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A comparative study of the mutagenicity of various types of tobacco products ☆

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Abstract

Toxicological data are an important aspect of tobacco product characterization. In this study, TPM (Total Particulate Matter) (three replicates) was collected from cigarettes [five brands, ISO conditions: puff volume, 35 mL; duration, 2 s; interval, 60 s (35/2/60)], cigars (two brands, 45/2/30), cigarillos (two brands, 35/2/60), bidis (two brands, 45/2/30), and pipe tobacco (two brands, 50/2/12). TPM was extracted from the Cambridge filter pad using dimethyl sulfoxide (DMSO). Smokeless tobacco (ST) (six brands) was extracted with DMSO using an ultrasonic homogenizer. Both types of extracts were filtered and stored at −80 °C. All extracts were analyzed for humectants, water and nicotine. Mutagenic activity was assessed per OECD guideline 471 using *Salmonella typhimurium* TA98+S9 and TA100+S9. TA98+S9 response (specific activity expressed as revertants/mg nicotine) was greatest for the cigarette fabricated with dark, air-cured tobaccos. Average product responses with TA98+S9 based on nicotine and relative to cigarettes (excluding dark tobacco) were cigars, 242%; cigarillos, 238%; bidis, 91%; and pipe tobacco, 44%. ST response was not significant for TA98+S9. Corresponding values for TA100+S9 were cigars, 189%; cigarillos, 155%; pipe tobacco, 130%; bidis, 114% and ST, 34%. ST TA100+S9 response ranged from a low of 501 to a high of 8547 revertants/mg nicotine, depending on ST composition.

Keywords: Ames; *Salmonella typhimurium*; Bidis; Chemistry; Chewing; Cigarettes; Cigars; Mutagenicity; Smoke; Snuff; Tobacco

1. Introduction

In the latter decades of the twentieth century, US cigarette consumption began to decline on account of increased health concerns and taxes. Conversely, sales of other smoking products such as cigars increased after declining for many years (Wehlburg, 1999). Sales of smokeless tobacco products, which had also declined, increased, particularly sales of wet snuff (Surgeon General, 1986). Part of this increase in the sales of noncigarette tobacco products was believed due to consumer perceptions that smokeless tobaccos and cigars were safer than cigarettes. The US Congress mandated warning labels on smokeless tobacco products in 1986. One of those warnings was, "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). Warnings were put on cigar products in 2000 (CDC, 2000).

While most experts see little difference in the health risks associated with smoking noncigarette tobacco products versus smoking cigarettes, 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 of the reasons for debate has been that there are many different kinds of smokeless tobacco products used worldwide. Traditionally, three types of smokeless tobacco products

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have been sold in North America: (1) chewing tobacco (looseleaf, plug, twist); (2) dry snuff; and (3) wet snuff (Surgeon General, 1986; Wahlberg and Ringberger, 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). Some have advocated use of smokeless tobacco products as a substitute for cigarettes for those who cannot or will not stop smoking (Levy et al., 2004). However, others feel that the health risks of such a proposition are too high and that only medicinal nicotine should be used (Hatsukami et al., 2004). While there is considerable epidemiological evidence supporting the use of certain smokeless tobacco products as has been pointed out by experts in the field (Rodu and Godshall, 2006; Nilsson, 2006; Rodu and Jansson, 2004; Bates et al., 2003), there is a lack of bioassays to distinguish smokeless tobacco products deemed less hazardous from those that could be more hazardous. In addition, it would also be desirable to have bioassays to compare the hazards of smokeless tobacco products with smoking products to assist public health officials in making policies on use of smokeless tobacco products. Furthermore, it would be desirable to be able to put the results of such bioassays on a common metric to compare all tobacco products whether smoking or not.

