7.5.4-2: UPDATE - CHEMISTRY AND CONSTITUENTS - LITERATURE SUMMARY

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

AFB_1	aflatoxin B_1
CPDB	Carcinogenic Potency Database
FDA	Food and Drug Administration
GC/MS	gas chromatography/mass spectrometry
GC-MS/MS	gas chromatography with tandem mass spectrometry
GC-TCD	gas chromatography with thermal conductivity detection
LC-ESI-MS	liquid chromatography-electrospray ionization mass spectrometry
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LOD	limit of detection
MRTPA	Modified Risk Tobacco Product Application
NNK	4-(methylnitrosamino-1-(3-pyridyl)-1-butanone
NNN	N′-nitrosonornicotine
ppb	parts per billion
rDNA	ribosomal DNA
ST	smokeless tobacco
TSNA	tobacco-specific nitrosamine
UHPLC-MS/MS	ultra–high-performance liquid chromatography with tandem mass spectrometry
U.S.	United States
VNA	volatile N-nitrosamine
WWB	wet weight basis

7.5.4-2. CHEMISTRY AND CONSTITUENTS LITERATURE SUMMARY

The United States (U.S.) Food and Drug Administration's (FDA's) Modified Risk Tobacco Product Application (MRTPA) Draft Guidance (2012) Section (B)(1) provides recommendations for data and information on product analyses to assess users' and nonusers' potential exposure to harmful substances.

Specifically, the U.S. FDA's MRTPA Draft Guidance recommends:

- "product analyses regarding the chemistry"
- "…product analyses [that] will facilitate FDA's understanding of the [candidate] product, the potential for exposure to harmful or potentially harmful constituents from use of the [candidate] product, and provide context for evaluating other data submitted in an MRTPA."

Product chemistry also informs the health effects of smokeless tobacco (ST) and the relevance/applicability of existing epidemiologic data to the ST products that are the subject of this application. This literature review section provides a product chemistry overview of the entire relevant ST category since publications containing product specific data are scarce. Specific product chemistry and batch-to-batch information are found in Section 3.1.

7.5.4-2.1.Literature Search and Review Process

A comprehensive literature review was conducted through December 2014 that reviewed, among other topics, the chemistry and constituents of smokeless tobacco (ST) (Section 7.5.1), and literature summaries were drafted in areas that are important in the assessment of an MRTP candidate. A second literature review was conducted for the period of December 08, 2014, to February 06, 2017, to update the original search. During the new search, 1,029 citations were identified, and, after applying predetermined inclusion and exclusion criteria, 165 articles were deemed to be within the scope of the review. In general, the in-scope articles were peer-reviewed and studied ST products commercially available in the U.S.

A keyword assignment exercise was performed to determine if any of those articles provide information about ST chemistry and constituents, and 14 articles that provide information regarding ST chemistry and constituents were identified. However, as new references became available after December 2014, they were, initially, included in the original narratives if they added new information. Of the 14 articles that provide information on ST chemistry and constituents in the updated search, two had already been included in the initial literature summary in Section 7.5.4-1, even though they were published after the cutoff date. A summary of the remaining 12 articles identified is provided in Table 7.5.4-2-1, and this section is intended to supplement the previous literature review (Section 7.5.4-1) to provide a current, updated literature review of ST chemistry and constituents.

7.5.4-2.2.Literature Review on Smokeless Tobacco Chemistry and Constituents

Of the 12 articles identified in the updated literature search, five provide data regarding carcinogens or other toxicants found in ST products. These five articles are further summarized in Section 7.5.4-2.2.1.

