57 > 43. The precursor ion 57 with the product 43 is common for numerous compounds including hydrocarbons, but it is absent in PAHs. The absence of ion m/z = 43 for each PAH was an indication that there was no interference from the matrix. Precision of the study was demonstrated by the low RSD% obtained by analyzing triplicate of both standards and samples. The RSD% values for standards were typically below 3% (except for the last two levels of dilution) and for samples RSD% was typically below 7–8%. The limit of quantitation (LOQ) and limit of detection for the method were not calculated, but the signal to noise *S/N* ratios were measured for all analytes using the lowest standards. LOQ values were all (except for perylene) below the concentration of the lowest standards, as indicated by the following *S/N* ratios: NP (99.9), ANY (181.7), ANE (61.7), FLR (167.5), PHE (190.4), ANT (132.8), 5-Me-ANT (64.8), FLT (74.9), PYR (48.3), BaA (50.9), CHR (43.6), 5-Me-CHR (33.4), 6,12-diMe-CHR (39.4), BbF (202.7), BkF (80.4), BeP (ND), BaP (48.0), PER (9.8) , IN1,2,3-cdPY (54.3), DBahA (103.8), BghiPE (66.0). The S/N > 10 for all the analytes (*S/N* = 10 for PER) indicated that all the quantitations are possible for levels as low as the lowest standard. Recovery of PAHs by extraction with methanol has been verified for BaP as previously described, and other studies also showed the complete extractability of PAHs in methanol (12, 13).

2.5. Procedure for the attempt to evaluate BaP levels in the water extract of moist snuff

The extraction of the moist snuff with the goal of measuring the BaP level in the extract was performed using a continuous extraction setup shown in Figure 2.
A sample of moist snuff was placed in a glass column with frit and stopcock. The outlet of the column was introduced in a container that was partially filled with 5 mL CH_2Cl_2 and with water.

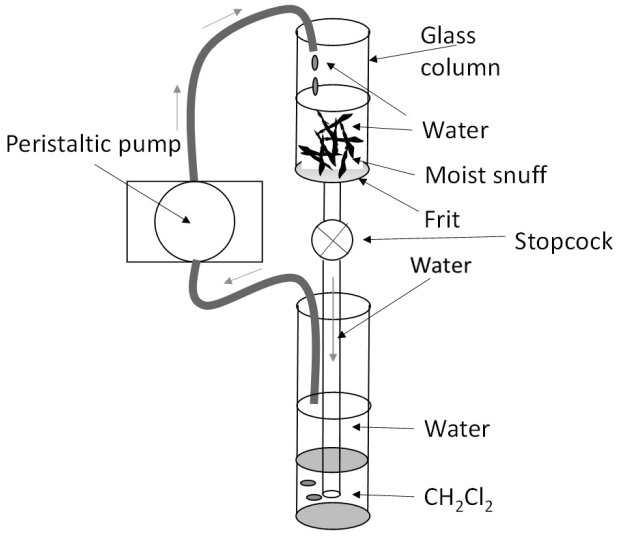

Figure 2. Setup for continuous washing the moist snuff with water and BaP extraction in CH_2Cl_2.

Using a peristaltic pump, about 20 mL water was recirculated for 24 h to wash the moist snuff. The water percolated through 5 mL of CH_2Cl_2. The flow rate of the water was set at 10 mL/min. After 24 hours, the volume of water washing the moist snuff was about 14.4 L. The CH_2Cl_2 was expected to extract the BaP from the water that percolated through the solvent. An aliquot of 1 mL CH_2Cl_2 was evaporated and the residue was re-dissolved in 1 mL methanol. The methanol solution was analyzed for BaP. The experiment was performed either on 5g moist snuff (as is) or on a sample of 5 g moist snuff spiked with 10 µg BaP.

2.6. Procedure for the evaluation of extraction efficiency of PAHs from moist snuff

The PAHs levels were first measured in the freeze-dried tobacco (result *Xi*) (*i* indicates a specific PAH). After that, extraction was performed on 500 mg of freeze-dried tobacco with 6 portions of 20 mL water (120 mL), or with 4 portions of 20 mL artificial saliva, followed by two portion of 20 mL water. The volumes of water and/or artificial saliva used for extraction were selected in large excess to those typically present in the human mouth (14). For extraction, the samples were shaken manually with the 20 mL liquid for 5 min before filtering. The extraction with water was performed at room temperature (~24 °C). For the extraction with artificial saliva, the saliva was heated at 37 °C before the extraction, but no precautions were taken to maintain this temperature during the extraction. After washing the moist snuff with 80 mL artificial saliva, two portions of 20 mL water were used to continue the washing and to eliminate the residual saliva from the samples. The resulting material (extracted moist snuff) was freeze-dried and weighed (result *W*). The level of PAHs remaining in the freeze-dried extracted moist snuff was measured (result *Yi*). The recovery after washing was calculated using the following formula:

$$Recovery\ (i)\ \% = \frac{W \times Yi}{500 \times Xi} \times 100 \qquad [2]$$

This procedure is schematically described for BaP in Figure 3.
The utilization of drying of the initial moist snuff and of the extracted snuff was necessary for the calculation of recovery, in order to relate the PAHs content to the solid part of the moist snuff before and after extraction. Part of the solids are water soluble and are extracted in water or saliva such that $W < 500$ mg.