There are no indications that use of smokeless tobacco products has been associated with smoking-related nonneoplastic lung disease such as chronic obstructive pulmonary disease (Anczak and Nogler, 2003). Furthermore, there is no environmental tobacco smoke (ETS) generated by use of smokeless tobacco. The debate has been focused on the relationships between smokeless tobacco use and various cancers. At least one expert has related all associations between smokeless tobacco and cancer to the tobacco specific nitrosamines (TSNAs) in the products (Nilsson, 2006). However, the TSNA levels in contemporary, commercial smokeless tobacco products as well as mainstream cigarette smoke are thought to be too low to contribute to the mutagenicity as measured by the Ames assay especially in the presence of nicotine (Brown et al., 2001; Grasso et al., 1996; Deaton, 1987). On the other hand, extracts of smokeless tobacco products have been found to be mutagenic (Nair et al., 2004; Niphadkar et al., 1996; Stamm et al., 1994; Jansson et al., 1991; Guttenplan, 1987). Some authors have reported correlations between results of various *in vitro* assays for mutagenicity such as the Ames *Salmonella*/microsome mutagenicity assay with the results on rodent carcinogenicity studies (Mortelmans and Zeiger, 2000; Kim and Margolin, 1999a). Thus, there have been numerous reports over the years of the Ames assay being used to determine and compare the mutagenicity of tobacco smoke condensates as noted in the reviews by DeMarini (2004), Massey (2002). In addition, and as noted above, several researchers had determined the Ames activity of extracts of smokeless tobacco products. However, none of these studies had been designed to compare conventional cigarette products, with other contemporary smoking products as well as contemporary smokeless tobacco products available in the North American market. In the study reported herein, a number of smoking and smokeless tobacco products were obtained and characterized both chemically and with the Ames assay. Furthermore, the results from the Ames assay have been put on a per-unit nicotine basis to permit comparison of smoking and smokeless products on a per unit nicotine basis.

2. Experimental

Smoking products evaluated included several types of filtered cigarettes (dark air-cured, blended, flue-cured), cigarillos, cigars, bidis (made in India), and pipe tobacco. Three types of smokeless tobacco products were evaluated: (1) pouched and loose wet snuff typical of products sold in US and Canada; (2) tableted and loose dry snuff reportedly made with specially cured, low-nitrosamine tobaccos; and (3) two types of smokeless tobacco products from India. The first of these was a gutkha, which is a sweetened mixture of tobacco, betel nut, lime, catechu and other ingredients popular in India. The second product is a chewing tobacco known as zarda. Zarda is a mixture of tobacco flakes, silver leaves, aromatic spices, and synthetic flavors. Zarda is generally chewed in various combinations with other materials such as betel quid or a mixture of areca nut and lime. Tobacco products were obtained at retail from domestic and international markets except for the CIM-7 (Canadian Industry Monitor, 100% flue-cured tobacco by Canadian Tobacco Manufacturers Council, Ottawa, Canada and provided free of charge) and the Kentucky KR2R4F reference cigarettes [Kentucky Tobacco Research and Development Center (formerly known as the Kentucky Tobacco and Health Institute), Lexington, KY 40546–0236, USA]. On receipt, they were stored at 4 °C until laboratory use. Smoking products were conditioned according to ISO 3402 prior to analyses (ISO, 1999). Cigarettes and cigarillos were smoked according to ISO 4387 (35 mL puff, 2-s puff duration, 60-s puff interval) using a Borgwaldt RM-20/CS smoking machine (ISO, 2000). More intensive smoking conditions were not used as they tend to give reduced mutagenicity values when values are expressed as revertants per unit weight of TPM (Roemer et al., 2004; Rickert et al., 2002).

Cigars (using Cerulean SM-400) and bidis (using Borgwaldt RM-20/CS) were smoked under similar conditions except that the puff volume was 45 mL and the puff interval was 30 s (Field et al., 2001). Pipe tobaccos were smoked according to the procedure described by Joza and co-workers with a 50 mL puff of 2-s duration taken every 12 s (Joza et al., 2001). Glycerin and propylene glycol contents of the TPM were measured in addition to water and nicotine. For the mutagenicity studies, TPM was dispersed in DMSO at a concentration of 10 mg TPM/mL, the mixture filtered

through sterile cheesecloth, and stored at −80 °C until just prior to use with the Ames assay.

Smokeless tobacco products were extracted with DMSO according to the following procedure. The tobacco product was dispersed in DMSO (1:9, w/v) using an ultrasonic homogenizer. The dispersion was then incubated at 37 °C for 21 h. The dispersion was then centrifuged and filtered. The extract was stored at −80 °C prior to assay. Nicotine content of the DMSO extract was determined. While it can be envisaged that the bacterial content of smokeless tobacco products (Brotzge, 1984; Fisher and Hill, 1990; Rubinstein and Pedersen, 2002; Warke et al., 1999) might lead to the formation of artifacts from bacterial growth during the extraction process, DMSO has significant antimicrobial activity (Basch and Gadebusch, 1968).