7.5.4-2.2.1. Carcinogens and Other Toxicants Found in Smokeless Tobacco Products

In 2016, 34 ST products (moist snuff, snus, dry snuff, and chewing tobacco) purchased in 2015 in the U.S., were measured for their N′-nitrosonornicotine (NNN) levels (Ammann, Lovejoy, Walters, & Holman, 2016). The range of the NNN levels were between 0.64 to 12.0 µg/g dry weight, with dry snuff having the highest levels of NNN (>5 µg/g dry weight). However, a general decrease in NNN levels was observed for the six moist snuff products that were analyzed in 2004 and then again in 2015. Additionally, there was a wide range of water content among various ST products tested, with dry snuff having the lowest water content (3.9% to 7.9%) and moist snuff having the highest water content (21.4% to 54.8%). In particular, the content of NNN in Copenhagen® Snuff Fine Cut purchased in 2015 was 3.94 µg/g dry weight (SD = 0.20), and the water content was 49.9% (SD = 1.7). Zitomer et al. (2015) assessed the concentration of the carcinogen aflatoxin B1 in 32 commercially available ST products in the U.S. (loose moist snuff, n = 12; pouched moist snuff, n = 4; dry snuff, n = 6; chewing tobacco, n = 7; and snus, n = 3). Aflatoxin B1 concentrations in ST products analyzed ranged from less than the limit of detection (0.007 ppb) to 0.271 ppb (dry mass). Aflatoxin B1 was most frequently detected in dry snuffs and chews, whereas all moist snuff products tested were below the limit of detection. However, the amounts of Aflatoxin B1 detected were low relative to the 20-ppb regulatory limit established by U.S. FDA for food and feeds.

Toxicants and carcinogens from tobacco products may affect not only the health of tobacco product users, but also other people who live in the same households. Whitehead et al. (2015) collected dust samples from homes of active smokers, ST products users, and nonuser of tobacco. The study showed that concentrations of NNN and 4-(methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), another carcinogen frequently found in tobacco products, were 4.8-fold and 6.9-fold higher, respectively, in homes of ST users than in nonusers of tobacco. Median myosmine/nicotine ratios (a novel indicator of the source of tobacco contamination in each home) were lower in homes of ST users than in homes of active smokers, a result that indicated that cigarette smoke was not the predominant source of tobacco constituents in homes of ST users. It was hypothesized that children living with ST users may be exposed to tobacco-specific carcinogens via contact with contaminated dust and household surfaces.

Lv et al. (2016) demonstrated a method to determine volatile N-nitrosamines (VNA) in tobacco and tobacco products. Using this method, with lower levels of detection varying by analyte between 0.03 and 0.15 ng/g, they were able to determine levels of six different VNAs in tobacco products. The levels of detected VNAs ranged from 0.4 to 30.7 ng/g.

In 2015, McAdam et al. (2015) showed the presence of acrylamide, one of the chemicals included in the Hazardous or Potentially Hazardous Constituent list generated by the U.S.

FDA. The average levels of acrylamide among various ST products were not significantly different, except for U.S. snus, which had on average greater levels than other types of ST. Typically, acrylamide levels increased by at least six-fold during manufacturing of a snus product, but then decreased during storage for up to 22 weeks. However, acrylamide exposure from contemporary ST products is small when compared with acrylamide exposure from dietary products.

7.5.4-2.2.2. Updated Findings

Information on chemistry and constituents of ST in the update literature review is consistent with that seen in the initial literature review. Although the conclusions from the initial literature review (Section 7.5.4-1) have not changed, there were two articles with novel results identified in the updated literature review. First, one article showed that acrylamide exposure from contemporary ST products is lower than acrylamide exposure from dietary products (McAdam et al., 2015). Second, people living with ST users may be exposed to NNN and NNK, well-known toxicants and carcinogens, found in dusts in homes of ST users (Whitehead et al., 2015).

A tabular summary of the ST chemistry and constituents literature review is presented in Table 7.5.4-2-1.

Table 7.5.4-2-1: Literature Review for Smokeless Tobacco – Chemistry and Constituents

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Seidenberg, Ayo-Yusuf, Ayo-Yusuf, & Rees, 2017)	Characteristics of "American Snus" and Swedish Snus Products for Sale in Massachussetts, USA	A total of 14 snus products made by American manufacturers Philip Morris USA,, R.J. Reynolds Tobacco Company, and US Smokeless Tobacco Company ("American snus") and 10 snus products made by Swedish Match North America ("Swedish snus") were considered. Objective: To examine the characteristics of "American snus" products, including nicotine levels, and compare with snus products that are more typical of Sweden.	ST products were analyzed for their pH, total nicotine, and unionized (free) nicotine levels.	"American snus" brands contained significantly lower concentrations of unionized nicotine, lower pH, and less moisture than "Swedish snus." Therefore, "American snus" products may have lower addiction potential than "Swedish snus," and may be more likely to be used dually with cigarettes.	Limitations: This study only analyzed snus products whose reports were submitted to the Massachusetts Department of Public Health for 2014.