3. RESULTS AND DISCUSSION

3.1. Moist snuff samples evaluated in the study and their moisture

The moist snuff samples evaluated in this study are listed in Table 3. The same table indicates the moisture of the samples. The moisture level was obtained by the weight difference between the sample (as is) and after freeze-drying.

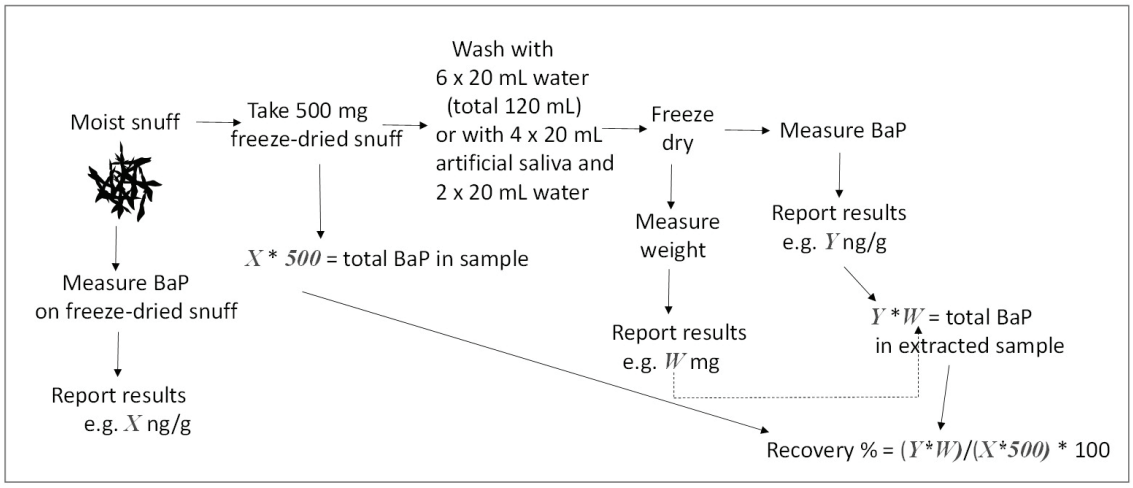

Figure 3. Work flow for the evaluation of extraction efficiency of BaP from moist snuff.

Table 3. Moist snuff samples and their moisture content.

No.	Moist snuff type	Moisture (%)
1	Red Man fine cut (natural)	49.23
2	Red Seal fine cut (natural)	53.69
3	Skoal wintergreen long cut	54.25
4	Longhorn fine cut (natural)	46.21
5	Grizzly fine cut (natural)	49.95
6	Grizzly wintergreen long cut	50.32
7	Kodiak wintergreen	50.16
8	Copenhagen fine cut (original)	49.05
9	Copenhagen mint long cut	54.09

3.2. Analysis of BaP in the moist snuff water extract

The moist snuff utilized for the evaluation of BaP in the extract was Kodiak wintergreen. The total amount of BaP in the sample was measured to 384.8 ng. The concentration of BaP in 1 mL methanol for 100% transfer was calculated to be about 77.0 ng/mL. The result measured by the HPLC procedure was below detection limit and the experiment was performed twice. The moist snuff was further spiked with 10 µg BaP for a total content of 10384.8 ng. The measured concentration of BaP in 1 mL methanol was 6.74 ng/mL corresponding to 33.7 ng BaP recovered from the initial amount of 5 g. This indicated that about 0.32% of BaP was transferred from the moist snuff to the CH_2Cl_2 solution. The extracted moist snuff was transferred to 20 mL scintillation vials and extracted with 10 mL methanol for 30 min. The amount of BaP measured in the sample was 9.94 µg. This level was equivalent to 95.7% recovery of the BaP. The recovery was very close to 100%, although a perfect mass balance was not possible. The result indicated that BaP from the moist snuff was not extracted by the water washing. Only one experiment was performed on continuous extraction with water of moist snuff spiked with BaP.

3.3. Levels of BaP in moist snuff measured by the HPLC procedure

The levels of benzo[*a*]pyrene (BaP) measured by the HPLC procedure in each sample reported in ng/g dry material (dry weight basis) are given in Table 4. All samples were analyzed in duplicate.

