Regulatory Toxicology and Pharmacology 53 (2009) 121–133

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Chemical and toxicological characterization of commercial smokeless tobacco products available on the Canadian market

W.S. Rickert [a,*], P.J. Joza [a], A.H. Trivedi [a], R.A. Momin [a], W.G. Wagstaff [a], J.H. Lauterbach [b]

[a] Labstat International ULC, 262 Manitou Drive, Kitchener, Ont., Canada N2C 1L3
[b] Lauterbach & Associates, LLC, 211 Old Club Court, Macon, GA 31210-4708, USA

ARTICLE INFO

Article history:
Received 25 September 2008
Available online 24 December 2008

Keywords:
Commercial smokeless tobacco products
Snuff
Snus
Plug
Mutagenicity
Genotoxicity
Cytotoxicity
Canada
GothiaTek®

ABSTRACT

Some health experts are recommending that smokers who refuse to quit or refuse to use nicotine replacement therapy (NRT) such as nicotine-containing chewing gum switch to certain types of smokeless tobacco products (STP) such as Swedish snus. Other health experts disagree citing the uncertainty in the composition of commercially available STP, the lack of governmental regulations to ensure that STP advertised to meet certain standards (i.e., GothiaTek®) do actually meet such standards, and the uncertainty that any STP can provide as safe as alternative to smoking as NRT. One reason for uncertainty is the dearth of detailed chemical and toxicological information on contemporary STP. Unlike the situation with cigarettes, there are few standardized methods for analytical and toxicological studies of STP. Consequently, the objective for this work was to characterize several types of STP available on the Canadian market using the modifications of the Official Health Canada chemical and toxicological methods developed for cigarettes. Moist snuff samples tested had TSNA and B[*a*]P levels somewhat above the GothiaTek® standard while samples of Swedish snus, low-moisture snuff, and US-style chewing tobacco did not. Use of *in vitro* assays to assess STP toxicity was of limited utility in distinguishing product types.

This study was funded by Health Canada under contract number H4133-060012/001/SS.

1. Introduction

In the latter third of the twentieth century, sales of smokeless tobacco products (STP) in the USA, particularly those of moist snuff, began to increase after years of decline (Surgeon General, 1986). According to the US Federal Trade Commission, annual sales of moist snuff increased from just over 36 million pounds in 1986 to more than 75 million pounds in 2005 (Federal Trade Commission, 2007). Data from the same report showed that the poundage of other types of STP decreased during the same period. The rapid increase in US sales of moist snuff apparently was surprising to one marketing expert. In 1999, Wyckham claimed that US STP manufacturers were targeting the Canadian market because of less than desirable business prospects in the USA (Wyckham, 1999). Wyckham also claimed that at the time, the mix of STP sold in Canada were similar to that in the USA, that all STP sold in Canada were imported from the US and other countries, after the Canadian subsidiary of US Smokeless Tobacco closed its plant in Montreal in 1980. However, STP sales did not increase in Canada as they did in the USA (Snider and Kaiserman, 2007).

Part of the increase in the sales of STP in USA was believed due to consumer perceptions that use of this type of product was safer than using cigarettes. The US Congress mandated warning labels on STP in 1986. One of those warnings is, "This product is not a safe alternative to smoking" (CDC, 2000). The basis for such warnings was evidence that use of smokeless tobacco products had been associated with oral cancer and other diseases and such products were addictive (Surgeon General, 1986). There has been increased debate about the health risks associated with smokeless tobacco products over the last decade as exemplified by Nilsson's risk assessment on snuff dipping (Nilsson, 1998). One reason for debate is that there are many different kinds of smokeless tobacco products in use worldwide (IARC, 2007). However, the products generally used in India, Africa, and the Middle East are different in manufacture/preparation, composition, and in means of consumption than the products used in United States and Sweden (Waterbor et al., 2004). However, those who claim that use of STP sold in North American and Europe can lead to oral cancer often confuse such products with those made in India, Africa, and the Middle East (Rodu and Jansson, 2004; Phillips et al., 2005; Nilsson, 2006; Weitkunat et al., 2007). The report by Hoffmann et al. on high levels of TSNAs in a brand of moist snuff introduced in the US market in 1989–1990 has also been used to support the association of TSNAs in STP with oral cancer (Hoffmann et al., 1991). Indeed,

* Corresponding author. Fax: +1 519 748 1654.
E-mail address: wrickert@labstat.com (W.S. Rickert).

0273-2300/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2008.12.004

the recent IARC report on smokeless tobacco comingled Swedish snus and other low-TSNA products manufactured in North America and Europe with high-TSNA STP made elsewhere. Furthermore, that report as well as the report on smokeless tobacco by the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks both used data on the chemical composition of STP that dated back to the 1980s to characterize the health risks of contemporary products (IARC, 2007; SCENIHR, 2008). One of the most recent reviews in this area limited consideration of the relative risks for esophageal cancer and pancreatic cancer to studies done in Denmark, Norway, and Sweden, but considered studies done in both North America and Asia in order to obtain an overall relative risk for oral cancer (Boffetta et al., 2008).

Traditionally, three types of smokeless tobacco products have been sold in North America: (1) chewing tobacco (loose-leaf, plug, twist); (2) dry snuff; and (3) wet snuff (Surgeon General, 1986; Wahlberg and Ringberger, 1999; Wyckham, 1999). Each of these three types of products is different, chemically and physically, from each other and each of them differs in many respects from smokeless tobacco products made outside North America (Nilsson, 2006, 1998; Rodu and Jansson, 2004; Wahlberg and Ringberger, 1999). Cigarette tobaccos and finished cigarette blends have been well characterized through extensive analytical studies over the years, and the results of those studies are available in journal articles and other documents in the public domain. However, there is relatively little available on the routine or detailed chemistries of tobaccos used in STP and on finished smokeless tobacco products. Much of the information available has centered on the tobacco specific nitrosamine (TSNA) content of STP (Wahlberg and Ringberger, 1999). There are at least two reasons for this. First, there has not been the focus by research organizations on STP and their components that there has been on cigarette products and their components. Second, some of the companies that produce STP have been smaller and less sophisticated technically than the major cigarette companies. Furthermore, except for the STP-TSNA issue, there have been few health issues that have received the high attention of those associated with smoking. For example, there are no concerns about users inhaling pyrolysis products from additives in STP as there is from smokers inhaling the pyrolysis products from additives on cigarette fillers. Also, many smokeless tobacco products use fermented tobaccos and/or other processing steps that change the chemical and physical properties of the tobacco so that it provides the necessary hedonic properties. The understanding of these processes and the resulting product chemistries, can require complicated scientific instrumentation and very experienced personnel that have generally only been available at sophisticated laboratories (Alford et al., 1989; Moldoveanu and Colby, 1990; Geiss et al., 1990; Clarke et al., 2006).

Therefore, we have very little to go on in terms of the detailed chemistry of contemporary smokeless tobacco products sold in North America and Europe. Contemporary data on a brand-by-brand basis are scarce. Furthermore, the situation is confounded by the fact that contemporary Southeast Asian STP can be purchased at some locations in North America and Europe (McNeill et al., 2006 this study). Perhaps the best summary of data currently available is that given in the recent IARC monograph on smokeless tobacco (IARC, 2007).

As noted by several authors, links with STP use and disease may be associated with trace-level components in the products such as TSNAs, heavy metals and benzo[*a*]pyrene (B[*a*]P). One company, Swedish Match, has published standards for the maximum levels of TSNAs, certain heavy metals, and other trace-level contaminates that it would allow in its snus products (Sundén, 2001). These standards are known collectively as the GothiaTek® standard (Swedish Match, 2001) and include standards for the tobaccos and ingredients used as well as the processing conditions for the manufacture of snus. Trace-level contaminants included on the GothiaTek® list include (limits in parentheses are on a dry-weight basis): Total TSNAs (10 ppm), *N*-nitrosodimethylamine (10 ppb), benzo[*a*]pyrene (20 ppb), nitrite (7 ppm), arsenic (0.5 ppm), cadmium (1 ppm), chromium (3 ppm), lead (2 ppm), nickel (4.5 ppm), and nitrite (7 ppm) (GothiaTek®, 2008). The GothiaTek® standard also states that products must meet the Swedish Match pesticide policy, but the details of that policy are not given. In any case, some but not all, experts on health effects of tobacco have suggested that contemporary Swedish snus (i.e., meeting GothiaTek® standards) is safe enough to be recommended by health authorities as an alternative to cigarettes (Levy et al., 2004). The GothiaTek® standard appears to have been adopted by British–American Tobacco (Williamson and Proctor, 2007; Williamson et al., 2007) and by the European Smokeless Tobacco Council (2007). However, others have suggested that compliance with the GothiaTek® standard may not be enough. Pappas and colleagues recently (2008) suggested that the list of metals used to characterize the toxicity of STP be expanded to include barium, beryllium, and cobalt. SCENIHR cited work showing the organic and aqueous extracts of STP were mutagenic and/or clastogenetic (SCENIHR, 2008; IARC, 2007; Rickert et al., 2007). The fact that such genotoxicity was found at all raises the question of other toxicants being present in addition to those included in the GothiaTek® standard. There is also a strong relationship between use of certain STP and oral mucosal lesions, particularly in Scandinavia where most users of STP have a characteristic "snuff-induced lesion" (Kallischnigg et al., 2008). That also points to toxicants in STP that are not covered by the GothiaTek® standard (Lauterbach, 2008). Therefore, one objective for the work reported here was to explore the toxicological properties of contemporary STP as measured by *in vitro* bioassays for cytotoxicity, clastogenicity, and mutagenicity. Another objective was to determine the levels of target analytes in the STP sold in Canada. Another purpose of this research was to begin building a market map of commercially available STP similar to the commercial cigarette market map reported by Counts and her colleagues (Counts et al., 2006). However, it should be noted that differences among the different types of STP included in this study, the method of use (e.g. oral versus nasal), and the typical amount of product used per day by a consumer make such mapping exercises more challenging than they are for cigarettes.

2. Materials and methods

2.1. Smokeless tobacco products (STP)

Seven types of smokeless tobacco products were evaluated: (1) fine-cut moist snuff reportedly made in US by U.S. Smokeless Tobacco Company (UST) and imported into Canada; (2) long-cut moist snuff also made by UST and imported; (3) pouched moist snuff also made by UST and imported; (4) low-moisture snuff reportedly manufactured by McChrystal's in UK and imported into Canada; (5) loose-leaf and plug chewing tobacco reportedly made in US by Swedish Match North America and imported into Canada, (6) pouched snus, reportedly made in Sweden and imported into Canada by Imperial Tobacco Canada; and (7) a gutkha-type product imported from India. The list of brands tested can be found in Table 1a and b. The products were picked up and delivered to the laboratory by agents for Health Canada. On receipt, the individual containers of products were labeled with a unique identifier and stored at 4 °C until they were ready to be prepared for analysis. Samples were prepared for analysis as follows. A minimum of 100 g of product was ground using a Robot Coupe 2 V batch processor unless the tobacco already is in a ground form (e.g., low moisture snuff, moist snuff). Before grinding or mixing, any non-tobacco material (e.g., pouch material in the case of pouched moist

Table 1a
Chemistry of moist snuff products (mean values on DWB except for percentage dry matter and pH).

