



Extraction of benzo[a]pyrene from moist snuff with water or artificial saliva (Part 2)

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Background

- Benzo[a]pyrene (BaP) is one of the 7 harmful/potentially harmful constituents (HPHC) reported to the FDA for smokeless tobacco products
- BaP is present in moist snuff at levels between 50 and 200 ng/g (dry basis).
- In order to be transferred to a human user, BaP must be extracted first into saliva, but BaP has very low solubility in water.
- A previous study analyzed the remaining BaP in moist snuff after extraction with water or with artificial saliva [1].
- The results showed that about 100% of BaP remains in the moist snuff extracted with water or saliva, but the results had a variability of about $\pm 10\%$.
- The present study is focused on the measurement of BaP transferred to water and artificial saliva during extraction of moist snuff samples.
- The results of the present study are not intended to be a substitute for clinical studies regarding PAHs transfer to human users from moist snuff.

[1] S. C. Moldoveanu, J. W. Marshall, T. H. Poole, *Beitr. Tabak. Intern.* 28 (5) (2019) 214-223.

Improvements in the extraction procedure

- The previous study used artificial saliva purchased from Pickering Laboratories (Cat. No. 1700-0305). This was replaced with complete artificial saliva containing mucin and enzymes [2] that better simulates natural saliva.
- Extraction was performed for 1 hour at 37 °C, using a mechanical agitator. This provides better control of the extraction temperature and extends the extraction time which was 30 min. in the previous study.
- Additional improvements were made to the analytical method, notably by using an internal standard in the determinations.

[2] Chou, C. C., and Hee, S. S. *Environ. Toxicol. Chem.*, 13, (1994) 1177-86.

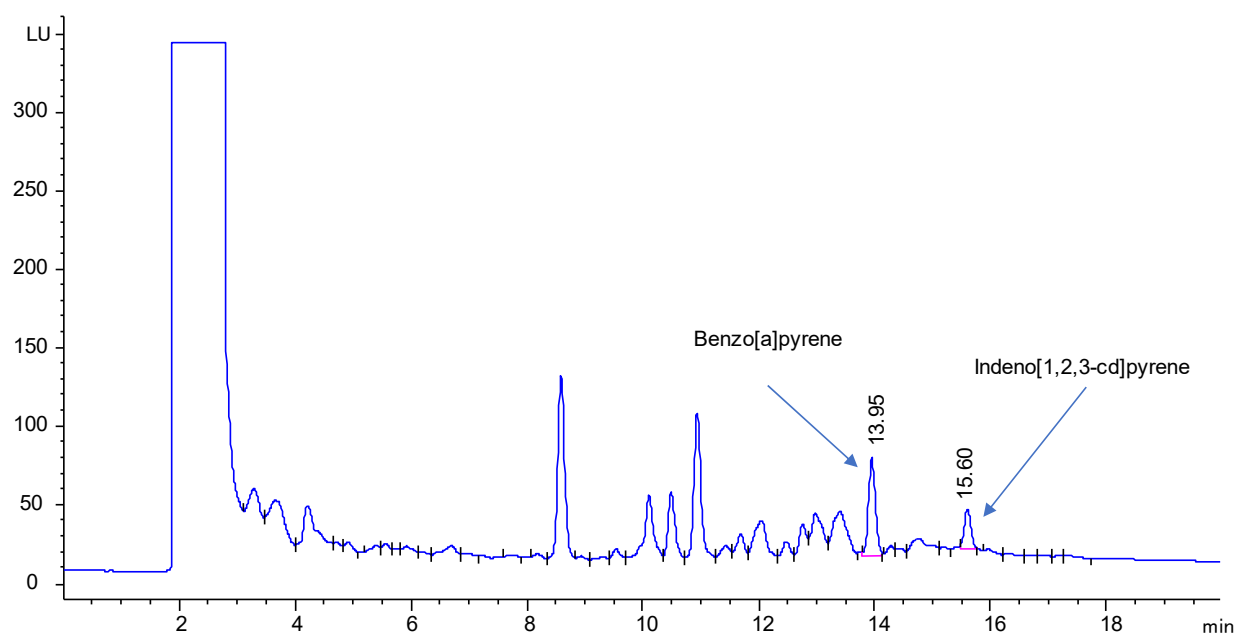
Analytical method summary for BaP measurement in moist snuff

- 500 ± 0.01 mg of moist snuff (as is) was precisely weighed in a 20 mL scintillation vial and extracted with 5 mL methanol for 30 min with a wrist action shaker.
- The methanol solution contained 200 ng/mL indeno[1,2,3-cd]pyrene as internal standard (I.S.)
- The solution from each extract was filtered through a $0.45 \mu\text{m}$ pore size PVDF filter and placed in 2 mL screw cap HPLC autosampler vials.
- The analysis of the extract was performed using an HPLC technique with fluorescence detection.
- The HPLC system was a 1290 Series instrument from Agilent (Wilmington, DE 19808).