The Ames assays were performed according to internationally accepted protocols (OECD, 1997). Only strains TA98 and TA100 were used and all assays were done with S9 activation with 20-min pre-incubation. The S9 (postmitochondrial supernatant in 0.154 M KCl) used for the Ames Assay was purchased from Molecular Toxicology Inc. (*a/k/a* Moltox Inc.). It came from the livers of male Sprague–Dawley rats induced with Aroclor 1254. Three replicate sets of assays were done for each extract. Mutagenic potency was estimated from the slope of the linear portion of the dose–response curve (Bernstein et al., 1982). The linear range used was 0–125 μg of TPM per plate in the case of smoking tobacco products. The linear range was 0–1389 μg of product per plate in the case of smokeless tobacco products. The corresponding nicotine concentrations were used to calculate slopes in terms of revertants per milligram nicotine. Thus the nicotine range used for the slope calculations was different for each different product.

For the smoking products, the dose–response curves obtained with TA98+S9 and TA100+S9 were very typical of those reported for tobacco smoke condensate. Condensates with a high relative proportion of nicotine gave lower slopes than those with lower proportions of nicotine. There was no significant dose–response 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. None of the dose–response data met the two-fold rule (Hamada et al., 1994; Cariello and Piegorsch, 1996), but gave overall positive slopes for the dose–response curves in the range of 0–1389 μg of product per plate. Therefore, the dose–response data were further evaluated with the SALM program (Kim and Margolin, 1999b). The SALM program was used with both the data from individual replicate extractions (e.g., three plates per dose level per replicate extract) and with the data from all replicates for a given set of extracts pooled (e.g., nine plates per dose level per sample). The SALM program provides a measure of relative potency and provides a statistical test for mutagenicity. The cited paper by Kim and Margolin and the references cited therein should be consulted for the statistical principles used by the SALM software.

3. Results

The results for the mainstream smoke chemical analyses are shown in Table 1. The results obtained are typical for the products analyzed. The TPM from the dark tobacco cigarette and from the CIM-7 flue-cured reference cigarette did not contain detectable glycerin or propylene glycol. This is consistent with what is known about the products. Likewise, the lack of propylene glycol in the TPM from the KY2R4F reference cigarette is consistent with the published formulation.

The results for the chemical analyses of the smokeless tobacco products are shown in Table 2. The two low-moisture snuff products contain lower percentages of nicotine than the percentage reported for the 1S2 reference dry snuff (NCSU, 2006). The presence of glycerin and menthol in both low-moisture products is also atypical of the 1S2. The two moist snuff products have nicotine concentrations typical of the 2S3 reference moist snuff (NCSU, 2006) and those reported for commercial products (Richter and Spierto, 2003).

Tables 3 and 4 give the results of the mutagenicity assays on TPM for TA98+S9 and TA100+S9, respectively. The data in Tables 3 and 4 are presented on a per milligram TPM basis. These data are presented so that readers may compare our results with others in the literature that are presented on a per milligram TPM basis. The results for the cigarette products are unremarkable and are well within the range of expected values for the types of cigarettes analyzed with the TPM generated under ISO conditions. The relative activity reported here of the TPM from the clove cigarette (kreteks) being less than that of the TPM from the KR2R4F confirms the findings in an unpublished INBIFO report (Roemer, 2002). Another unpublished INBIFO report covered the mutagenicity of the TPM from cigarillos (Gomm et al., 1996) and that study showed that the mutagenicity of cigarillo TPM was similar to that of the KY1R4F reference cigarette.

Tables 5 and 6 show the data for TA98+S9 and TA100+S9, respectively, when estimated per milligram of delivered nicotine for each of the smoking products. As expected, products with air-cured (burley, dark air-cured) tobaccos gave a higher number of revertants per milligram of nicotine than did the flue-cured products. This finding is believed due to the relatively higher amounts of nitrogen-containing, mutagenic pyrolysis products found in the TPM of air-cured tobaccos than found in flue-cured tobaccos.