Sample ID	Brand/style	Sample type	Ammonia (ug/g)	Propylene Glycol (mg/g)	Glycerol (mg/g)	Dry matter (%)	Nicotine (mg/g)	Nitrate (mg/g)	pH
080202	Copenhagen fine cut	Fine cut moist snuff	6981	BDL	BDL	46.3	30.8	28.9	7.55
080207	Skoal fine cut Wintergreen	Fine-cut wintergreen flavored moist snuff	6695	BDL	BDL	46.9	29.5	28.6	7.52
080201	Copenhagen long cut	Long cut moist snuff	14,830	BDL	BDL	46.1	27.6	30.4	7.68
080212	Skoal long cut straight	Long cut moist snuff	6207	BDL	BDL	46.6	28.2	34.1	7.60
080208	Skoal long cut apple	Long-cut fruit-flavored moist snuff	12,889	9.70	BDL	46.8	23.7	34.1	7.26
080190	Skoal long cut berry	Long-cut fruit-flavored moist snuff	7459	3.92	BDL	46.8	24.9	30.5	7.38
080209	Skoal long cut cherry	Long-cut fruit-flavored moist snuff	6150	6.95	BDL	47.4	27.3	30.1	7.37
080191	Skoal long cut citrus	Long-cut fruit-flavored moist snuff	12,689	6.72	BDL	46.9	23.9	30.0	7.26
080211	Skoal long cut peaches	Long-cut fruit-flavored moist snuff	12,581	23.4	BDL	46.8	27.5	30.3	6.97
080192	Skoal long cut mint	Long-cut mint-flavored moist snuff	6043	BDL	BDL	46.6	30.4	29.1	7.47
080193	Skoal long cut spearmint	Long-cut mint-flavored moist snuff	6743	BDL	BDL	46.8	31.2	27.7	7.48
080194	Skoal long cut vanilla	Long-cut vanilla-flavored moist snuff	12,319	9.73	BDL	46.7	27.1	28.4	7.24
080219	Rooster Wintergreen	Long-cut wintergreen-flavored moist snuff	13,022	BDL	BDL	46.2	27.5	27.4	7.81
080210	Skoal long cut classic	Long-cut wintergreen-flavored moist snuff	6385	BDL	BDL	46.8	26.4	28.3	7.54
080213	Skoal long cut wintergreen	Long-cut wintergreen-flavored moist snuff	6135	BDL	BDL	46.9	30.4	31.4	7.44
080206	Skoal Berry Pouches	Pouched fruit-flavored moist snuff	10,007	NQ	BDL	47.8	24.3	36.1	7.62
080204	Skoal bandit mint	Pouched mint-flavored moist snuff	9156	BDL	BDL	52.1	30.2	32.8	7.55
080203	Copenhagen pouches	Pouched moist snuff	7472	BDL	BDL	47.5	22.6	28.7	8.19
080205	Skoal bandit wintergreen	Pouched wintergreen-flavored moist snuff	9867	BDL	BDL	48.5	26.7	32.2	7.62

NQ, not quantifiable; BDL, below detection limit.

Table 1b
Chemistry of other smokeless tobacco products (mean values on DWB except for percentage dry matter and pH).

Sample ID	Brand/style	Sample type	Ammonia (ug/g)	Propylene Glycol (mg/g)	Glycerol (mg/g)	Dry matter (%)	Nicotine (mg/g)	Nitrate (mg/g)	pH
080200	du Maurier Freshmint	Swedish snus mint-flavored	694	16.2		70.8	23.1	14.3	7.39
080199	du Maurier Original	Swedish snus	657	16.6		73.9	18.1	14.0	7.39
080218	McChrystal's Apricot	Low-moisture fruit flavored snuff	152	BDL	BDL	88.2	6.27	5.53	9.46
080198	McChrystal's Mild Lemon	Low-moisture fruit flavored snuff	302	BDL	BDL	82.7	6.62	4.88	9.43
080196	McChrystal's Raspberry	Low-moisture fruit flavored snuff	174	23.3	BDL	85.7	5.91	4.72	9.40
080197	McChrystal's Violet	Low-moisture lavender snuff	217	BDL	BDL	84.1	5.47	4.91	9.45
080216	McChrystal's Menthol	Low-moisture mentholated snuff	203	BDL	BDL	80.9	8.62	6.79	9.56
080217	McChrystal's Original	Low-moisture snuff	114	BDL	BDL	83.3	9.02	6.27	9.68
080215	Red Man	US-style chewing tobacco - loose-leaf	2663	10.6	27.7	78.4	8.86	7.18	5.85
080214	Apple Plug	US-style chewing tobacco -plug	1285	15.9	7.10	82.9	13.9	8.76	4.90
080220	Manikchand	Indian-style chewing tobacco	214	BDL	BDL	94.2	2.44	BDL	8.25

NQ, not quantifiable; BDL, below detection limit.

snuff, plug wrap in the case of plug chewing tobacco) is removed. The ground sample is sieved through a 4-mm sieve, if necessary. The non-tobacco elements are ground, sieved separately, and then recombined with the rest of the sample. After thorough mixing, samples were stored in double-wrapped plastic bags at 4 °C until needed for analysis.

2.2. Chemical analysis

The Health Canada methods mandated for the analysis and yearly reporting of STP constituents were used with the exception of nicotine. This was determined using the CDC method (CDC, 1997). Other methods were: (1) Official Method T-302, Determination of Ammonia in Whole Tobacco; (2) Official Method T-304, Determination of Humectants in Whole Tobacco; (3) Official Method T-306, Determination of Nickel, Lead, Cadmium, Chromium, Arsenics, Selenium and Mercury in Whole Tobacco; (4) Official Method T-307, Determination of Benzo[*a*]Pyrene in Whole Tobacco; (5) Official Method T-308, Determination of Nitrate from Whole Tobacco; (6) Official Method T-309, Determination of Nitrosamines in Whole Tobacco; (7) Official Method T-310, Determination of Whole Tobacco pH; (8) Official Method T-311, Determination of Triacetin in Whole Tobacco; (9) Official Method T-312, Determination of Sodium Propionate in Whole Tobacco; and (10) Official Method T-313, Determination of Sorbic Acid in Whole Tobacco. All Health Canada tobacco methods are available from the Health Canada web site. The URL is http://www.hc-sc.gc.ca/hl-vs/tobacta-bac/legislation/reg/indust/index_e.html (accessed June 12, 2008). The moisture contents of the tobacco samples were determined with AOAC Official Method 966.02, Moisture in Tobacco, Gravimetric Method (AOAC, 1990).

2.3. Preparation of extracts of STP for toxicological assays

Samples for toxicological assays were prepared as described previously and then extracted with DMSO (dimethylsulfoxide) according to the following procedure. A portion of a smokeless tobacco sample (25 g) was dispersed in DMSO (225 mL to give 1:9 *w/v)* using an ultrasonic homogenizer. Three dispersions (extracts) of each brand-style were prepared. Each dispersion was then incubated at 37 °C for 21 h and homogenized for a second time. Each dispersion was then centrifuged and ultra-filtered to ensure the removal of microorganisms. All extract solutions were tested for sterility prior to storage and stored at −80 °C prior to assay. Nicotine content of

the DMSO extract was determined by taking a 100 μL aliquot of the extract, transferring it to a GC autosampler vial, adding 1000 μL of TNC extracting solution (cf., Health Canada Method T-115), capping the vial, and analyzing for nicotine as per Method T-115.

In addition, a subset of four brands was extracted using three solvents: DMSO, DCM (dichloromethane), and artificial saliva (van Ruth et al., 2001). Solvent-substitution was required for the DCM extracts to be assayed in the bacterial mutation assay as well as in the *in vitro* cell culture assays since DCM has been shown to be mutagenic in both bacterial and cell culture assays. Due to the mutagenicity of DCM and its poor solubility in water, DCM was substituted with (DMSO) using a modification of the procedure reported by Stamm et al. (1994). The DCM extract was concentrated by evaporation of the solvent at a constant temperature of 37 °C under an atmosphere of nitrogen gas using a Zymark TurboVap concentrator. The dried DCM extract was solvated in DMSO at a ratio of 1:9 (g of tobacco product per mL DMSO). The final extracts were filter-sterilized using sterile disposable syringes and 0.2 μm pore-size filters (e.g., Millex-LG 0.2 μm PTFE 25 mm Filter, Millipore Catalog # SLLG025SS) and were stored at −80 °C until used for bioassays.

2.4. Determination of mutagenicity

The mutagenicity assays on the extracts of the STP were carried out using Health Canada Official Method T-501 (Health Canada, 2004a) and as further described by Rickert et al. (2007). The linear dose range was 0–5560 μg of product per plate (as-is basis). The tester strains used were TA98, TA100, TA102, TA1535, and TA1537. Metabolic activation with rat-liver S-9 was used. Several different approaches were used for evaluation of the data as will be discussed in Section 3.

2.5. Determination of cytotoxicity

The cytotoxicity assays on the extracts of the STP were done using Health Canada Official Method T-502 (Health Canada, 2004b), which is based on the Neutral Red cytotoxicity assay developed by Borenfreund and Puerner (1985). The DMSO extracts as described in Section 2.3 were used in place of the mainstream smoke extracts as specified in the method. The DMSO extracts from only twelve of the thirty-one brands were used. The DCM and saliva extracts were also evaluated for four of the brands. All but one tobacco sample lacked sufficient cytotoxicity to produce the typical sigmoid-shaped dose–response curve.

2.6. Determination of genotoxicity

The genotoxicity assays on the extracts of the STP were carried out using Health Canada Official Method T-503 (Health Canada, 2004c), which is based on the *in vitro* micronucleus assay developed by Van Hummelen and Kirsch-Volders (1990). The DMSO extracts as described in Section 2.3 were used in place of the mainstream smoke extracts as specified in the method. As with the Neutral Red assay, only twelve of the thirty-one brands were assayed; and the DCM and saliva extracts were evaluated for four of those brands. The *in vitro* micronucleus assay is now considered a validated assay instead of just a screening method (Corvi et al., 2008).

3. Results and discussion

3.1. Bases for reporting results

A major challenge in evaluating smokeless tobacco products (STP) is the reporting of analytical and toxicological data on a basis that allows for comparison of products of widely differing compositions, widely differing moisture contents, and widely differing nicotine contents. Ideally, comparisons among products would be based on biomarker concentrations reflective of dose and toxicity. However, that is not practical because of the magnitude of the studies that would be needed and the number of analytes that might be measured. Another approach would be to ratio all chemistry and toxicology data to a product's nicotine yield or content. This is the approach we used when comparing the mutagenicity of smoke condensate from cigarettes and other smoking products with the mutagenicity of extracts of smokeless tobacco products (Rickert et al., 2007). However, this approach has a serious drawback when comparing smokeless tobacco products where the method of use is different.