Table 4. Level of BaP ng/g on a dry weight basis (values for *X*) in the analyzed moist snuff samples.

No.	Moist snuff type	BaP * (ng/g)	RSD (%)
1	Red Man fine cut (natural)	61.80	1.28
2	Red Seal fine cut (natural)	95.78	0.62
3	Skoal wintergreen long cut	109.31	0.39
4	Longhorn fine cut (natural)	62.02	0.77
5	Grizzly fine cut (natural)	77.72	0.53
6	Grizzly wintergreen long cut	148.15	0.27
7	Kodiak wintergreen	154.42	0.49
8	Copenhagen fine cut (original)	89.28	0.98
9	Copenhagen mint long cut	87.68	1.26

* dry weight basis (*X*)

3.4 Results for BaP in moist snuff washed with water

The results for the BaP measured in the moist snuff washed with 120 mL water (six portions of 20 mL) are given in Table 5. The table indicates the dry-weight residual *W* after washing the initial 500 mg freeze-dried snuff, the levels *Y* of BaP measured in the washed and dried material, as well as the recovery for BaP. The loss of weight after washing is caused by the dissolution of soluble components from the moist snuff. The recoveries were calculated using formula [2] with *X* values from Table 4.

As shown in Table 5, the recovery percentage of BaP from the moist snuff samples after washing with water is around 100% (96.3% to 109.6%), indicating that a negligible amount of BaP can be extracted from the moist snuff with water.

3.5. Results for BaP in moist snuff washed with artificial saliva (and water)

The results for the BaP measured in the moist snuff washed with 80 mL saliva (four portions of 20 mL) and 40 mL water (two portions of 20 mL) are indicated in Table 6.

Table 5. Weight after washing (dry) (values for *W*), level of BaP on a dry-weight basis for the snuff samples washed with water (values for *Y*), and % recoveries after washing.

No.	Moist snuff type washed with 120 mL water	Residual weight (mg) (*W*)	BaP (ng/g) *	RSD (%)	Recovery (%)
1	Red Man fine cut (natural)	259.9	119.74	0.07	100.72
2	Red Seal fine cut (natural)	302.18	152.64	0.40	96.32
3	Skoal wintergreen long cut	265.39	213.82	2.44	103.82
4	Longhorn fine cut (natural)	270.74	122.02	0.11	106.52
5	Grizzly fine cut (natural)	261.17	160.94	0.62	108.17
6	Grizzly wintergreen long cut	277.14	291.23	0.08	108.96
7	Kodiak wintergreen	260.85	324.47	0.67	109.62
8	Copenhagen fine cut (original)	263.31	170.81	0.71	100.76
9	Copenhagen mint long cut	281.42	169.88	0.63	109.05

* dry weight basis (*Y*)

Table 6. Weight after washing (dry) (values for *W*), level of BaP on a dry-weight basis for the snuff samples washed with saliva and water (values for *Y*), and % recoveries after washing.

No.	Moist snuff type washed with 80 mL saliva and 40 mL water	Residual weight (mg) (*W*)	BaP (ng/g) *	RSD (%)	Recovery (%)
1	Red Man fine cut (natural)	260.51	119.73	0.85	100.94
2	Red Seal fine cut (natural)	292.69	174.04	0.45	106.37
3	Skoal wintergreen long cut	274.08	215.11	0.93	107.87
4	Longhorn fine cut (natural)	249.41	125.03	0.16	100.56
5	Grizzly fine cut (natural)	286.03	145.63	0.01	107.20
6	Grizzly wintergreen long cut	277.33	287.45	0.13	107.62
7	Kodiak wintergreen	250.84	333.18	0.24	108.24
8	Copenhagen fine cut (original)	261.98	169.38	0.48	99.41
9	Copenhagen mint long cut	265.48	171.03	0.32	103.57

* dry weight basis (*Y*)

The table indicates the dry weight residual *W* after washing, the levels *Y* of BaP measured in the washed and dried material, as well as the calculated recoveries for BaP.
As shown in Table 6, the recovery percentage of BaP from the moist snuff samples after washing with saliva and water is also around 100% (99.4% to 108.3%), indicating that a negligible amount of BaP can be extracted from the moist snuff with artificial saliva.