One of the more interesting findings from this research was that under the conditions of the assays, the extracts of the smokeless tobacco products were not mutagenic with TA98+S9. Table 7 shows the results of the mutagenicity assays with TA100+S9 on the extracts of the smokeless

Table 1
Smoke analyses

Sample	Weight (mg/unit)		Puff count (per unit)		MS TPM (mg/unit)		CO (mg/unit)		Water (mg/unit)		Nicotine (mg/unit)		Tar (mg/unit)		Propylene glycol (mg/unit)		Glycerol (mg/unit)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Flue-cured cigarette	1112	10	10.7	0.5	24.6	1.8	15.5	0.4	4.24	0.45	1.49	0.12	18.9	1.2	BDL	BDL	2.12	0.13
Clove cigarette	789	12	7.45	0.19	11.0	0.0	6.80	0.2	0.76	0.02	0.74	0.01	9.51	0.05	0.151	0.007	0.834	0.043
Dark tobacco cigarette	969	6	6.54	0.13	12.6	0.6	14.6	0.6	1.00	0.22	0.45	0.01	11.2	0.4	NQ	NQ	NQ	NQ
CIM-7	972	5	7.99	0.21	14.6	0.4	12.4	0.3	1.53	0.09	1.03	0.02	12.0	0.4	BDL	BDL	BDL	BDL
KR2R4F	1076	5	8.79	0.25	10.9	0.3	12.1	0.5	0.80	0.04	0.75	0.02	9.33	0.26	NQ	NQ	0.947	0.027
Cigarillo 1	1075	33	11.6	0.4	49.2	3.3	52.3	1.7	8.32	0.85	2.28	0.09	38.6	2.4	0.310	0.025	0.400	0.013
Cigarillo 2	1077	9	12.0	0.7	58.2	3.8	52.7	2.9	12.0	1.3	2.7	0.02	43.5	2.5	BDL	BDL	BDL	BDL
Bidi 1	371	16	13.9	1.8	50.1	8.7	19.5	2.3	11.8	0.9	2.12	0.39	36.2	8.4	BDL	BDL	BDL	BDL
Bidi 2	459	8	16.4	2.5	43.2	12.7	18.1	3.0	11.3	4.1	2.23	0.41	29.6	8.2	BDL	BDL	BDL	BDL
Cigar 1	3415	199	35.8	1.1	229	32	NM	NM	64.4	12.2	9.52	0.34	155	20	2.16	0.20	BDL	BDL
Cigar 2	4309	51	50.5	9.3	215	11	NM	NM	63.6	9.8	6.28	0.06	146	1	1.93	0.14	BDL	BDL
Pipe tobacco 1	1501	1	80.3	25.0	150	21	NM	NM	52.5	2.8	5.34	0.77	91.8	17.7	4.92	0.45	0.734	0.22
Pipe tobacco 2	1501	1	82.9	49.8	198	25	NM	NM	73.5	10.7	6.27	1.03	118	15	1.30	0.36	15.4	3.2

Unit equals one cigarette, cigarillo, bidi, cigar, or portion of pipe tobacco.
Means and standard deviations presented are from three sample replicates.
BDL means below detection limit.
NM means not measured.
NQ means result obtained was below the limit of quantitation for the method.

Table 2
Smokeless tobacco analyses[a]

Sample	Country of origin	Menthol[b] (mg/g)		Nicotine[b] (mg/g)		Glycerol[b] (mg/g)		Dry matter (%)		Moisture (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tableted low-moisture snuff	US	5.94	0.07	5.71	0.34	0.314	0.044	96.7	0.2	3.29	0.24
Low-moisture wintergreen snuff	US	6.44	0.15	9.17	0.06	0.561	0.025	95.7	0.2	4.34	0.23
Gutkha	India	7.60	0.18	2.10	0.11	1.97	0.18	92.7	0.3	7.34	0.30
Long-cut fruit-flavored moist snuff	US	BDL	BDL	14.3	0.1	BDL	BDL	48.5	0.1	51.5	0.1
Pouched moist snuff	US	BDL	BDL	13.3	0.4	BDL	BDL	49.4	0.2	50.6	0.2
Zarda chewing tobacco	India	21.8	0.5	26.7	1.0	46.3	1.0	87.5	0.0	12.5	0.0

BDL means below detection limit.
[a] All data reported on an "as is" basis; Means and standard deviations presented are from three sample replicates.
[b] The menthol, nicotine, and glycerol contents of the products were estimated from analysis of the DMSO extracts.