Nicotine from nasal ingestion of low-moisture nasal snuff is not believed to enter the gastric system, as is the case with much of the nicotine from smokeless tobacco products used in the oral cavity (Russell et al., 1981, 1983; Schneider et al. 1996; Laugesen, 2007). This could make results expressed on a per-unit-nicotine basis misleading as users of oral moist snuff need to insert much more product in the oral cavity to get the same effect as an experience user of nasal snuff can get from a pinch. Therefore, unless otherwise specified, the results presented in the report are on a "dry-weight basis" (DWB). The full dataset is included in the supplementary materials so that researchers can express the data in other ways should they chose to do so.

3.2. Results from determinations of major components

Tables 1a and 1b show the results from the determination of some of the major components in the STP sampled. To facilitate product comparisons, these tables as well as Tables 2a and 2b are further divided by product class: (1) fine-cut moist snuff; (2) long cut moist snuff; (3) pouched moist snuff; (4) Swedish snus; (5) low-moisture snuff; (6) US-style chewing tobaccos; and (7) STP manufactured in India. These tables do not show the results for all analytes determined as some such as triethylene glycol and triacetin were not found in any of the western-style STP sampled [triacetin was found in the one product from India (Manikchand Gutkha) at about 80 ppm]. In a couple of cases, an analyte was found in only one sample. For example, propionate was found in raspberry-flavored low-moisture snuff at about 400 ppm (calculated as sodium propionate). Reportedly, propionic acid salts have been used as a preservative in raspberry extract (Nova's Bakery, 2008). These salts are also commonly found in bakery products. Sorbate was found in the sample of US-style loose-leaf chewing tobacco at about 1150 ppm (calculated as sorbic acid). Sorbic acid derivative have historically been used a preservatives in some tobacco products and in some food products. Glycerol, a common tobacco humectant, was found in both samples of US-style chewing tobacco but in none of the other samples. Propylene glycol was also found in those same two chewing tobacco samples, where its function, based on the level found, was likely that of a humectant. Several of the low-moisture snuff and moist snuff samples were found to contain propylene glycol, but at a generally lower level. In such cases, the propylene glycol may have been used as a flavor-carrier in place of ethanol although at the levels found, it could also have humectant properties.

Next, we will discuss the endogenous tobacco constituents such as ammonia, nitrate, and nicotine. Levels of these constituents can be indicative of the tobacco blends and processes used. Most tobaccos used for STP are air-cured tobaccos. These tobaccos tend to be high in nitrate. In addition, most tobaccos used in STP are fermented to some degree, which is known to reduce the carbohydrate and protein contents of tobaccos. Thus, analytes such as nitrate may show higher levels than expected. Nitrate

values alone allow separation of the products into three classes: (1) moist snuffs and related such as pouched snus; (2) low-moisture snuffs; and (3) other products. For example, if one discounts the added sugars and humectants to the two US-style chewing tobaccos, then they would have nitrate levels approaching those of the moist snuffs. Another parameter is product ammonia. Ammonia can come from three sources: (1) endogenous ammonia (air-cured tobaccos generally higher than flue-cured tobaccos); (2) ammonia generated from the fermentation of proteins; and (3) added ammonium salts, such as ammonium carbonate, used to adjust product pH. However, ammonia can also be lost through: (1) processing such as tobacco drying; (2) reaction with carbohydrates and other reducing substances in the product; and (3) migration through packaging during distribution and storage. Thus, for example, we do not know if moist snuff products with over 10,000-ppm ammonia are really different from those with less than 10,000-ppm ammonia, but over 1000-ppm ammonia. The products with less than 1000-ppm ammonia are almost exclusively the low moisture snuffs. Together with the nitrate data, the ammonia levels suggest that the low-moisture snuffs were made with predominately flue-cured tobaccos. Moisture-adjusted nicotine data again show a split between the moist-snuff products and the low-moisture snuffs with the former having higher levels than the latter. This is likely a design feature based on the differences in how the two types of products are used. In addition, the moisture levels found in the low-moisture snuff products were higher than the moisture levels typically found in so-called dry snuff products such as the 1S2 reference smokeless tobacco product (North Carolina Agricultural Research Service, 2006).

3.3. Results from trace component determinations

Most of the alleged health effects of STP usage are reportedly due to the trace-level components in STP. Much attention has been given to levels of TSNAs, particularly, NNK; B[*a*]P (benzo[*a*]-pyrene), and heavy metals in the STP. Data for the sample set are shown in Tables 2a and 2b. Again, these data split along product types, but for reasons that may not be completely obvious. For example, moist snuff products have higher levels of TSNAs than do low-moisture snuffs. The difference is believed due to fermentation conditions and levels of product nitrate (a source of the nitrite needed for TSNA synthesis). However, B[*a*]P levels tend to be higher in moist snuff products than they are in low-moisture snuff. Moist snuff products are generally formulated with fire-cured tobaccos because of the unique flavor characteristics generated by the pyrolysis of lignin in the hardwood used to fuel the curing fires (Leffingwell and Alford, 1997). Fire-cured tobaccos pick-up B[*a*]P from the burning wood smoke used during the curing (Hoffmann et al., 1986). Tobaccos that have been cured by other methods would have B[*a*]P only from environmental contamination of the leaf surfaces and/or inadvertent exposure to combustion fumes during processing. Heavy metal levels in STP have also been thought to contribute to diseases associated with STP use. The levels reported here are typical of those reported by others. The full chemistry dataset including mean values and standard deviations are shown in Parts A and B of the Supplementary data.

3.4. Results from the cytotoxicity assays

The cytotoxicity data are shown in Part C of the Supplementary data and graphically in Fig. 1a (DMSO extracts only). Only a subset of the main sample set (11 products) was used for this assay. The relative amount of tobacco in a product after taking into account water, humectants, and flavors may be an important factor in product cytotoxicity. The alternate extraction conditions were used to determine if extraction with dichloromethane (DCM) or artificial saliva would extract bioactive constituents that would not be efficiently extracted by DMSO. Fig. 1b and c show the dose–response curves for a smaller subset (4 samples) of the samples that was used for assays with the dichloromethane and artificial saliva extracts, respectively. No significant dose–response relationship could be modeled for the dichloromethane and saliva extracts except for the Manikchand Gutkha (080220). The artificial saliva extracts of that sample were not quite as cytotoxic as the corresponding DMSO extracts. We do not have an explanation for the cytotoxicity of the DMSO and saliva extracts of the gutkha sample.

3.5. Results from the clastogenicity assays

The results from the *in vitro* micronucleus assays are shown in Part D of the Supplementary data and graphically in Figs. 2 and 3 for the DMSO extracts with the 3h−S9 and 3h+S9 conditions, respectively. Only a subset of the main sample set (11 products) was used for this assay. Most of the assays did not reach the 50% cytotoxicity target (Corvi et al., 2008). As with the cytotoxicity assays, the main driver of activity could be the proportion of tobacco in a given product. When the 3-hour minus S-9 conditions were used with the saliva extract of the Manikchand Gutkha (080220) sample, a higher level of micronuclei were observed than with the other extraction conditions. This elevation was not observed with the 3-hour plus S-9 condition nor was it seen in the DMSO extracts (Figs. 2 and 3). The increased clastogenicity may be caused by reactive oxygen species formed from the reaction of polyphenols and other endogenous constituents in the product with the slaked lime additive (Nair et al., 2004).

3.6. Results from mutagenicity assays

The results of the Ames assays are shown in Part E of the Supplementary data. In many cases, no significant dose-dependent response was observed when the DMSO extracts of the smokeless tobacco products were assayed with TA98 + S9. The dose–response curves for the DMSO extracts of the smokeless tobacco products had shallow and somewhat variable slopes for the assays with TA100 + S9 and TA102 + S9. None of the dose–response data met the twofold rule (Hamada et al., 1994; Cariello and Piegorsch, 1996). However, there is considerable controversy on what constitutes a positive Ames result; and one of the better treatises on this subject is the work by Kim and Margolin (1999). However, the practical significance of slopes of about 20 revertants/mg of dry product is debatable.

4. Discussion of results

The main objective of this study was to determine the levels of analytes in STP's with mandated Canadian reporting requirements. Currently this information is not available in the public domain. Another objective was to compare the cytotoxicity, genotoxicity, and mutagenicity results obtained on extracts of these products. A third objective was the collection of data for product mapping. There was no intent of correlating the results of the chemical tests and bioassays with the epidemiology reported for use of various western-style STP in terms of neoplastic (Nilsson, 1998, 2006; Rodu and Jansson, 2004; Weitkunat et al., 2007); non-neoplastic diseases (Asplund, 2003; Asplund et al., 2003; Kallischnigg et al., 2008); and both disease categories for nasal snuff (Sapundzhiev and Werner, 2003). All but one of the products ana-

lyzed were contemporary western-style products. However, we found little in the way of chemical and toxicological data to support the epidemiological evidence that had been gathered decades in the past.