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Ammann et al., 2016)	A Survey of N'-Nitrosonornicotine (NNN) and Total Water Content in Select Smokeless Tobacco. Products Purchased in the United States in 2015	A total of 34 ST products (moist snuff, n = 18; snus, n = 4; dry snuff, n = 4; and chewing tobacco, n = 8) were collected. Objective: To measure the levels of NNN and water content of a select number or ST products sold in the U.S. in 2015.	ST products were analyzed for NNN levels and water content using LC-MS/MS and GC-TCD methods, and compared with previous studies.	The range of the NNN levels of the examined ST products was between 0.64-12.0 µg/g dry weight. Dry snuff had the highest levels of NNN (>5 µg/g dry weight). There is a general decrease in NNN levels for the same six moist snuff products analyzed in 2004 as compared with the moist snuff products analyzed in 2015. The water content of the ST products ranged from 3.92% to 54.8%. Copenhagen® Original Fine Cut purchased in 2015: NNN content: 3.94 ±0.20 □g dry weight. Water content: 49.9% ±1.7%	Limitations: There are no data on other ST product classes.

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Han et al., 2016)	Bacterial Populations Associated with Smokeless Tobacco Products	A total of 90 samples, representing 15 common ST products (of four major types), were purchased in Little Rock, AR, and Washington, DC, in November 2012, March 2013, and July 2013. Objective: To identify the bacterial microorganisms that were present in ST products in two geographically distinct locations and to determine the effect of temporal changes on the microbial communities present in ST products.	Bacterial populations were evaluated using culture, pyrosequencing, and denaturing gradient gel electrophoresis.	Moist-snuff products exhibited higher levels of bacteria and diversity of bacterial populations than snus and some chewing tobacco products. Species found by culturing were *Bacillus pumilus*, *B. licheniformis*, *B. safensis*, and *B. subtilis*, members of the genera *Oceanobacillus*, *Staphylococcus*, and *Tetragenococcus*. Pyrosequencing analysis identified the genera *Tetragenococcus*, *Carnobacterium*, *Lactobacillus*, *Geobacillus*, *Bacillus*, and *Staphylococcus*. Several species identified are found to cause opportunistic infections and are able to reduce nitrates to nitrites, which may be an important step in the formation of carcinogenic tobacco-specific N´-nitrosamines.	Limitations: (1) For each type of product, a small cross-section of the available products was tested. However, efforts were made to include a variety of products and manufacturers to minimize this potential concern; (2) There was a lack of positive cultures from the snus samples.
(Lv et al., 2016)	Determination of nine volatile N-nitrosamines in tobacco and smokeless tobacco products by dispersive solid-phase extraction with gas chromatography and tandem mass spectrometry	Five replicates of samples were treated by dispersive solid-phase extraction using 1 g of primary secondary amine and 0.5 g of carbon. Objective: To determine nine VNAs in tobacco and ST products.	Samples were analyzed by GC-MS/MS for nine VNAs.	The recoveries for targets ranged from 84% to 118%, with <16% relative standard deviations at three spiking levels of 0.5, 1.25, and 2.5 ng/g. The limits of detection ranged from 0.03 to 0.15 ng/g. The presence of six nitrosamines in the range of 0.4-30.7 ng/g was detected.	Limitations: The source of sample used in the experiment is unclear.