Table 3
Summary of TA98+S9 assays for smoking products, slope values in terms of revertants per milligram TPM

Sample	TPM dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–125	1279	92	1151	62	1062	133	1164	82
Clove cigarette	0–125	1334	86	1264	56	1347	63	1315	58
Dark tobacco cigarette	0–125	2191	116	2641	133	2722	132	2518	87
CIM-7	0–125	1240	44	1040	24	1090	77	1123	45
KR2R4F	0–125	2299	114	2477	280	2213	92	2330	97
Cigarillo 1	0–125	2364	139	2588	176	2262	80	2405	83
Cigarillo 2	0–125	2407	153	2525	112	2593	82	2508	77
Bidi 1	0–125	904	39	990	12	940	54	945	29
Bidi 2	0–125	961	38	1039	68	1062	60	1021	45
Cigar 1	0–125	1585	35	1928	92	1816	58	1776	58
Cigar 2	0–125	1683	55	1888	71	2103	98	1892	76
Pipe tobacco 1	0–125	380	36	441	25	338	25	386	26
Pipe tobacco 2	0–100	283	25	315	49	209	49	269	32

Table 4
Summary of TA100+S9 assays for smoking products, slope values in terms of revertants per milligram TPM

Sample	TPM dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All Replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–125	635	37	666	18	678	27	660	27
Clove cigarette	0–125	555	47	611	18	519	32	562	55
Dark tobacco cigarette	0–125	770	37	525	43	674	101	656	54
CIM-7	0–125	543	29	502	36	568	26	538	26
KR2R4F	0–125	826	78	724	54	653	37	734	87
Cigarillo 1	0–125	653	35	728	29	568	14	650	23
Cigarillo 2	0–125	690	27	819	48	614	20	708	73
Bidi 1	0–125	482	28	469	23	500	35	484	31
Bidi 2	0–125	537	26	425	44	606	23	523	27
Cigar 1	0–125	659	27	589	15	522	43	590	21
Cigar 2	0–125	635	30	654	32	600	25	629	20
Pipe tobacco 1	0–125	337	31	371	23	475	33	394	21
Pipe tobacco 2	0–125	409	25	412	41	451	9	424	20

Table 5
Summary of TA98+S9 assays for smoking products, slope values in terms of revertants per milligram nicotine

Sample	Nicotine dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–8	21,167	1516	19,055	1025	17,573	2198	19,265	1361
Clove cigarette	0–8	19,901	1281	18,856	831	20,100	935	19,619	861
Dark tobacco cigarette	0–4	60,938	3226	73,439	3705	75,696	3680	70,024	2417
CIM-7	0–9	17,590	623	14,758	344	15,471	1093	15,940	643
KR2R4F	0–9	33,237	1645	35,814	4055	31,992	1336	33,681	1404
Cigarillo 1	0–6	50,899	3003	55,722	3783	48,693	1727	51,771	1789
Cigarillo 2	0–6	51,644	3289	54,161	2403	55,624	1755	53,810	1652
Bidi 1	0–5	21,391	923	23,409	288	22,229	1285	22,343	690
Bidi 2	0–7	18,207	727	19,695	1297	20,135	1129	19,346	848
Cigar 1	0–5	37,526	825	45,663	2184	42,993	1365	42,061	1367
Cigar 2	0–4	57,729	1895	64,745	2431	72,126	3366	64,867	2594
Pipe tobacco 1	0–4	10,640	994	12,335	687	9446	693	10,807	723
Pipe tobacco 2	0–4	8945	796	9957	1564	6624	1537	8509	1007

tobacco products. This table shows the slopes of the linear portions of the dose–response curves and the associated error terms. The units for the slopes are revertants per milligram of product nicotine. Table 8 shows the results of using the SALM program on the mutagenicity data. Again, the results are presented on a nicotine-weight, not product-weight basis. Three values are provided for each replicate: (1) potency value, (2) fit p-value, and (3) mutagenicity p-value. The potency value is similar but not the same as the slope of the linear portion of the dose–response curve. Differences in the shape of the dose–response curve can result in potency values that differ from those estimated

Table 6
Summary of TA100+S9 assays for smoking products, slope values in terms of revertants per milligram nicotine