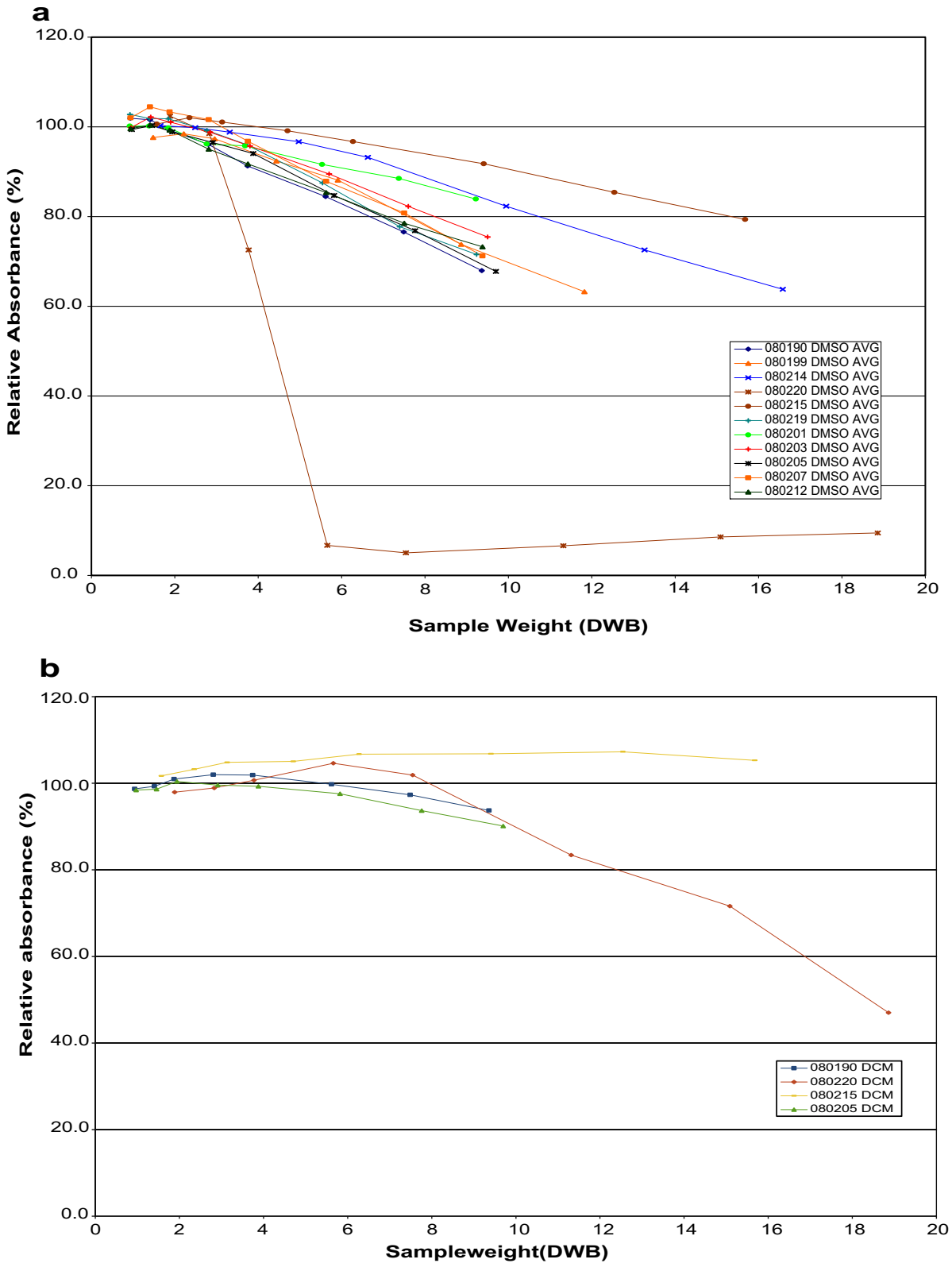

Fig. 1. (a) NRU dose–response curve, which is a plot of Relative Absorbance (%) versus Sample Weight (μg DWB), for the DMSO extracts of the smokeless tobacco samples. The lowest trace (indicating highest cytotoxicity) is for the sample of Manikchand Gutkha. (b) NRU dose–response curve, which is a plot of Relative Absorbance (%) versus Sample Weight (μg DWB), for the DCM extracts of the smokeless tobacco samples. The lowest trace (indicating highest cytotoxicity) is for the sample of Manikchand gutkha. (c) NRU dose–response curve, which is a plot of Relative Absorbance (%) versus Sample Weight (μg DWB), for the artificial saliva extracts of the smokeless tobacco samples. The lowest trace (indicating highest cytotoxicity) is for the sample of Manikchand Gutkha.

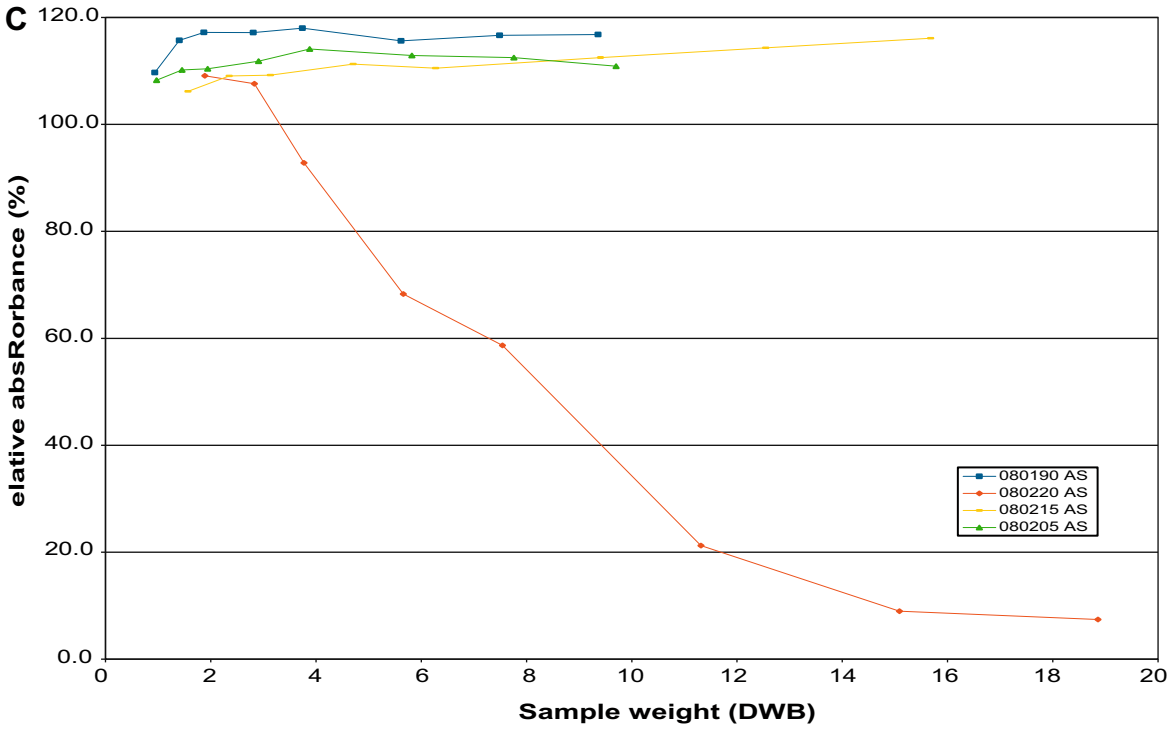

Fig 1. *(continued)*

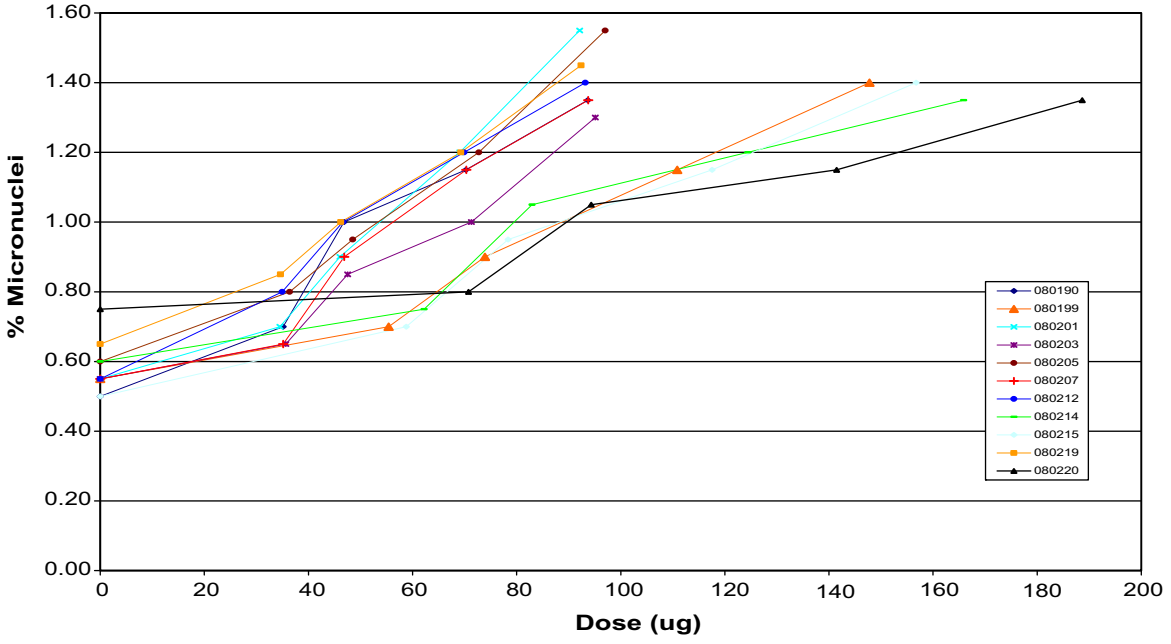

Fig. 2. Dose–response curve for formation of micronuclei with the DMSO extracts smokeless tobacco products in the 3h-S9 part of the assay. Dose is based on dry sample weight in the extracts. The lowest trace (indicating lowest clastogenicity) is for the sample of Manikchand Gutkha.

4.1. Influence of choice of products included in the sample set on overall outcomes

The sample set consisted of fifteen traditional moist snuff products (all manufactured by US Smokeless Tobacco Manufacturing Co. (UST) of Nashville, TN; and imported into Canada; four pouched moist snuff products (all manufactured by UST); two pouched snus-type products reportedly made in Sweden and distributed by Imperial Tobacco Canada; six low-moisture snuff products manufactured in Leicester, England by McChrystal's and imported into Canada; and two US-style chewing tobaccos made by Swedish Match, NA, in Owensboro, KY, and imported into Canada. In addition, the sample set included a sample of Indian Gutkha, which is atypical of western-style STP.

There is no reason to suspect major differences in the basic product composition among similar products from the same

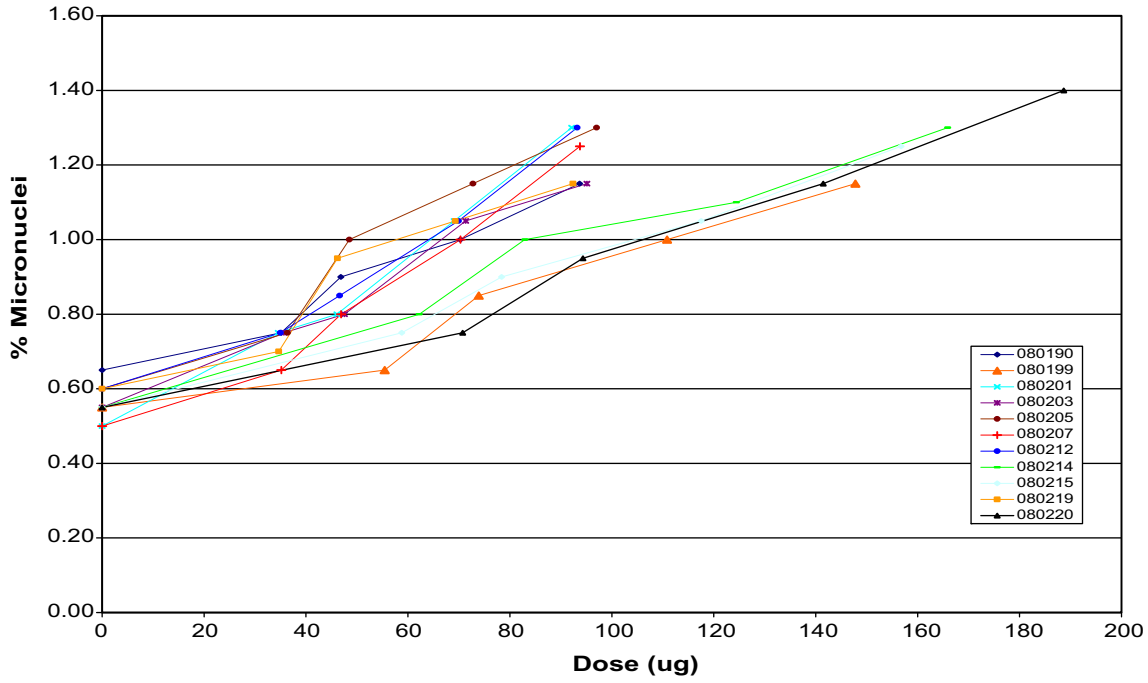

Fig. 3. Dose–response curve for formation of micronuclei with the DMSO extracts smokeless tobacco products in the 3h + S9 part of the assay. Dose is based on dry sample weight in the extracts. The lowest trace (indicating lowest clastogenicity) is for the sample of Manikchand Gutkha.

manufacturer. For example, all the Skoal-brand long-cut flavored moist snuffs were likely prepared from the same blend or very similar blends. Thus, excursions in basic chemical properties and the results of toxicological assays should not be expected. Likewise, little difference in basic composition and toxicological properties should be expected between fine-cut and long-cut products from the same manufacturer. When excursions in the data are found, they may be indicative of loss of product integrity after a product is manufactured, errors in the laboratory, and/or variations in product composition inherent with STP. Thus, the application of statistical tests to compare one product with another may be inappropriate unless one has done sufficient sampling and testing to understand normal production variation and the extent to which it is reflected in the laboratory results. In reviewing the data in this report, it must be remembered that most STP are not sterile; and microbial growth may occur after the product is packaged. Furthermore, all products with the exception of the one product from India, contain sufficient moisture to support microbial growth in the retail package.

