Characterization of Smokeless Tobacco Products Extracted with Different Solvents for In Vitro Testing

Jingjie Zhang,[1] Utkarsh Doshi,[1] Jyoti Thaikoottathil,[1*]
Russell L. Wolz,[2] K. Monica Lee[1]

[1]Altria Client Services LLC, Richmond, VA 23219, USA
[2]Enthalpy Analytical, Richmond, VA
*Former employee

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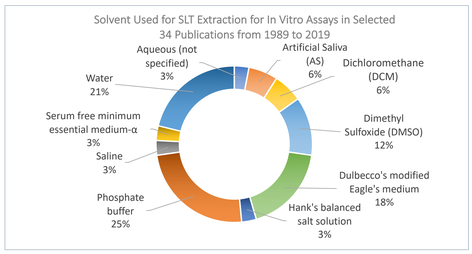
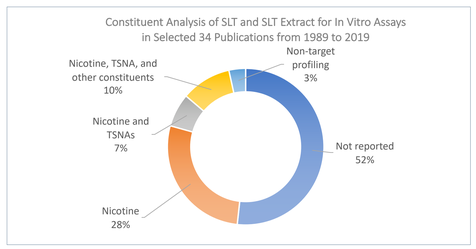
ABSTRACT

Unlike cigarettes, there are no standardized methods available for preparing extracts from smokeless tobacco (SLT) products for in vitro toxicological evaluation. Methods are available for Harmful and Potentially Harmful Constituents (HPHCs) characterization but they often differ from methods for in vitro studies in which limited types of solvents can be used. Additionally, the extracts tested in vitro are not typically characterized for constituent levels, making it difficult to interpret the observed response. The purpose of this study was to characterize extracts from two CORESTA reference SLT products: CRP1.1 (Swedish style snus pouch) and CRP 2.1 (American-style loose moist snuff) using solvents that are routinely used for in vitro testing (ethanol, DMSO, and artificial saliva). We compared the extraction efficiency of each solvent based on selected analytes (nicotine, tobacco-specific nitrosamines (TSNAs), nitrate, and benzo[a]pyrene (B[a]P)). Reference products were first characterized with analyte-specific methods. Nicotine and TSNAs in CRP 1.1 and CRP 2.1 and B[a]P in CRP 2.1 were generally comparable with literature values, while B[a]P in CRP 1.1 (0.5 ng/g) was lower than the CORESTA-reported value of 0.7 ng/g. The reference products were then extracted at up to 20% w/v concentration in each solvent for 2 hours at 37°C or 24 hours at ambient temperature, and the extraction efficiency was reported as percent recovery compared to the analytical reference values. In general, the percent recovery of analytes ranged from 55-103% for different solvents. This study suggests that characteristics of extracts prepared for in vitro studies are dependent on the extraction method. Therefore, it is imperative that appropriate test article characterization should accompany any in vitro toxicological evaluation of SLT products.

INTRODUCTION



There are no standardized methods available for preparing extracts from SLT products for in vitro toxicological evaluation.



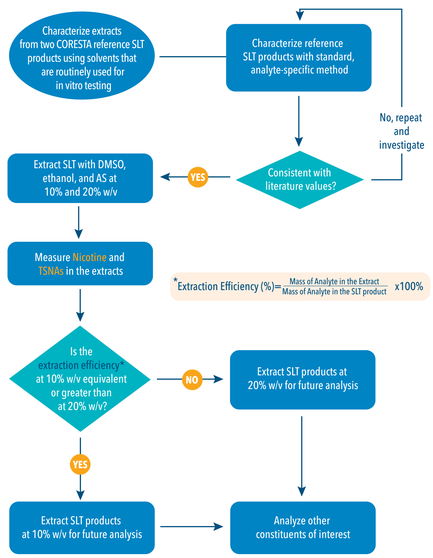
The extracts tested in vitro are not typically characterized for constituent levels.