Sample	Nicotine dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–8	10,505	606	11,028	299	11,230	445	10,921	453
Clove cigarette	0–8	8274	700	9115	270	7740	474	8376	815
Dark tobacco cigarette	0–4	21,400	1027	14,609	1198	18,741	2812	18,250	1488
CIM-7	0–9	7709	407	7130	514	8060	374	7633	365
KR2R4F	0–9	11,934	1123	10,464	779	9440	535	10,613	1252
Cigarillo 1	0–6	14,066	756	15,673	626	12,229	296	13,989	502
Cigarillo 2	0–6	14,809	575	17,580	1035	13,175	434	15,188	1563
Bidi 1	0–5	11,407	656	11,092	553	11,822	830	11,440	733
Bidi 2	0–7	10,172	491	8050	833	11,493	431	9905	503
Cigar 1	0–5	15,597	641	13,937	344	12,367	1009	13,967	507
Cigar 2	0–4	21,764	1041	22,431	1089	20,563	849	21,586	670
Pipe tobacco 1	0–4	9425	874	10,395	656	13,284	912	11,035	588
Pipe tobacco 2	0–4	12,935	793	13,031	1283	14,273	298	13,413	642

Table 7
Mutagenicity of extracts of smokeless tobacco products with TA100+S9, slope values in terms of revertants per milligram of nicotine

Sample	Nicotine dose range μg/plate	Country of origin	Replicate 1		Replicate 2		Replicate 3		All replicates	
			Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Tableted low-moisture snuff	0–8	US	6270	990	4760	661	4202	560	5077	463
Low-moisture wintergreen snuff	0–13	US	1421	394	1369	705	2801	194	1863	354
Gutkha	0–3	India	13,902	985	4163	995	7577	1300	8547	1714
Long-cut fruit-flavored moist snuff	0–20	US	1120	146	1585	112	1182	151	1296	119
Pouched moist snuff	0–19	US	1615	170	1990	128	2265	172	1957	117
Zarda chewing tobacco	0–37	India	554	84	286	206	663	138	501	94

by other methods (Kim and Margolin, 1999b). The fit *p*-value describes the quality of the fit between the experimental data and the fitted model: the better the fit, the higher the *p*-value. The mutagenicity *p*-value describes the probability that the potency value is greater than zero. A mutagenicity *p*-value of less than 0.05 indicates mutagenicity. The major differences in results between the two methods for estimating mutagenic potency is that the SALM software indicated that two of the gutkha replicates and one of the low-moisture wintergreen snuff replicates had non-significant slopes while significant slopes were found with the other estimation procedure. An inspection of the dose–response curves showed that in those cases, the dose–response curves did not rise with increasing dose in the expected manner.

The dose–response curves for the extracts of the smokeless tobacco products with TA100+S9 are shown in Figs. 1–4. As noted earlier, none of the dose–response curves (see Figs. 1–4) showed a twofold increase in revertants per plate over background levels; and thus we used the SALM program to provide another estimate of mutagenic potency.

In Fig. 1, the dose–response curves for the two low-moisture snuff products are shown. The legend terms are as follows: (1) TLMS, tableted low-moisture snuff; (2) LMWGS, low-moisture wintergreen snuff. R1, R2 and R3 are the identifiers for the replicate samples. Except for a single step-change in the dose–response curve o Replicate 1 of the extract of the LMWGS, there is no monotonically rising increase in response with increase in dose. This could be the reason the SALM program reported no significant mutagenic potency for that replicate. The dose–response curves for the moist snuff products are shown in Fig. 2. LCFFMS, long-cut fruit-flavored moist snuff; and PMS, pouched moist snuff. These two products appeared to give the most consistent dose–response curves of the products included in this study. This consistency may stem from the fact that both products have high consumer acceptance and are believed to be made in large factories.

Fig. 3 shows the dose–response curves for the Guthka extracts with TA100+S9. Replicate 1 showed significant mutagenicity while the other two replicates did not show significant mutagenicity with the SALM program although slopes could be calculated from the linear parts of the dose–response curves. The dose–response curves for the extracts of Zarda with TA100+S9 are shown in Fig. 4. These curves indicate much more cytotoxicity than did the other samples. This increased cytotoxicity may have been due to the additives to the tobacco.

It is important to note that the SALM software uses all points in the dose–response curve unless the internal error-checking routines delete them as likely errors (Kim and Margolin, 1999b). While use of the twofold rule could result in the conclusion that none of the snuff extracts were mutagenic, the results from the SALM program show that all the samples were mutagenic with TA100+S9.