4.2. GothiaTek® Standard and results from this study

Several public health experts have stated that STP that meets the GothiaTek® standard (http://www.gothiatek.com/templates/start.aspx?page_id=84) for limits on trace contaminants such as TSNAs, heavy metals, etc., should be suitable as smoking substitutes (Levy et al., 2004). While the GothiaTek® standard was designed for Swedish snus, public health authorities seem to apply it more widely. Table 3 shows the GothiaTek® standard limits for such trace contaminants when the values are expressed on a dry-weight basis and expressed is the same units that are used in Tables 2a and 2b. We did not analyze the STP in this sample set for nitrite and for the volatile nitrosamine, nitrosodimethylamine (NDMA) which are not on the mandated Health Canada list of reportable constituents. Three classes of products in the current data set met the GothiaTek® standard for the analytes we determined: (1) the two brand-styles of Swedish snus; (2) the two brand-styles of chewing tobacco (made by the Swedish Match, NA, which is part of the Swedish Match that developed the GothiaTek® standard); and (3) the McChrystal's low-moisture snuffs. All of the UST samples failed to meet the GothiaTek® limits, as most had total TSNA contents in excess of 10,000 ng/g and all had B[*a*]P contents in excess of 20 ng/g. Fig. 4 illustrates the relationships among analyte levels in the two Swedish-type snus samples and the analyte-level ranges for the other products in this study. Fig. 4 shows that for most analytes, the snus samples contained lower concentrations of the GothiaTek® analytes than did the other samples. The three exceptions are cadmium, nickel, and chromium. The cadmium is believed to be taken in from the soil by the growing tobacco plants (Rickert and Kaiserman, 1994). Nickel and chromium could also come from the soil, but they could also come from the equipment that cuts and grinds the tobacco into the correctly sized particles. Fig. 5 depicts the percentage contribution of individual heavy metal to the total measured metals in each type of STP.

4.3. Results from the determinations of product chemistries

The results we obtained for the moist snuff samples are similar to those reported for the 1S3 and 2S3 reference moist snuffs (IARC, 2007). The analytical data reported for the 1S1 and 2S1 chewing tobacco samples are similar to what we reported for the analytes we measured and for the formulas as posted on the manufacturer's web site (http://www.swedishmatch.com/en/Ourbusiness/Chewing-tobacco/Ingredients-in-chewing-tobacco/). Some may consider the McChrystal's snuff products to be dry snuff as exemplified by the 1S2 reference dry snuff. However, the analytical data shows them to be quite different although they are intended for nasal use. First, the moisture levels are higher and the alkaloid and nitrate levels lower than those of the 1S2 reference dry snuff. Second, the blends likely contain less high-nitrate, fire-cured tobaccos than were used to prepare the 1S2 reference dry snuff. Data from analyses of nasal snuffs similar to the McChrystal's can be found on the SmokeLess New Zealand web site (2007). Much of the information

Table 2a
Trace-level Contaminants in Moist Snuff Products (mean values on DWB).

Sample ID	Brand/style	Sample type	Cd (ng/g)	Cr (ng/g)	Ni (ng/g)	Pb (ng/g)	As (ng/g)	Se (ng/g)	B[*a*]P (ng/g)	NNN (ng/g)	NAT (ng/g)	NAB (ng/g)	NNK (ng/g)	Total TSNA (ng/g)
080202	Copenhagen fine cut	Fine cut moist snuff	1030	1303	1417	412	281	75.0	83.2	5818	5657	557	1694	13726
080207	Skoal fine cut wintergreen	Fine-cut wintergreen flavored moist snuff	939	1231	1339	419	359	82.2	71.3	4896	4036	313	1263	10508
080201	Copenhagen long cut	Long cut moist snuff	865	868	1191	318	237	NQ	79.5	4892	3889	289	1658	10728
080212	Skoal long cut straight	Long cut moist snuff	875	880	1186	311	320	NQ	70.4	5483	4246	366	1806	11901
080208	Skoal long cut apple	Long-cut fruit-flavored moist snuff	904	912	1312	349	289	62.0	44.1	4458	3383	294	1548	9683
080190	Skoal long cut berry	Long-cut fruit-flavored moist snuff	806	937	1201	327	254	NQ	80.1	5679	4070	311	2141	12201
080209	Skoal long cut cherry	Long-cut fruit-flavored moist snuff	967	900	1232	341	241	NQ	76.1	5169	4188	262	1558	11176
080191	Skoal long cut citrus	Long-cut fruit-flavored moist snuff	914	1120	1344	360	314	NQ	49.9	4277	2949	NQ	1587	8814
080211	Skoal long cut peaches	Long-cut fruit-flavored moist snuff	998	964	1262	340	276	NQ	33.1	5561	3861	315	1938	11675
080192	Skoal long cut mint	Long-cut mint-flavored moist snuff	933	928	1199	313	229	NQ	74.0	5983	4564	399	2255	13202
080193	Skoal long cut spearmint	Long-cut mint-flavored moist snuff	976	837	1167	335	247	NQ	73.7	5619	4517	339	1837	12311
080194	Skoal long cut vanilla	Long-cut vanilla-flavored moist snuff	980	1144	1363	384	293	NQ	48.3	4409	3618	NQ	1514	9542
080219	Rooster wintergreen	Long-cut wintergreen-flavored moist snuff	905	974	1341	357	366	NQ	36.7	3864	3516	NQ	1454	8835
080210	Skoal long cut classic	Long-cut wintergreen-flavored moist snuff	889	797	1145	302	218	NQ	64.2	6253	6033	397	1874	14557
080213	Skoal long cut wintergreen	Long-cut wintergreen-flavored moist snuff	975	879	1212	334	280	NQ	62.8	5512	4145	316	2055	12029
080206	Skoal berry pouches	Pouched fruit-flavored moist snuff	967	1035	1451	383	301	66.4	48.3	5048	3826	324	1639	10837
080204	Skoal bandit mint	Pouched mint-flavored moist snuff	1086	1085	1462	410	318	66.6	21.1	6782	4504	416	2496	14198
080203	Copenhagen pouches	Pouched moist snuff	924	1027	1225	389	260	NQ	80.6	4223	3527	310	992	9052
080205	Skoal Bandit wintergreen	Pouched wintergreen-flavored moist snuff	1068	1416	1627	399	292	64.3	21.1	5943	4093	350	2111	12497

NQ, not quantifiable; BDL, below detection limit.

Table 2b
Trace-level Contaminants in Moist Snuff Products (mean values on DWB).

Sample ID	Brand/style	Sample type	Cd (ng/g)	Cr (ng/g)	Ni (ng/g)	Pb (ng/g)	As (ng/g)	Se (ng/g)	B[*a*]P (ng/g)	NNN (ng/g)	NAT (ng/g)	NAB (ng/g)	NNK (ng/g)	Total TSNA (ng/g)
080200	du Maurier Freshmint	Swedish snus mint-flavored	994	1575	1446	242	175	159	1.59	1214	905	NQ	NQ	2119
080199	du Maurier Original	Swedish snus	967	1985	1536	233	143	153	2.08	1212	831	NQ	456	2499
080218	McChrystal's apricot	Low-moisture fruit flavored snuff	300	1307	1509	931	356	NQ	11.8	865	617	NQ	469	1951
080198	McChrystal's mild lemon	Low-moisture fruit flavored snuff	320	1964	1957	627	437	BDL	18.6	922	600	NQ	491	2013
080196	McChrystal's raspberry	Low-moisture fruit flavored snuff	319	2186	2045	690	373	NQ	17.9	849	571	NQ	452	1872
080197	McChrystal's violet	Low-moisture lavender snuff	316	1756	1769	827	392	NQ	17.0	958	591	NQ	487	2036
080216	McChrystal's menthol	Low-moisture mentholated snuff	365	1580	1905	833	396	NQ	17.1	1487	870	139	715	3211
080217	McChrystal's original	Low-moisture snuff	344	1727	1841	1202	388	NQ	16.4	1310	941	NQ	785	3036
080215	Red man	US-style chewing tobacco- loose-leaf	478	714	844	301	168	84.6	BDL	1021	619	BDL	NQ	1640
080214	Apple plug	US-style chewing tobacco -plug	528	1210	1712	365	238	82.0	NQ	2179	829	NQ	378	3385
080220	Manikchand	Indian-style chewing tobacco	BDL	818	1016	NQ	NQ	BDL	183	797	NQ	NQ	NQ	797

NQ, not quantifiable; BDL, below detection limit.

Table 3
GothiaTek® standard data expressed on a dry-weight basis.

Component	Limit (DWB)	Component	Limit (DWB)
Nitrite (ng/g)	7000	Lead (ng/g)	2000
TSNA (ng/g)	10000	Arsenic (ng/g)	500
NDMA (ng/g)	10	Nickel (ng/g)	4500
BaP (ng/g)	20	Chromium (ng/g)	3000
Pesticides	According to the Swedish Match pesticide policy	Cadmium (ng/kg)	1000

Source: http://www.gothiatek.com/templates/start.aspx?page_id=84 accessed July 31, 2008.

available in journal articles and governmental reports on the composition of contemporary STP and the trace contaminants in those products has been summarized in the recent IARC monograph on smokeless tobacco products (IARC 2007). However, as the experiments by Pappas and his fellow scientists showed, the entire amount of each trace metal that can be determined experimentally probably is not all bioavailable, as it could not be fully extracted under conditions of simulated human use (Pappas et al., 2008). Their findings were along the same line as those reported by Maier, Bray, and Pories almost twenty years earlier (Maier et al., 1989). However, all these data together do not account for the potential of these products to cause disease with the possible exception of dental conditions associated with the high sugar levels in US-style chewing tobaccos. Indeed, the biochemistry of the oral mucosal lesions (leukoplakia) associated with use of moist snuff and snus is not well understood although such lesions are reported to disappear when use of such products is stopped (Kallischnigg et al., 2008).