Analytical method summary for BaP measurement in moist snuff (cont.)

- Separation was performed on a Zorbax Eclipse XDB-C18 column, 4.6 x 250 mm 5 Micron, from Agilent.
- The gradient for the mobile phase started with 25% water and 75% acetonitrile for 0.5 min, then to 100% acetonitrile at 12.0 min (linear), holding at 100% acetonitrile for 6 min. At 18 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 20 min).
- Detection of BaP was accomplished using fluorescence with excitation at 378 nm and emission at 405 nm and after 15 min the excitation was changed to 370 nm and emission to 460 nm for the detection of indeno[1,2,3-cd]pyrene.
- The injection volume was 20 μ L.
- Photomultiplier gain for the Agilent 1290 FLD was set at 13.

Typical chromatogram of a moist snuff sample



Extraction with water or artificial saliva of moist snuff

- 5.0 + 0.05 g of moist snuff was precisely weighed in 250 mL flasks.
- 100 mL water or 100 mL artificial saliva was added to the flask.
- The flasks were placed in a water bath at 37 °C with rotary agitation capability and the samples were extracted for 1 hour. The agitation was performed at 50 rpm.
- The water/saliva solutions were filtered and placed in separatory funnels.
- To the solution in a funnel was added 5 mL cyclohexane, 20 µL I.S. solution in methanol containing 10 µg/mL indeno[1,2,3-cd]pyrene, and 5 mL saturated solution of NaCl that was used for salting out effect.
- The funnels were agitated for 15 min on a wrist action shaker.
- After the water/cyclohexane layers were separated, the cyclohexane layer was placed in a 20 mL scintillation vial.
- Cyclohexane was evaporated at 65 °C under a mild current of air until dry (about 20 min).
- The evaporated samples were re-dissolved in 1 mL methanol by placing the vials in a sonic bath for about 1 min.
- The generated solution in methanol was filtered through 0.45 µm PVDF filters and placed in 2 mL GC vials. These samples were analyzed by the same HPLC procedure as used for moist snuff analysis.

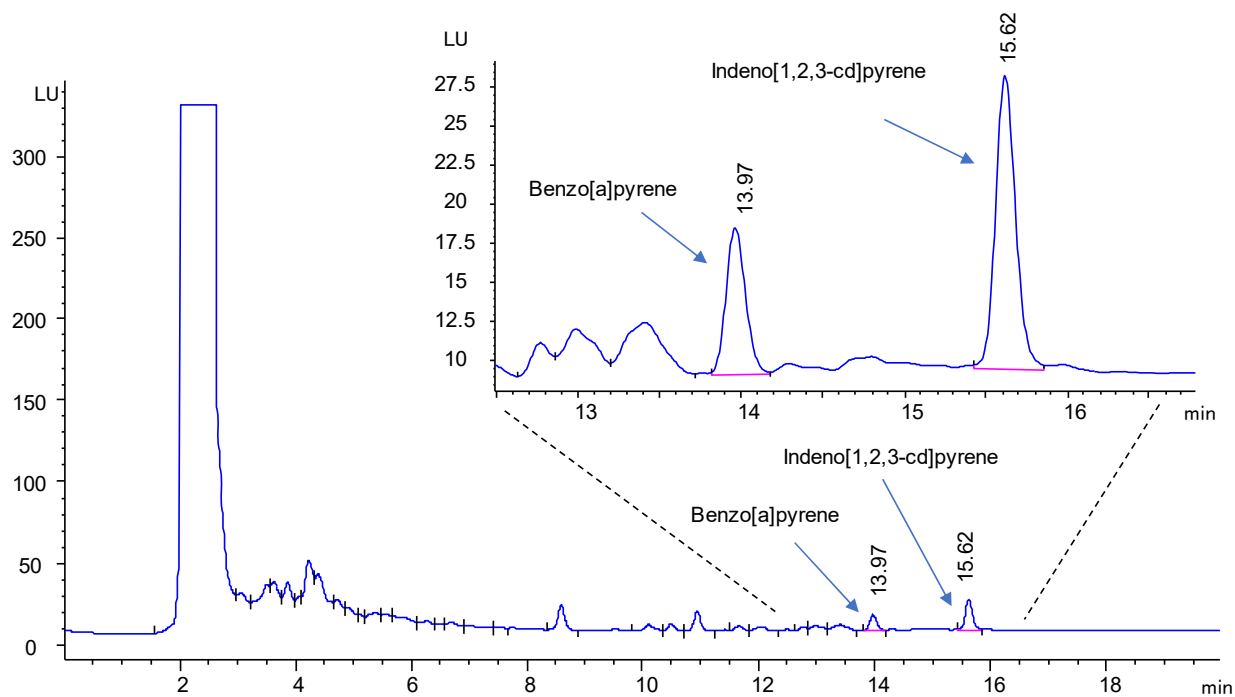
Composition of artificial saliva [2]