Table 8
mutagenicity of extracts of smokeless tobacco products with TA100+S9 estimated with SALM program, potency values in terms of revertants per milligram of nicotine

Sample	Nicotine dose range (μg/plate)	Replicate 1			Replicate 2			Replicate 3			All replicates		
		Potency value	Fit *p*-value	Mutagenicity *p*-value	Potency value	Fit *p*-value	Mutagenicity *p*-value	Potency value	Fit *p*-value	Mutagenicity *p*-value	Potency value	Fit *p*-value	Mutagenicity *p*-value
Tableted low-moisture snuff	0–32	11,887	0.147	0.000	9156	0.329	0.000	9729	0.767	0.000	10,275	0.026	0.000
Low-moisture wintergreen snuff	0–51	200	0.192	0.084	2953	0.376	0.000	6981	0.989	0.000	5415	0.783	0.000
Gutkha	0–12	30,049	0.946	0.000	NS	0.332	0.500	31	0.10	0.480	20,579	0.030	0.000
Long-cut fruit-flavored moist snuff	0–80	514	0.526	0.000	3698	0.987	0.000	366	0.156	0.000	2876	0.964	0.000
Pouched moist snuff	0–74	3533	0.486	0.000	4000	0.775	0.000	3893	0.818	0.000	3778	0.188	0.000
Zarda chewing tobacco	0–149	1056	0.961	0.000	1163	0.176	0.000	1159	0.747	0.000	1130	0.840	0.000

Table 9 shows the ratios of the mutagenic potencies for TA98+S9 and TA100+S9 for the TPM from smoking products and pipe tobaccos. Two items of note: (1) products made with air-cured tobaccos gave higher values for the TA98+S9/TA100+S9 ratio than did all flue-cured products; and (2) the two tobacco blends smoked in pipes had values of that ratio that were less than unity. However, that latter finding may be the result of the use of pipes as opposed to the tobacco blend as pipes when compared with tubular smoking articles (e.g., cigarettes, cigars) have been shown to give reduced biological activity with the same blend (Billimoria, 1975). Similar ratios have been noted by other researchers (Sato et al., 1977; Cerna and Angelis, 1985).

4. Discussion

Mutagenicity generally is not an acceptable attribute for a product intended for human consumption. However, some foods and beverages (e.g., broiled meat and coffee) show some evidence of mutagenic activity (Sugimura, 2000). It may be argued that nutrition is essential for life and some foods need to be cooked to minimize the levels of pathogens and toxins, and mutagens arising from such cooking may not present an unreasonable risk of disease. On the other hand, items consumed for pleasure, such as tobacco, preferably should not be mutagenic. Conventional tobacco smoking products produce mutagenic smoke as shown by the experimental data in this article and references cited herein. Even electrically heated cigarettes produce mutagenic TPM albeit at much lower potency than conventional products (Tewes et al., 2003). Therefore, there has been an increased focus on converting smokers who will not quit to users of smokeless tobacco products (Levy et al., 2004). However, not all smokeless tobacco products present the same risk to the user (Rodu and Jansson, 2004). Furthermore, much of the literature on health risks of smokeless tobacco products has focused on TSNAs and NNK, in particular (Nilsson, 2006; Rodu and Jansson, 2004). In addition, the concentrations of TSNAs in smokeless tobacco products are reportedly below the limit of detection for the Ames assay (Grasso et al., 1996). Furthermore, endogenous polyphenolic compounds have been shown to be antimutagenic towards NNK (Miller et al., 1996). Thus, mutagenicity observed is likely caused by yet unidentified compounds that are mutagenic with TA100+S9, but not TA98+S9. For example, some aldehydes and dicarbonyl compounds that have been reported in tobacco products are active with TA100+S9 but not TA98+S9 (Dillon et al., 1998; Aeschbacher et al., 1989). Pyrolysis products from plant materials and carbohydrates have been reported to be mutagenic with TA100+S9 but not TA98+S9 (Derache, 1982; Kitts et al., 2006). Certain Maillard reaction products, which may be created during the curing of tobacco and in the manufacture of tobacco products, could result in mutage-