3.6. Extension of extraction study to other PAHs

The same type of measurements as those for BaP were performed for a number of PAHs present in moist snuff. The only difference was that the PAHs measurement was carried out using the GC/MS/MS method. The PAHs evaluated regarding extractability in water or in artificial saliva (and water) did not include naphthalene (NP), acenaphthylene (ANY), and acenaphthene (ANE). The levels measured for these compounds in the freeze-dried moist snuff were very low in comparison with the levels reported in the literature (3). The analysis of moist snuff samples before freeze-drying, also showed higher levels of NP, ANY and ANE as compared to those measured in the freeze-dried material. On the other hand, the analysis of the other PAHs (which involves extraction) generated the same results for sample analyzed before freeze-drying (and corrected for the moisture content) and after freeze-drying. It was concluded that during the freeze-drying process NP, ANY and ANE are partially lost by evaporation, while the extraction and analysis of the other PAHs is not affected. The levels of BaP measured by the GC/MS/MS method were in very good agreement with those obtained by the LC/fluorescence method. Also, the values obtained for the levels of various PAHs were in good agreement with the levels reported in the literature (3). The recovery percentages for different PAHs after extraction with 120 mL water or with 80 mL artificial saliva and 40 mL water as obtained using formula [2] are indicated in Figure 4.
The results shown in Figure 4 indicate that basically there is no difference between the extraction of moist snuff with water and the extraction with artificial saliva. The extraction efficiency is different for lighter PAHs as compared to that of heavier ones. While compounds such as fluorene, phenanthrene, anthracene, and 5-methylanthracene showed a relatively good extractability, compounds such as benzo[*a*]pyrene, dibenz[*a,h*]anthracene, etc., were not extracted at any measurable level. For BaP this result was a confirmation of the same result obtained separately using a different method of analysis. An intermediate group including fluoranthene, pyrene, and benz[*a*]anthracene showed some extractability in the conditions of this *in vitro* experiment. The recovery percentages results showed relatively large differences between different moist snuff samples. These differences are caused by multiple factors including analytical results variability, and possibly differences in the matrix composition of different types of

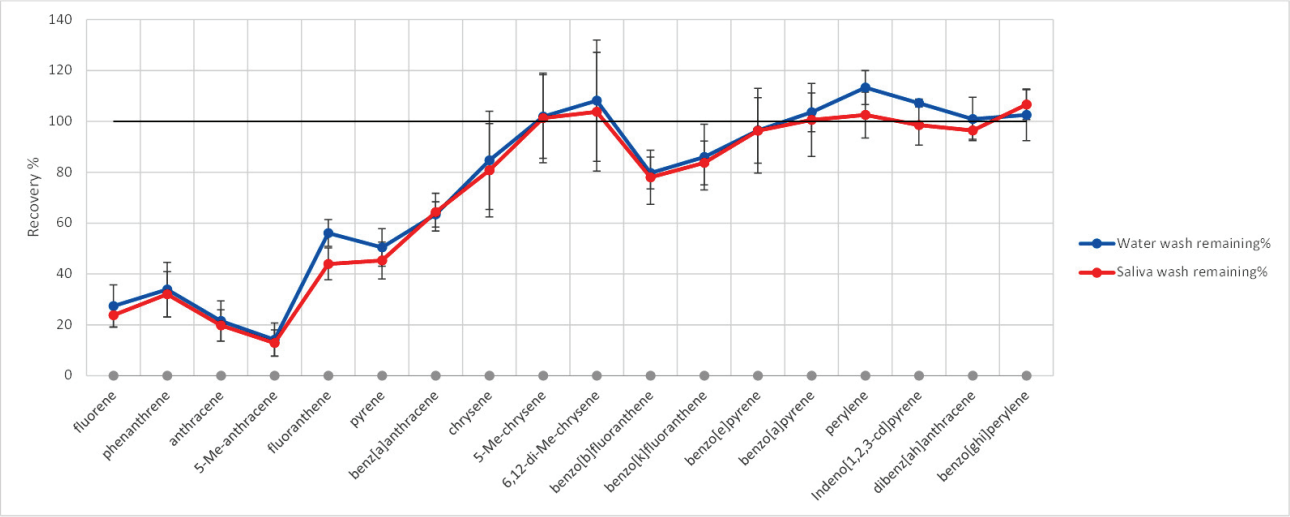

Figure 4. Recoveries % for different PAHs after extraction with 120 mL water or with 80 mL artificial saliva and 40 mL water. The error bars indicate relative % differences obtained between different types of moist snuff samples.

snuff that may influence in part the PAHs extractability. The study did not attempt any correlation of *in vitro* extractability with the results from clinical studies regarding bioavailability of PAHs from moist snuff. However, these results should be used as cautionary information in that context as the biomarker for one PAH cannot necessarily be used as a biomarker for another PAH (particularly as the molecular weights of the precursor PAHs differ), since their bioavailabilities can be very different.

4. CONCLUSIONS

The present study evaluated *in vitro* extractability of various PAHs from moist snuff, when the extracting agent is water or artificial saliva. The results indicated that no difference can be seen between the extraction with water and the extraction with artificial saliva. The extraction efficiency was higher for lighter PAHs while the heavier PAHs seemed to not be extracted at all in the conditions of the experiment.

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