4.4. Results from the in vitro assays for cytotoxicity, mutagenicity, and clastogenicity

One approach toxicologists have for understanding the properties of complex mixtures is the use of broad-based *in vitro* assays such as the Ames assay for mutagenicity, the micronucleus assay for clastogenicity, and the Neutral Red assay for cytotoxicity. Such assays have been successfully used to characterize the toxicological properties of cigarette smoke (CORESTA *in vitro* Toxicology Task Force, 2007). However, when we applied them to extracts of STP, the results were only a small fraction (less than 10%) of those observed for extracts of mainstream cigarette smoke condensate. In the case of mutagenicity, for example, low activities of STP extracts are consistent with the finding by Curvall and colleagues that the mutagen levels in snuff users' urines were no higher than those of in the urines of nonusers, and abstinent snuff users (Curvall et al., 1987).

We as well as other researchers have used the Ames assay to characterize the mutagenic potential of various extracts of STP (Rickert et al., 2007 and references cited therein). In our previous study, which only used six products and only DMSO as the solvent, we found weak and variable dose–response curves. We chose to express the mutagenicity in terms of revertants per milligram of nicotine in the extracts so that we could compare the results to those obtained with smoke condensate. However, this approach was not very satisfactory for comparing the results from STP that differed greatly in nicotine and water contents and relative amount of tobacco per unit product weight.

A review of the cytotoxicity dose–response curves (Fig. 1a–c) expressed on a dry-weight basis helped clarify the situation as other approaches for expressing the amount of sample used (as-is weight, weight per unit nicotine) resulted in dose–response curves that did not appear reasonable. As noted earlier, different types of STP can differ greatly in moisture content. More importantly, they differ even more in the actual tobacco content. Using data from the ingredients section of the Swedish Match web site (Swedish Match, 2008), we estimated the relative amounts of tobacco in various types of STP. For example, the two US-style chewing tobaccos are only about 39% (Apple plug, 080214) and 28% (Red Man, 080215) tobacco. The dose–response curve for 080215 shows less toxicity than does then one for 080214. The water in these products does not contribute to bioactivity, and it is likely that the additives and processing aides other than sodium chloride and other inorganic salts do not contribute to the bioactivity. Swedish Match nasal snuffs that may be competitive with the McChrystal's products contain about 60% tobacco with the remainder being water and additives. Swedish Match moist snuffs sold in North America, and which are in direct competition with the UST moist snuff products, are around 35% tobacco. While on a percent tobacco basis, one would expect similar cytotoxicities from moist snuff and from US-style chewing tobacco, extracts of the former are more cytotoxic than are those of the latter. The observed differences may be due to alkaloid content, salt content, and endogenous tobacco components such as nitrate and nitrite. However, given the success in using dry-weight basis to explain the cytotoxicity data, we also used it for the other bioassays.

The results of the *in vitro* micronucleus assays showed a similar pattern in the dose–response curves (Figs. 2 and 3). While there has been another report of *in vitro* clastogenicity caused by STP (Jansson et al., 1991), this is apparently the first report of the use of DMSO as the main extraction solvent. One interpretation of these results is that on a dry-weight basis, moist snuff is more clastogenetic than the US-style chewing tobaccos. Concentration data for certain analytes such as NNK appeared to correlate with the results of the *in vitro* micronucleus assay. While such correlations were statistically significant, we do not feel that the data reported here are sufficient to conclude that the given analyte caused the biological effect. Another explanation of the results is that the high salt content of the moist snuffs was enough to disturb the osmality of the test solution or that there are phenomena at work that can give false positives (Kirkland et al., 2007). The alkalinity of the culture medium, which can be increased by the alkaline nature of the product being tested, can also give false positives in the *in vitro* micronucleus assay (Kirkland and Müller, 2000). Finally, it must be remembered that the relative amount of micronuclei generated by the STP extracts was small in comparison with what would be expected for cigarette smoke condensate.

Based on our previous work, we thought that the Ames assays for the determination of mutagenicity of the STP extracts would be routine. However, it was not. The mutagenic potencies were weak and variable as in our previous study (Rickert et al., 2007). Based on the twofold rule, none of the extracts gave a positive response with TA98 + S9 as expected. The situations with TA100 + S9 and TA102 + S9 were much more complex in that some replicates of some samples had monotonically increasing dose–response curves while other replicates of the same samples did not [in those cases, Dunnett's *t*-test would not yield significant increases ($P < 0.01$) at two consecutive doses and such findings would argue against mutagenicity (Kirkland and Dean, 1994)]. One approach to the problem would be to combine replicates as suggested by Brusick et al. (1992). The argument against this approach were results within a given type of STP (i.e., all low-moisture snuffs, all long-cut moist snuffs, etc.) were inconsistent with some brand styles showing mutagenicity and others not. Furthermore, these inconsistencies were not consistent with the TA100 + S9 and TA102 + S9 results (e.g., TA100 + S9 results would be negative but TA102 + S9 results would be positive). This leads one to suspect that unknown interferences and/or unique product characteristics were responsible for the inconsistent results. Two potential sources of interference are bacterial metabo-

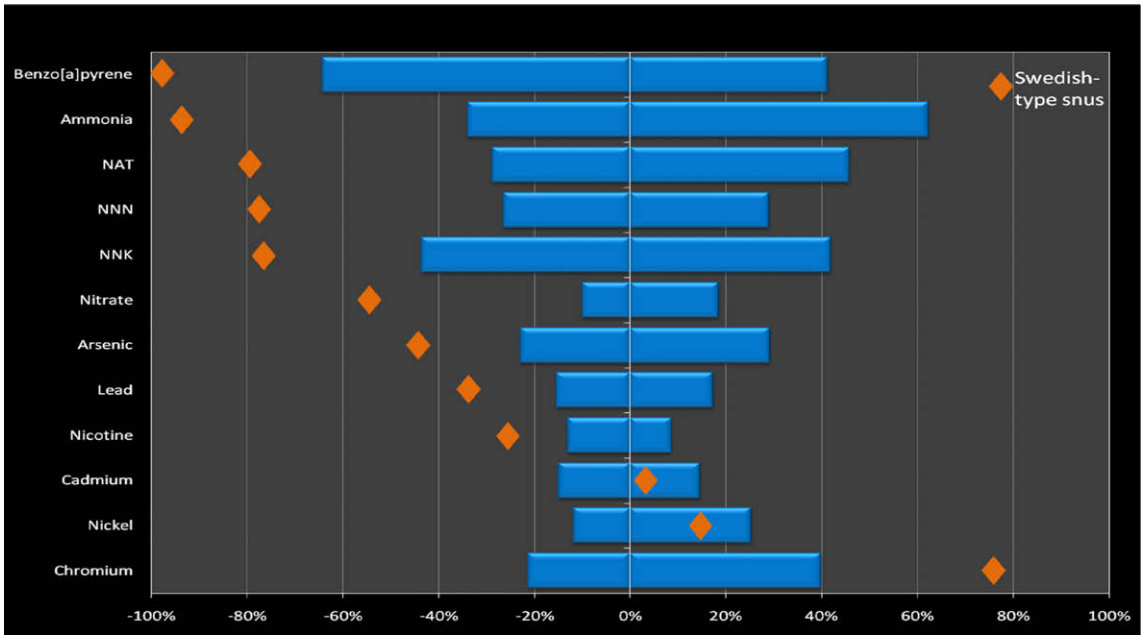

Fig. 4. Market map for several analytes measured on products in this study. Orange-colored diamonds show mean values for the Swedish-type snus products. Blue-colored bars show range of values for the other products in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

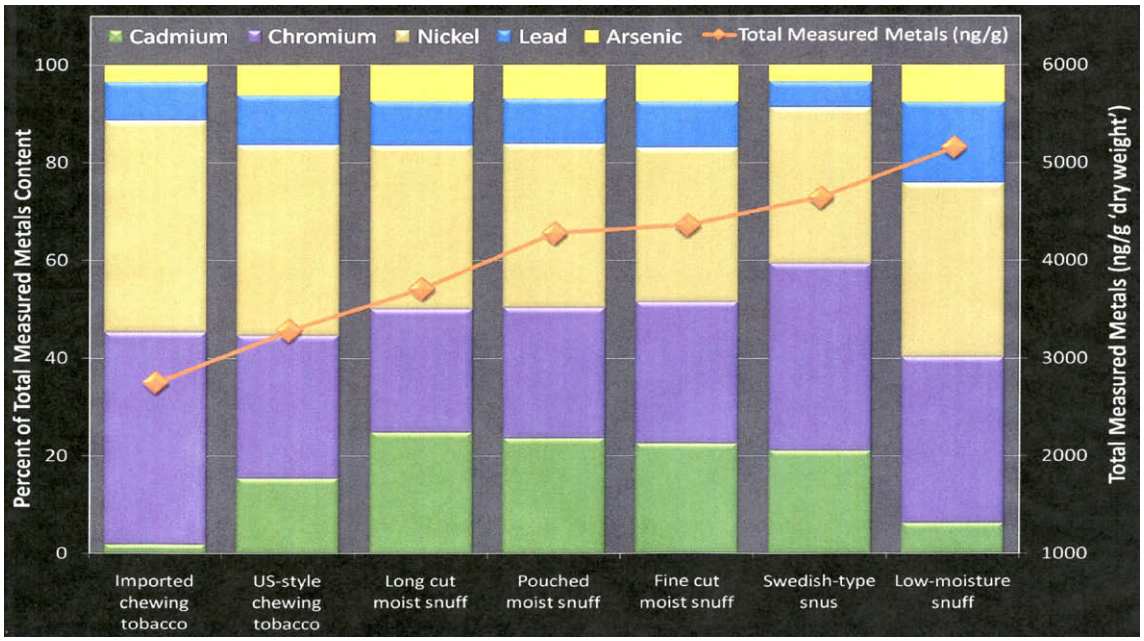

Fig. 5. Relative percent distribution (colored bars) and total content (orange-colored line) of the measured heavy metals in the different types of smokeless tobacco products used in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lites in the products before extraction and filtration of the extracts for sterilization, and nitrate. The presence of bacteria in smokeless tobacco products is well known (Rubinstein and Pedersen, 2002; Fisher and Hill, 1990; Brotzge, 1984). All of the products tested likely contained nitrite. Nitrite is known to give positive results in the Ames assay (Prival et al., 1991). The products also contained nitrate, which could be reduced to nitrite along with the formation of mutagenic dicarbonyl compounds. These dicarbonyl compounds are mutagenic with TA100 and TA102 (Levin et al., 1984; Nagao et al., 1986; Dillon et al., 1998). Such reactions may explain the mutagenicity observed in the two samples for which all three replicates (DMSO extracts with TA102 + S9) of each gave plausible dose–response curves. The two samples were the Manikchand Gutkha (080220) and the Skoal Long Cut Cherry moist snuff (080209). The former is reported to contain reactive oxygen species (Nair et al., 2004). The latter may contain compounds in the cherry flavor that result in mutagens when applied to the moist snuff.