| Component | Source | Content in 1 L water * |
|------------------|---------------------------------|------------------------|
| Potassium | Potassium chloride | 0.95 g/L |
| Sodium | Sodium chloride | 1.4 g/L |
| Calcium | Calcium chloride dihydrate | 0.37 g/L |
| Magnesium | Magnesium chloride hexahydrate | 0.21 g/L |
| Phosphate | Di-potassium hydrogen phosphate | 0.84 g/L |
| Urea | Urea | 0.09 g/L |
| Glucose | D-(+)-Glucose | 0.2 g/L |
| Mucin | Gastric Mucin III | 2.7 g/L |
| Alpha-amylase | | 100,000 units/L |
| Lysozyme | | 750 units/L |
| Acid phosphatase | | 4 units/L |

*Note: The solution was filtered before the addition of mucin and enzymes.

[2] Chou, C. C., and Hee, S. S. *Environ. Toxicol. Chem.*, 13, (1994) 1177-86.

Typical chromatogram of a BaP analysis in the water extract of moist snuff



Recovery study for a water sample spiked with BaP

| Added BaP ng | Analyzed BaP ng | Recovery % |
|--------------|-----------------|------------|
| 5.0 | 5.104 | 102.09% |
| 5.0 | 5.048 | 100.96% |
| 5.0 | 4.954 | 99.08% |
| 0.156 | 0.162 | 104.04% |
| 0.156 | 0.147 | 94.04% |
| 0.156 | 0.157 | 100.58% |

Moist snuff samples evaluated in this study and their moisture %.

| | Moist snuff type | Moisture % | RSD% |
|---|--------------------------------------|------------|------|
| 1 | Kodiak Premium Wintergreen * | 50.05 | 0.42 |
| 2 | Grizzly Long Cut Premium Wintergreen | 50.74 | 2.62 |
| 3 | Grizzly Fine Cut Premium Wintergreen | 50.35 | 0.97 |
| 4 | Copenhagen Snuff Original Fine Cut | 53.19 | 2.11 |
| 5 | Copenhagen Long Cut Mint | 54.02 | 1.65 |
| 6 | Skoal Long Cut Classic Wintergreen | 53.60 | 1.40 |
| 7 | Red Seal Natural Fine Cut | 53.56 | 0.04 |
| 8 | Long Horn Fine Cut Natural | 50.70 | 0.00 |
| 9 | Red Man Moist Snuff Fine Cut Natural | 47.13 | 1.23 |

*Note: The first eight moist snuff samples were purchased from the market in May 2019 and stored at ambient conditions. The Red Man Fine Cut Natural sample was purchased in January 2018 and was kept in a freezer at -20 °C.

Levels of BaP ng/g on a dry weight basis for moist snuff

| | Moist snuff type | BaP ng/g | RSD% |
|---|--------------------------------------|----------|------|
| 1 | Kodiak Premium Wintergreen | 152.82 | 0.82 |
| 2 | Grizzly Long Cut Premium Wintergreen | 121.09 | 0.50 |
| 3 | Grizzly Fine Cut Premium Wintergreen | 85.98 | 1.61 |
| 4 | Copenhagen Snuff Original Fine Cut | 83.50 | 1.89 |
| 5 | Copenhagen Long Cut Mint | 59.63 | 2.80 |
| 6 | Skoal Long Cut Classic Wintergreen | 99.11 | 0.59 |
| 7 | Red Seal Natural Fine Cut | 73.88 | 0.48 |
| 8 | Long Horn Fine Cut Natural | 45.82 | 1.10 |
| 9 | Red Man Moist Snuff Fine Cut Natural | 65.60 | 2.42 |

Levels of BaP ng/g (snuff) detected in 100 mL water extract from 5 g moist snuff.