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Miao et al., 2016)	High-Intensity Sweeteners in Alternative Tobacco Products	Sixteen tobacco products were purchased from stores in New Haven, CT (snus, n = 4; moist snuffs, n = 5; electronic cigarette cartridges, n = 5; electronic cigarette refill liquids, n = 2; electronic refill liquid, n = 1; and dissolvable tobacco product, n = 1). Sugar-free confectionary products (n = 4) and sugar-free beverages (n = 2) were purchased from area stores. Objective: To quantify added sweeteners in ST products, a dissolvable product, electronic cigarette liquids and to compare with sweetener levels in confectionary products.	Products were analyzed for sweetener content using LC-ESI-MS method.	All ST products contained synthetic high-intensity sweeteners, with snus and dissolvables exceeding levels in confectionary products (25-fold). All snus samples contain sucralose, and most also contained aspartame. All moist snuff samples contained saccharin, the dissolvable sample contained sucralose and sorbitol, and electronic cigarette liquids contained mostly ethyl maltol.	None.

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Tyx et al., 2016)	Characterization of Bacterial Communities in Selected Smokeless Tobacco Products Using 16S rDNA Analysis	U.S.-made tobacco products (dry and moist snuff, n = 6) were obtained in the Atlanta area, and Sudanese toombak (n = 2) samples were obtained from stores in Sudan. Objective: To study bacterial presence in U.S.-made dry snuff, moist snuff, and Sudanese toombak using next generation sequencing.	Products were analyzed for bacterial presence by sequencing their 16S rDNA.	A total of 33 bacterial families from four phyla (Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes) were identified. U.S.-produced dry snuff products were dominated by Firmicutes, whereas toombak samples contained mainly Actinobacteria and Firmicutes. Dry snuff contained more diverse bacteria than moist snuff. The nitrate-reducing capability that contributes to the formation of carcinogenic nitrosamines comes from the following: the *Corynebacterium, lactobacillus*, *Staphylococcus* spp., and *Enterobacteriaceae* family (in dry snuff); *Staphylococcu*s spp (in moist snuff); and *Corynebacterium* and *Staphylococcus* spp. (in toombak).	Strength: This study provides information about the types of bacteria present in ST products (U.S.-made, and Sudanese toombak) that potentially may be harmful due to their nitrite production that may result in increased TSNA levels and the presence of proinflammatory biomolecules and endotoxins in fermented products. Limitations: The study (1) used shorter 16S sequences to identify bacterial species and (2) did not analyze any fungal presence in ST products (which are also known to contribute to the formation of toxins or carcinogens in ST products).

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Borgida et al., 2015)	Assessing Constituent Levels in Smokeless Tobacco Products: A New Approach to Engaging and Educating the Public	A total of 397 subjects (73 cigarette users and 324 nonsmokers; mean age 34.07 years; 46.1% male) were presented information online about two constituent dimensions of ST products: nicotine and/or toxicity. Objective: To provide accurate information about the constituents in nicotine-containing products to help tobacco users in making informed decisions about product choices.	Subjects completed measures of knowledge and tobacco health risks before and after exposure to the information.	The levels of free nicotine in the products included in the development of the scale ranged from 0.5 to 8.7 mg/g product, averaging 3.7 (±1.5) mg/g. The sum of NNN and NNK varied from 0.4 to 14.6 µg/g product, averaging 3.0 (±1.6) µg/g. Subjects increased their knowledge that toxicity contributes to disease risk and nicotine contributes to addiction, that ST products vary in their levels of nicotine and toxicity, and that ST and cigarette products have higher toxicity than medicinal nicotine replacement therapies.	None.
(Halstead, Watson, & Pappas, 2015)	Electron Microscopic Analysis of Surface Inorganic Substances on Oral and Combustible Tobacco Products	Commercially available cigarette and ST products were obtained between 2010 and 2014. Objective: To analyze constituents of cigarette and ST products using scanning electron microscopy with energy dispersive x-ray spectroscopy.	Inorganic constituents of tobacco products were analyzed using scanning electron microscopy with energy dispersive x-ray spectroscopy.	Aluminum silicates, silica, and calcium compounds were common inorganic particulate constituents of tobacco products.	Limitations: It is uncertain whether aluminum silicate particles on ST products pose any significant health risk. The number of cigarettes and ST products analyzed is unknown.