Most STP products contain tobaccos that were fermented to one degree or another and these fermentations can result in the conversion of proteins to amino acids (both D and L) and peptides, which can further react with sugars and carbonyl compounds to

give Maillard reaction products (Ali et al., 2006). These Maillard reaction products are known to be both weak clastogens and weak mutagens (Powrie et al., 1981, 1986; Dills, 1993). Such compounds may be the cause of the observed genotoxicity in the STP extracts. For example, Wei and coworkers reported that the nonvolatile products from the reaction of starch and glycine had a mutagenic potential with TA100 + S9 of about 600 revertants per milligram (Wei et al., 1981). Further work will be needed to understand the causes of the mutagenicity found in many of the assays and to determine if special procedures are needed to achieve consistent results with DMSO extracts of STP.

4.5. Are chemical measurements or one of the three in vitro assays a better predictor of product toxicity than the other assays used?

The purpose of this research was not to compare the predictive powers of the bioassays or the use of chemical measurements versus bioassays. However, do the data tell us anything that might explain the epidemiology associated with the use of contemporary smokeless tobacco products manufactured in the United States and Great Britain? Recent epidemiological studies (Sponsiello-Wang et al., 2008; Boffetta et al., 2008; Kallischnigg et al., 2008; Weitkunat et al., 2007; Luo et al., 2007) continue to show use of contemporary products gives much lower relative risks for disease than associated with smokeless products made and used elsewhere. The presence of snuff-induced lesions (SIL) occurs frequently among users of snus and moist snuff users (Kallischnigg et al., 2008). Perhaps the situation may be best stated by the following quote from Kallischnigg and co-workers.

"In Scandinavia, users have a near 100% prevalence of the characteristic SIL, a lesion which appears not to occur in non-users, though direct evidence from the available publications is limited to one study [20]. In the USA, the types of lesion studied are much more varied. There many snuff users do not have a lesion and some non-users do, but prevalence is much higher in users than in non-users, with reported odds ratios of up to almost 100 [34], and frequency dose-related to daily usage." In the quoted text, Reference 20 refers to Rolandsson et al., 2005, and Reference 34 refers to Ernster et al., 1990.

If we assume that Kallischnigg and colleagues are correct and that the main pathology observed from contemporary STP use is SIL, then there is little likelihood of any correlations, with the exception of nicotine and pH among the data reported herein and any of the epidemiology associated with contemporary STP. It appears that a more promising approach may be to consider the research of Furie and colleagues which suggests that SIL is caused by bacteria and their metabolites that are present in most smokeless tobacco products (Rubinstein and Pedersen, 2002; Furie et al., 2000).

5. Conclusions

In this study, we have used test methodology originally designed for cigarette smoke and cigarette smoke condensates, modified it for use on smokeless tobacco products, and determined the levels of toxicants and bioactivity in such products on the 'current' Canadian market. Many of the products had toxicant levels below or near the levels specified in the GothiaTek® standard. Attempts to use bioassays of cytotoxicity, clastogenicity, and mutagenicity to distinguish among the different types of STP tested were not overly successful because of weak inherent activity and the possibility of yet to be identified interferences present in the products. Consequently, it is likely the procedures currently in place for the study of smoked tobacco products will require further investigation before they can be applied routinely to STP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yrtph.2008.12.004.

References

Alford, E.D., Colby, D.S., Moldoveanu, S.C., 1989. Analytical results regarding direct silylation of the wet snuff. Brown & Williamson File Note, November 3, 1989. Available from: <http://legacy.library.ucsf.edu/tid/xtj51f00> (accessed 26.07.2008).

Ali, H., Pätzold, R., Brückner, H., 2006. Determination of L- and D-amino acids in smokeless tobacco products and tobacco. Food Chemistry 99, 803–812.

AOAC, 1990. AOAC Official Method 966.02, Moisture in Tobacco, Gravimetric Method. AOAC International, Gaithersburg, MD.

Asplund, K., Nasic, S., Janlert, U., Stegmayr, B., 2003. Smokeless tobacco as a possible risk factor for stroke in men: a nested case-control study. Stroke 34, 1754–1759.

Asplund, K., 2003. Smokeless tobacco and cardiovascular disease. Prog. Cardiovasc. Dis. 45, 383–394.

Boffetta, P., Hecht, S., Gray, N., Gupta, P., Straif, K., 2008. Smokeless tobacco and cancer. Lancet Oncol. 9, 667–675.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett. 24, 119–124.

Brotzge, K., 1984. Microbial examination of pipe, snuff, & chewing tobacco products Fall/Winter, 1983. Brown & Williamson Report, June 22, 1984. Available from: <http://legacy.library.ucsf.edu/tid/zcj41f00/> (accessed 31.01.2007).

Brusick, D.J., Ashby, J., de Serres, F.J., Lohman, P.H., Matsushima, T., Matter, B.E., Mendelsohn, M.L., Moore 2nd, D.H., Nesnow, S., Waters, M.D., 1992. International Commission for Protection against Environmental Mutagens and Carcinogens. A method for combining and comparing short-term genotoxicity test data: preface. A report from ICPEMC Committee 1. Mutat. Res. 266, 1–6.

Cariello, N.F., Piegorsch, W.W., 1996. The Ames test: the two-fold rule revisited. Mutat. Res. 369, 23–31.

CDC, 2000. The Surgeon General's report on reducing tobacco use: warning label fact sheet. Available from: <http://www.cdc.gov/tobacco/sgr/sgr_2000/warninglabel.pdf/> (accessed 31.01.2007).

CDC, 1997. Protocol to measure the quantity of nicotine contained in smokeless tobacco products manufactured, imported, or packaged in the United States. Federal Register 62, 24116–24119.

Clarke, M.B., Bezabeh, D.Z., Howard, C.T., 2006. Determination of carbohydrates in tobacco products by liquid chromatography-mass spectrometry/mass spectrometry: a comparison with ion chromatography and application to product discrimination. J. Agric. Food Chem. 54, 1975–1981.

CORESTA *in vitro* Toxicology Task Force, 2007. Report on interlaboratory study of the in vitro toxicity of particulate matter from four cigarettes (Ames, Neutral Red Cytotoxicity and Micronucleus Assays). Available from: <http://www.coresta.org/Reports/IVT_TF_Report_Particulate_Matter_Tox.pdf>

Corvi, R., Albertini, S., Hartung, T., Hoffmann, S., Maurici, D., Pfuhler, S., van Benthem, J., Vanparys, P., 2008. ECVAM retrospective validation of *in vitro* micronucleus test (MNT). Mutagenesis 23, 271–283.

Counts, M.E., Hsu, F.S., Tewes, F.J., 2006. Development of a commercial cigarette "market map" comparison methodology for evaluating new or non-conventional cigarettes. Regul. Toxicol. Pharmacol. 46, 225–242.

Curvall, M., Romert, L., Norlen, E., Enzell, C.R., 1987. Mutagen levels in urine from snuff users, cigarette smokers, and non tobacco users-a comparison. Mutat. Res. 188, 105–110.

Dillon, D., Combes, R., Zeiger, E., 1998. The effectiveness of Salmonella strains TA100, TA102 and TA104 for detecting mutagenicity of some aldehydes and peroxides. Mutagenesis 13, 19–26.

Dills Jr., W.L., 1993. Protein fructosylation: fructose and the Maillard reaction. Am. J. Clin. Nutr. (5 Suppl.), 779S–787S.

Ernster, V.L., Grady, D.G., Greene, J.C., Walsh, M., Robertson, P., Daniels, T.E., Benowitz, N., Siegel, D., Gerbert, B., Hauck, W.W., 1990. Smokeless tobacco use and health effects among baseball players. JAMA 264, 218–224.

European Smokeless Tobacco Council, 2007. Science and Harm reduction. Available from: <http://estoc.org/ScienceAndHarmReduction.asp> (accessed 25.07.2008).

Federal Trade Commission, 2007. Federal Trade Commission Smokeless Tobacco Report for the Years 2002–2005. Available from: <http://www.ftc.gov/reports/tobacco/02-05smokeless0623105.pdf> (accessed 27.07.2008).

Fisher, P.R., Hill, J.A., 1990. Microbial examination of pipe, snuff, and chewing tobacco products Spring/Summer 1990. Brown & Williamson Report, August 7, 1990. Available from: <http://legacy.library.ucsf.edu/tid/hzt03f00/> (accessed 31.01.2007).

Furie, M.B., Raffanello, J.A., Gergel, E.I., Lisinski, T.J., Horb, L.D., 2000. Extracts of smokeless tobacco induce pro-inflammatory changes in cultured human vascular endothelial cells. Immunopharmacology 47, 13–23.

Geiss, V.L., Wofford, J.C., Moldoveanu, S.C., 1990. Analytical results on free and total amino acids in several wet snuff samples. Brown & Williamson File Note, October 8, 1990. Available from: <http://legacy.library.ucsf.edu/tid/lvv21f00> (accessed 26.07.2008).

GothiaTek®, 2008. GothiaTek Standard, Product Requirements, GothiaTek® limits for undesired components. Available from: http://www.gothiatek.com/templates/start.aspx?page_id=84 (accessed 31.07.2008).