METHODS

- **Test Articles: two reference SLT products**
  - -CRP1.1 (Swedish style snus pouch)
  - -CRP 2.1 (American-style loose moist snuff)
- **Solvent**
  - -Ethanol, DMSO, and AS
- **Extraction Condition**
  - -2 hr at 37°C
  - -24 hr at ambient temperature
  - -10% w/v vs. 20% w/v

Constituents of Interest	
TSNAs	NNK, NNN, NAB, NAT
B[a]P	Benzo[a]pyrene
Nitrate	Nitrate
Nicotine	Nicotine

METHODS



CONCLUSIONS

- We first measured selected constituents (nicotine, TSNAs, B[a]P, and nitrate) in CRP1.1 and CRP2.1 using standard methods. The results were overall consistent with literature values.
- Solvents commonly used in in vitro assays (DMSO, AS, and ethanol) were used to extract CRP1.1 and CRP2.1. The extraction efficiency of the selected constituents varied with different solvents, constituents, and extraction conditions, ranging from 55% to 103%.
- This study suggests that characteristics of extracts prepared for in vitro studies are dependent on the extraction method. Therefore, it is imperative that appropriate test article characterization should accompany any in vitro toxicological evaluation of SLT products.

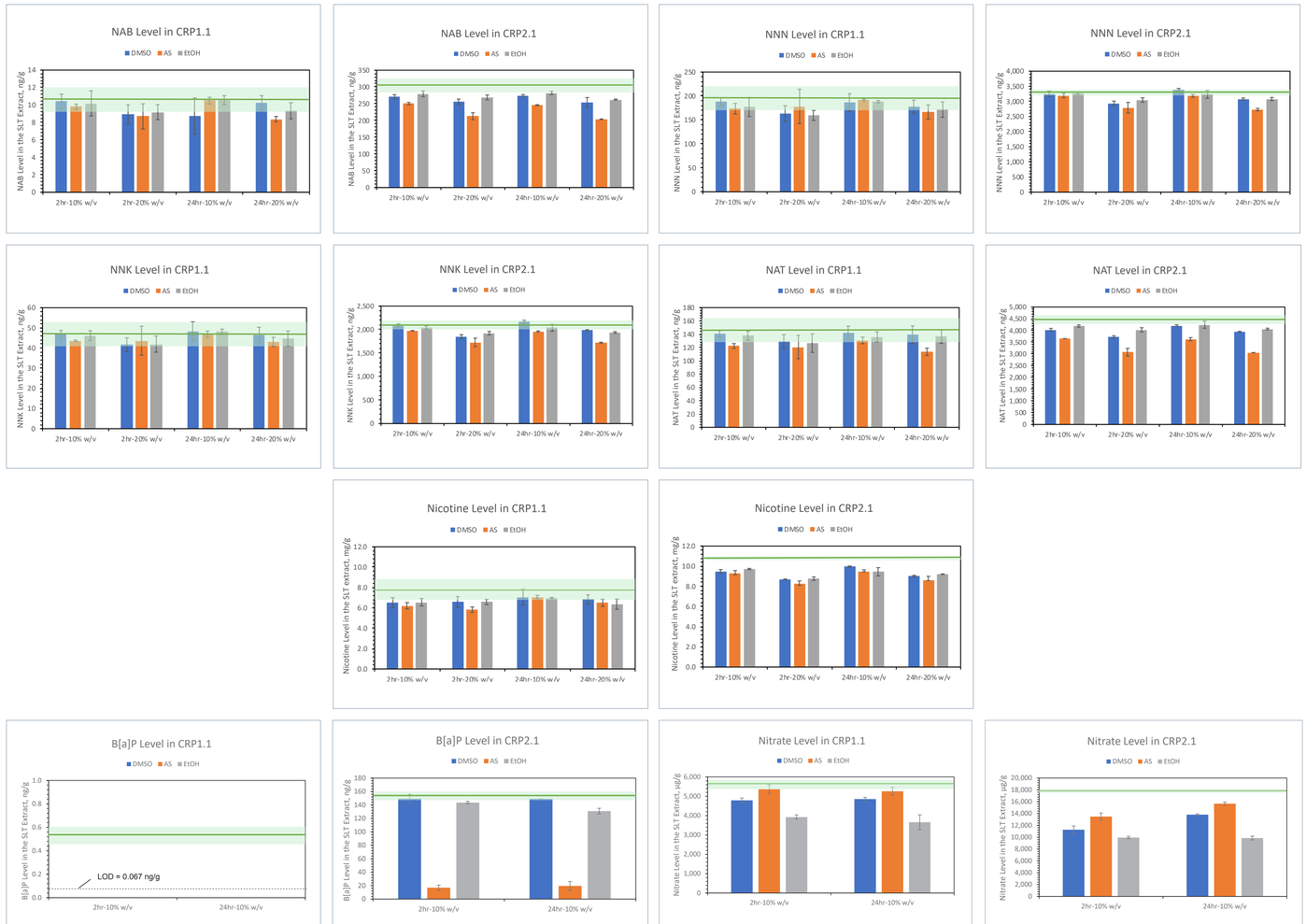
MAJOR REFERENCES

1. Smokeless tobacco sub-group technical report: CORESTA reference products 2016 analysis. 2017. CORESTA. https://www.coresta.org/sites/default/files/technical_documents/main/STS-105-CTR_2016-CRP-AnalysisWG4_Jan2017.pdf
2. Wan, J., Johnson, M., et al. Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol Biomarkers Prev. 2009;18(12): 3263-3304

RESULTS

Analysis of selected constituents extracted with DMSO, AS, and ethanol and comparison to the standard methods