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(McAdam et al., 2015)	The acrylamide content of smokeless tobacco products	ST products (n = 74) representing 90% of 2010 market share in the U.S. and Sweden were included. Objective: To identify the concentrations of acrylamide in ST products from the U.S. and Sweden.	ST products were examined for their acrylamide concentrations.	Acrylamide concentrations ranged from 62 to 666 ng/g WWB. The average concentrations of acrylamide (WWB) by type of ST were not significantly different, except for U.S. snus. Acrylamide concentrations were significantly and positively correlated with pH, but not with levels of either reducing sugars or ammonia nitrogen. Concentrations of acrylamide were increased by at least six fold during manufacture of a snus sample and then decreased during storage for up to 22 weeks. Acrylamide generation in tobacco occurs at lower temperatures, but at longer time scales than found with food production.	None.
(Whitehead et al., 2015)	Tobacco Alkaloids and Tobacco-Specific Nitrosamines in Dust from Homes of Smokeless Tobacco Users, Active Smokers, and Nontobacco Users	Dust samples were collected from homes occupied by ST users (n = 6), active smokers (n = 6), and nonusers of tobacco (n = 20). Objective: To study the effect on children of indirect exposure to tobacco constituents (collected from dust samples)	Dust samples were analyzed for five TSNA and five tobacco alkaloids using LC-MS/MS.	Concentrations of NNN and NNK were 4.8- and 6.9-fold higher, respectively, in homes of ST users than in homes of nonusers of tobacco. Median myosmine/nicotine ratio (indicator of the source of tobacco contamination) was lower in homes of ST users (1.8%) than in homes of active smokers (7.7%). Children living with ST users may be exposed to carcinogenic TSNA.	Limitations: (1) Small number of samples of dust collected from ST users homes (n = 6), (2) variability in the type of vacuum cleaners used, and (3) variability in vacuum cleaning habits in different homes.

Author	Title	Study Methods	Primary Study Measurements and Endpoints	Author's Findings Related to ST Chemistry and Constituents	Comments
(Zitomer et al., 2015)	Determination of Aflatoxin B_1 in Smokeless Tobacco Products by Use of UHPLC-MS/MS	A total of 32 ST products (produced by eight manufacturers) were obtained from retail stores in Washington, DC, and Atlanta, GA (loose moist snuff, n = 12; pouched moist snuff, n = 4; dry snuff, n = 6; chewing tobacco, n = 7; and snus, n = 3). Objective: To detect and quantify AFB_1 in ST products available in the U.S. using UHPLC-MS/MS method.	ST products were analyzed for AFB_1 using UHPLC-MS/MS method.	AFB_1 LOD was 0.007 ppb and concentrations in ST products analyzed ranged from <LOD to 0.271 ppb (dry mass). AFB_1 was most frequently detected in dry snuffs and chews, whereas all moist snuff products tested were below LOD. The amounts of AFB_1 detected were low relative to the 20-ppb regulatory limit established by U.S. FDA for food and feeds.	Limitations: This study only tested moist and dry snuffs, snus, and chews, but did not test other product classes, such as dissolvables due to incompatibility with the methods used.
(Lisko, Stanfill, & Watson, 2014)	Quantitation of ten flavor compounds in unburned tobacco products	A number of Southeast Asian and U.S. tobacco products were evaluated. Objective: To develop a versatile method to measure the concentrations of 10 common flavor chemicals found in various tobacco products.	Products were analyzed for their 10 flavor compounds using a new GC/MS method.	Excellent linearity, accuracy, and precision were achieved for all flavor analytes measured. Results were within typical ranges for U.S. snuff products: "The mint flavored snuff contained appreciable levels of eucalyptol (218 µg/g), menthol (3,240 µg/g), and ethyl salicylate (1,770 µg/g). Smaller, but measurable, levels of camphor, methyl salicylate, and pulegone were also present in the mint product. The wintergreen snuff varieties exhibited high levels of methyl salicylate (9,860 µg/g)."	Limitations: Many of the flavor compounds found in U.S. tobacco products are not included in the current analyte panel.

7.5.4-2.3.Literature Cited

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