Hamada, C., Wada, T., Sakamoto, Y., 1994. Statistical characterization of negative control data in the Ames Salmonella/microsome test. Environ. Health Perspect. 102 (Suppl. 1), 115–119.
Health Canada, 2004a. Bacterial reverse mutation assay for mainstream tobacco smoke, Official method T-501, second ed. A copy of this method can be obtained by sending an e-mail to TRR_RRRT@hc-sc.gc.ca or calling 1-866-318-1116.
Health Canada, 2004b. Neutral Red Uptake Assay for Mainstream Tobacco Smoke, Official method T-502, second ed. A copy of this method can be obtained by sending an email to TRR_RRRT@hc-sc.gc.ca or calling 1-866-318-1116.
Health Canada, 2004c. In Vitro Micronucleus Assay for Mainstream Tobacco Smoke, Official method T-503, second ed. A copy of this method can be obtained by sending an email to TRR_RRRT@hc-sc.gc.ca or calling 1-866-318-1116.
Hoffmann, D., Djordjevic, M.V., Brunnemann, K.D., 1991. New brands of oral snuff. Food Chem. Toxicol. 29, 65–68.
Hoffmann, D., Harley, N.H., Fisenne, I., Adams, J.D., Brunnemann, K.D., 1986. Carcinogenic agents in snuff. J. Natl. Cancer Inst. 76, 435–437.
IARC, 2007. Smokeless tobacco and some tobacco-specific *N*-nitrosamines. IARC Monogr. Eval. Carcinog. Risks Hum. 89, 1–592.
Jansson, T., Romert, L., Magnusson, J., Jenssen, D., 1991. Genotoxicity testing of extracts of a Swedish moist oral snuff. Mutat. Res. 261, 101–115.
Kallischnigg, G., Weitkunat, R., Lee, P.N., 2008. Systematic review of the relation between smokeless tobacco and non-neoplastic oral diseases in Europe and the United States. BMC Oral Health 8, 13.
Kim, B.S., Margolin, B.H., 1999. Statistical methods for the Ames Salmonella assay: a review. Mutat. Res. 436, 113–122.
Kirkland, D.J., Aardema, M., Banduhn, N., Carmichael, P., Fautz, R., Meunier, J.R., Pfuhler, S., 2007. In vitro approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive in vitro genotoxicity results. Mutagenesis 22, 161–175.
Kirkland, D.J., Müller, L., 2000. Interpretation of the biological relevance of genotoxicity test results: the importance of thresholds. Mutat. Res. 464, 137–147.
Kirkland, D.J., Dean, S.W., 1994. On the need for confirmation of negative genotoxicity results *in vitro* and on the usefulness of mammalian cell mutation tests in a core battery: experiences of a contract research laboratory. Mutagenesis 9, 491–501.
Laugesen, M., 2007. Snuffing out cigarette sales and the smoking deaths epidemic. N. Z. Med. J. 120 (1256), U2587.
Lauterbach, J.H., 2008. Are we focusing on the right toxicants for assessing the toxicological properties of smokeless tobacco products? 236th ACS National Meeting, Philadelphia, PA, August 17–21, 2008. Washington, DC, American Chemical Society TOXI-94.
Leffingwell, J.C., Alford, E.D., 1997. Phenolic aroma constituents of Kentucky fired-cured tobaccos. Available from: <http://www.leffingwell.com/firecured.htm> (accessed 27.08.2008).
Levin, D.E., Marnett, L.J., Ames, B.N., 1984. Spontaneous and mutagen-induced deletions: mechanistic studies in Salmonella tester strain TA102. Proc. Natl. Acad. Sci USA 81, 44574461.
Levy, D.T., Mumford, E.A., Cummings, K.M., Gilpin, E.A., Giovino, G., Hyland, A., Sweanor, D., Warner, K.E., 2004. The relative risks of a low-nitrosamine smokeless tobacco product compared with smoking cigarettes: estimates of a panel of experts. Cancer Epidemiol. Biomarkers Prev. 13, 2035–2042.
Luo, J., Ye, W., Zendehdel, K., Adami, J., Adami, H.O., Boffetta, P., Nyrén, O., 2007. Oral use of Swedish moist snuff (snus) and risk for cancer of the mouth, lung, and pancreas in male construction workers: a retrospective cohort study. Lancet 369, 2015–2020.
Maier, R.H., Bray, J.T., Pories, W.J., 1989. Trace element status of some commercial smokeless tobaccos. J. Toxicol. Environ. Health 28, 171–181.
McNeill, A., Bedi, R., Islam, S., Alkhatib, M.N., West, R., 2006. Levels of toxins in oral tobacco products in the UK. Tob. Control 15, 64–67.
Moldoveanu, S.C., Colby, D.S., 1990. Current DS Scan analytical work for monitoring the fermentation of the wet snuff. Brown & Williamson File Note, February 19, 1990. Available from: <http://legacy.library.ucsf.edu/tid/pvv21f00> (accessed 27.07.2008).
Nagao, M., Fujita, Y., Wakabayashi, K., Nukaya, H., Kosuge, T., Sugimura, T., 1986. Mutagens in coffee and other beverages. Environ. Health Perspect. 67, 89–91.
Nair, U., Bartsch, H., Nair, J., 2004. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. Mutagenesis 19, 251–262.
Nilsson, R., 2006. De minimus non curat lex—virtual thresholds for cancer initiation by tobacco specific nitrosamines—prospects for harm reduction by smokeless tobacco. Int. J. Occup. Med. Environ. Health 19, 6–35.
Nilsson, R., 1998. A qualitative and quantitative risk assessment of snuff dipping. Regul. Toxicol. Pharmacol. 28, 1–16.
North Carolina Agricultural Research Service, 2006. The Reference Smokeless Tobacco Products and analytical data on them are available from the Tobacco Analytical Lab, Crop Science Dept., Box 8604, North Carolina State University, Raleigh, NC 27695–8604.
Nova's Bakery, 2008. Nova's Bakery product ingredient list. Available from: <http://novasbakery.com/ingredients.htm> (accessed 18.06.2008).
Pappas, R.S., Stanfill, S.B., Watson, C.H., Ashley, D.L., 2008. Analysis of toxic metals in commercial moist snuff and Alaskan iqmik. J. Anal. Toxicol. 32, 281–291.
Phillips, C.V., Wang, C., Guenzel, B., 2005. You might as well smoke; the misleading and harmful public message about smokeless tobacco. BMC Public Health 5, 31.
Powrie, W.D., Wu, C.H., Molund, V.P., 1986. Browning reaction systems as sources of mutagens and antimutagens. Environ. Health Perspect. 67, 47–54.
Powrie, W.D., Wu, C.H., Rosin, M.P., Stich, H.P., 1981. Clastogenic and mutagenic activities of Maillard reaction model systems. J. Food Sci. 46, 1433–1438 (1445).
Prival, M.J., Simmon, V.F., Mortelmans, K.E., 1991. Bacterial mutagenicity testing of 49 food ingredients gives very few positive results. Mutat. Res. 260, 321–329.
Rickert, W.S., Wright, W.G., Trivedi, A.H., Momin, R.A., Lauterbach, J.H., 2007. A comparative study of the mutagenicity of various types of tobacco products. Regul. Toxicol. Pharmacol. 48, 320–330.
Rickert, W.S., Kaiserman, M.J., 1994. Levels of lead, cadmium, and mercury in Canadian cigarette tobacco as indicators of environmental change: results from a 21 year study (1968–1988). Environ. Sci. Technol. 28, 924–927.
Rodu, B., Jansson, C., 2004. Smokeless tobacco and oral cancer: a review of the risks and determinants. Crit. Rev. Oral Biol. Med. 15, 252–263.
Rolandsson, M., Hellqvist, L., Lindqvist, L., Hugoson, A., 2005. Effects of snuff on the oral health status of adolescent males: a comparative study. Oral Health Prev. Dent. 3, 77–85.
Rubinstein, I., Pedersen, G.W., 2002. Bacillus species are present in chewing tobacco sold in the United States and evoke plasma exudation from the oral mucosa. Clin. Diagn. Lab. Immunol. 9, 1057–1060.
Russell, M.A., Jarvis, M.J., Feyerabend, C., Fernö, O., 1983. Nasal nicotine solution: a potential aid to giving up smoking? Br. Med. J. (Clin. Res. Ed.) 286, 683–6844.
Russell, M.A., Jarvis, M.J., Devitt, G., Feyerabend, C., 1981. Nicotine intake by snuff users. Br. Med. J. (Clin. Res. Ed.) 283, 814–817.
Sapundzhiev, N., Werner, J.A., 2003. Nasal snuff: historical review and health related aspects. J. Laryngol. Otol. 117, 686–691.
SCENIHR, 2008. Scientific opinion on the Health Effects of Smokeless Tobacco Products, 6 February, Scientific Committee on Emerging and Newly Identified Health Risks, European Commission, Health & Consumer Protection DG Directorate C: Public Health and Risk Assessment Unit C7—Risk Assessment Office: B232 B-1049 Brussels.
Schneider, N.G., Lunell, E., Olmstead, R.E., Fagerstrom, K.O., 1996. Clinical pharmacokinetics of nasal nicotine delivery. A review and comparison to other nicotine systems. Clin. Pharmacokinet. 31, 65–80.
SmokeLess New Zealand, 2007. Nasal snuff—chemical analyses. Available from: <http://www.smokeless.org.nz/nasalsnuff_analyses.pdf> (accessed 7.07.2008).
Snider, J., Kaiserman, M.J., 2007. Smokeless tobacco: use and perceived risks in Canada. Abstract POS1-56, Abstracts 2007 meeting of Society for Research on Nicotine and Tobacco. Available from: <http://www.srnt.org/meeting/2007/pdf/onsite/2007SRNTAbstracts-FINAL.pdf> (accessed 7.08.2008).
Sponsiello-Wang, Z., Weitkunat, R., Lee, P.N., 2008. Systematic review of the relation between smokeless tobacco and cancer of the pancreas in Europe and North America. BMC Cancer 2008 8, 356.
Stamm, S.C., Zhong, B.Z., Whong, W.Z., Ong, T., 1994. Mutagenicity of coal-dust and smokeless-tobacco extracts in Salmonella typhimurium strains with differing levels of *O*-acetyltransferase activities. Mutat. Res. 321, 253–264.
Sundén, L., 2001. Swedish Match AGM Address 2001, April 24, 2001. Available from: <http://www.swedishmatch.com/en/Corporate-Governance/Annual-General-Meeting/AGM-adress-and-minutes/AGM-address-2001/> (accessed 23.05.2008).
Surgeon General, 1986. The Health Consequences of Using Smokeless Tobacco. Surgeon General's Advisory Committee on the Health Consequences of Using Smokeless Tobacco. NIH Publication No. 86-2874, United States Public Health Service.
Swedish Match, 2001. GothiaTek Swedish Snus, Introduction, September 25, 2001. Available from: <http://web.archive.org/web/20010925150823/http://www.gothiatek.com/>.
Swedish Match, 2008. Brand-by-brand list. Available from: <http://www.swedishmatch.com/en/Our-business/Snuff-and-snus/Ingredients-in-snuff/By-brand-list/> (accessed 26.08.2008).
Van Hummelen, P., Kirsch-Volders, M., 1990. An improved method for the 'in vitro' micronucleus test using human lymphocytes. Mutagenesis 5, 203–204.
van Ruth, S.M., Grossmann, I., Geary, M., Delahunty, C.M., 2001. Interactions between artificial saliva and 20 aroma compounds in water and oil model systems. J. Agric. Food Chem. 49, 2409–2413.
Wahlberg, I., Ringberger, T., 1999. Smokeless tobacco. In: Davis, D.L., Nielsen, M.T. (Eds.), Tobacco Production, Chemistry, and Technology. Blackwell Science, Malden, MA, pp. 452–460.
Waterbor, J.W., Adams, R.M., Robinson, J.M., Crabtree, F.G., Accordt, N.A., Gilliland, M.J., 2004. Disparities between public health educational materials and the scientific evidence that smokeless tobacco use causes cancer. J. Cancer Educ. 19, 17–28.
Wei, C.I., Kitamura, K., Shibamoto, T., 1981. Mutagenicity of Maillard browning products obtained from a starch—glycine model system. Food Cosmet. Toxicol. 19, 749–751.
Weitkunat, R., Sanders, E., Lee, P.N., 2007. Meta-analysis of the relation between European and American smokeless tobacco and oral cancer. BMC Public Health 7, 334.
Williamson, J., Proctor, C., 2007. Should the health community promote smokeless tobacco (snus): Comments from British American Tobacco. PLoS Med. 4(10), e300. 10.1371/journal.pmed.0040300 (accessed 25.07.2008).
Williamson, J., Massey, E.D., Digard, H., 2007. Constituent comparison in smokeless tobacco products used in Europe. Poster presented at the 9th Annual Society for Research on Nicotine and Tobacco Europe Conference, Madrid, Spain, October 3–6, 2007.
Wyckham, R.G., 1999. Smokeless tobacco in Canada: deterring market development. Tob. Control 8, 411–420.