Green Line: Mean values of selected constituents analyzed with the standard methods | Green Shade: Mean ± 1 SD (N = 3)



Analysis of selected constituents with standard methods and comparison to the literature

Constituents	CRP1.1 (CORESTA, 2017)[a]	CRP1.1 Analyzed at Enthalpy Analytical[b]	CRP2.1 (CORESTA, 2017)[c]	CRP2.1 Analyzed at Enthalpy Analytical[b]
Nicotine (mg/g)	7.6[d]	7.7 ± 0.4	10.7[d]	10.9 ± 0.02
Total TSNAs (NNN, NNK, NAT, NAB)[c]	0.391	0.400	9.950	10.17
NNN (ug/g)	0.19	0.196 ± 0.012	3.391	3.308 ± 0.039
NNK (ug/g)	0.052	0.046 ± 0.003	2.059	2.098 ± 0.039
NAT (ug/g)	0.14	0.146 ± 0.009	4.237	4.460 ± 0.080
NAB (ug/g)	0.009	0.011 ± 0.001	0.265	0.305 ± 0.010
B[a]P (ng/g)	0.716	0.532 ± 0.033	143.9	154.9 ± 2.6

[a]Mean as reported.
[b]Mean ± 1 SD (standard deviation); 3 replicates.
[c]The sum of means of NNN, NNK, NAT, and NAB.
[d]Originally reported as 0.762% and 1.069%, respectively.

Percent recovery of analytes compared to standard method (10% [w/v] extraction) (all values in %)

CRP1.1				
Analytes	Extraction Time	DMSO	AS	Ethanol
Nicotine	2 hr	84.5	80.6	85.0
	24 hr	91.6	91.8	90.6
NAT	2 hr	96.2	83.6	94.1
	24 hr	97.5	89.3	92.9
NAB	2 hr	97.7	91.9	95.6
	24 hr	82.0	98.6	98.9
NNK	2 hr	99.9	93.0	98.4
	24 hr	102.8	99.9	102.5
NNN	2 hr	95.8	88.4	90.3
	24 hr	95.2	97.4	95.9
B[a]P	2 hr	< LOD	< LOD	< LOD
	24 hr	< LOD	< LOD	< LOD
Nitrate	2 hr	85.1	95.3	69.7
	24 hr	86.3	93.4	65.1

CRP2.1				
Analytes	Extraction Time	DMSO	AS	Ethanol
Nicotine	2 hr	87.0	84.9	89.1
	24 hr	91.2	86.9	86.6
NAT	2 hr	90.5	82.0	94.0
	24 hr	94.3	81.4	95.3
NAB	2 hr	88.7	82.1	91.9
	24 hr	90.2	80.6	92.6
NNK	2 hr	99.5	94.2	97.5
	24 hr	103.3	93.4	97.1
NNN	2 hr	98.3	96.6	98.9
	24 hr	102.4	96.2	97.7
B[a]P	2 hr	96.3	10.9	92.7
	24 hr	96.2	12.6	84.5
Nitrate	2 hr	63.2	75.7	55.8
	24 hr	77.5	87.9	55.3