| | Moist snuff type | BaP ng/g | RSD% |
|---|--------------------------------------|----------|------|
| 1 | Kodiak Premium Wintergreen | 2.08 | 3.39 |
| 2 | Grizzly Long Cut Premium Wintergreen | 1.22 | 4.63 |
| 3 | Grizzly Fine Cut Premium Wintergreen | 1.45 | 7.42 |
| 4 | Copenhagen Snuff Original Fine Cut | 1.36 | 5.16 |
| 5 | Copenhagen Long Cut Mint | 0.81 | 6.78 |
| 6 | Skoal Long Cut Classic Wintergreen | 1.29 | 2.40 |
| 7 | Red Seal Natural Fine Cut | 0.78 | 1.32 |
| 8 | Long Horn Fine Cut Natural | 0.78 | 2.67 |
| 9 | Red Man Moist Snuff Fine Cut Natural | 0.83 | 1.10 |

Percent BaP extracted in 100 mL water from the initial level in moist snuff (5 g)

| | Moist snuff type | % BaP from initial using water extraction |
|---|--------------------------------------|---|
| 1 | Kodiak Premium Wintergreen | 1.36 |
| 2 | Grizzly Long Cut Premium Wintergreen | 1.01 |
| 3 | Grizzly Fine Cut Premium Wintergreen | 1.69 |
| 4 | Copenhagen Snuff Original Fine Cut | 1.62 |
| 5 | Copenhagen Long Cut Mint | 1.36 |
| 6 | Skoal Long Cut Classic Wintergreen | 1.30 |
| 7 | Red Seal Natural Fine Cut | 1.05 |
| 8 | Long Horn Fine Cut Natural | 1.70 |
| 9 | Red Man Moist Snuff Fine Cut Natural | 1.27 |

Levels of BaP ng/g (snuff) detected in 100 mL artificial saliva extract from 5 g moist snuff

| | Moist snuff type | BaP ng/g | RSD% |
|---|--------------------------------------|----------|------|
| 1 | Kodiak Premium Wintergreen | 3.65 | 0.70 |
| 2 | Grizzly Long Cut Premium Wintergreen | 2.63 | 1.42 |
| 3 | Grizzly Fine Cut Premium Wintergreen | 2.49 | 1.94 |
| 4 | Copenhagen Snuff Original Fine Cut | 2.79 | 2.35 |
| 5 | Copenhagen Long Cut Mint | 1.69 | 4.09 |
| 6 | Skoal Long Cut Classic Wintergreen | 2.00 | 4.66 |
| 7 | Red Seal Natural Fine Cut | 2.02 | 3.77 |
| 8 | Long Horn Fine Cut Natural | 1.79 | 2.96 |
| 9 | Red Man Moist Snuff Fine Cut Natural | 2.46 | 2.97 |

Percent BaP extracted in 100 mL artificial saliva from the initial level in moist snuff (5 g)

| | Moist snuff type | % BaP from initial using artificial saliva extraction |
|---|--------------------------------------|---|
| 1 | Kodiak Premium Wintergreen | 2.39 |
| 2 | Grizzly Long Cut Premium Wintergreen | 2.17 |
| 3 | Grizzly Fine Cut Premium Wintergreen | 2.89 |
| 4 | Copenhagen Snuff Original Fine Cut | 3.34 |
| 5 | Copenhagen Long Cut Mint | 2.83 |
| 6 | Skoal Long Cut Classic Wintergreen | 2.02 |
| 7 | Red Seal Natural Fine Cut | 2.73 |
| 8 | Long Horn Fine Cut Natural | 3.92 |
| 9 | Red Man Moist Snuff Fine Cut Natural | 3.75 |

Conclusions

- A previous study on BaP extraction from moist snuff with water or artificial saliva indicated that a large part of the BaP remains in the solid material and it is not extracted in the liquid.
- The results regarding the remaining BaP in the solid extracted moist snuff had a variability of about $\pm 10\%$ which did not allow for a precise evaluation of how much BaP was extracted.
- In the new study, artificial saliva was made to better mimic real saliva.
- The extraction of BaP in 100 mL water from 5 g moist snuff was between 0.78 to 2.08 ng/g, which represented between 1.0% to 1.7% from the initial level of BaP in the moist snuff samples.
- Extraction of BaP in 100 mL artificial saliva from 5 g moist snuff was between 1.69 to 3.65 ng/g, which represented between 2.0% to 3.9% from the initial level of BaP.
- Processing with artificial saliva increased BaP extraction approximately two-fold, although the level of BaP in the extract still remained very low (less than 4%).