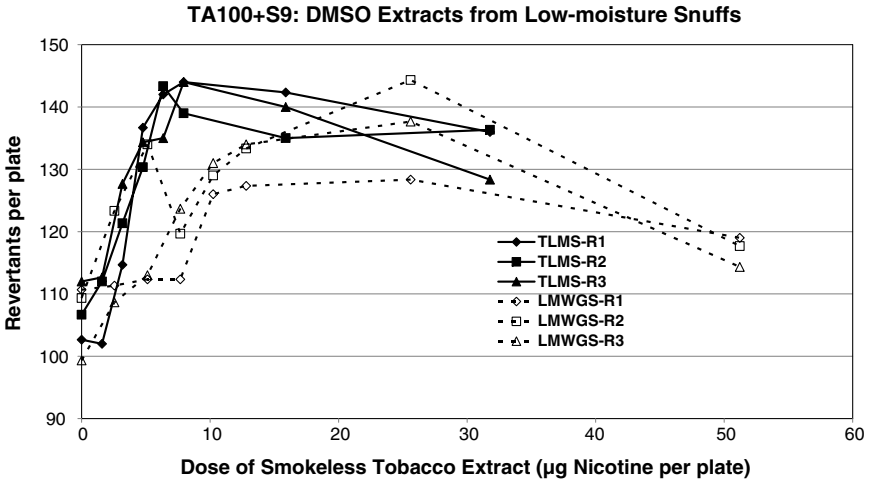

Fig. 1. Dose–response curve for TA100+S9: DMSO extracts from low-moisture snuffs.

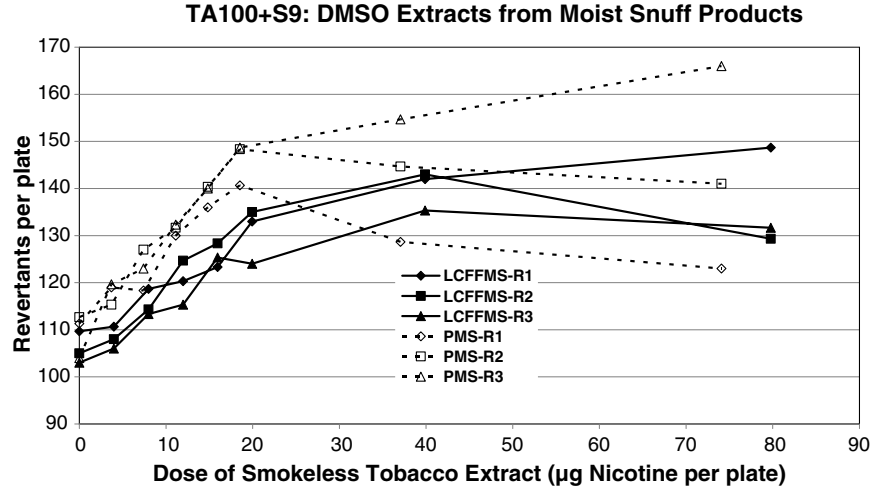

Fig. 2. Dose–response curve for TA100+S9: DMSO extracts from moist snuff products.

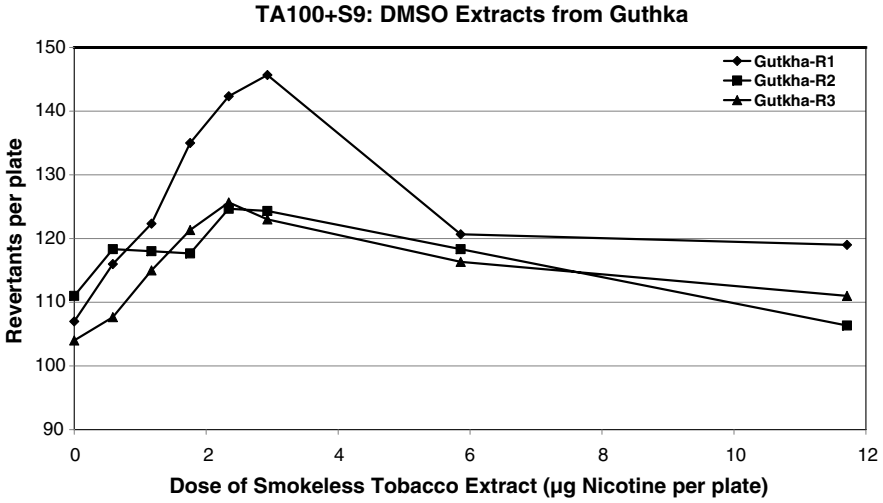

Fig. 3. Dose–response curve for TA100+S9: DMSO extracts from guthka.

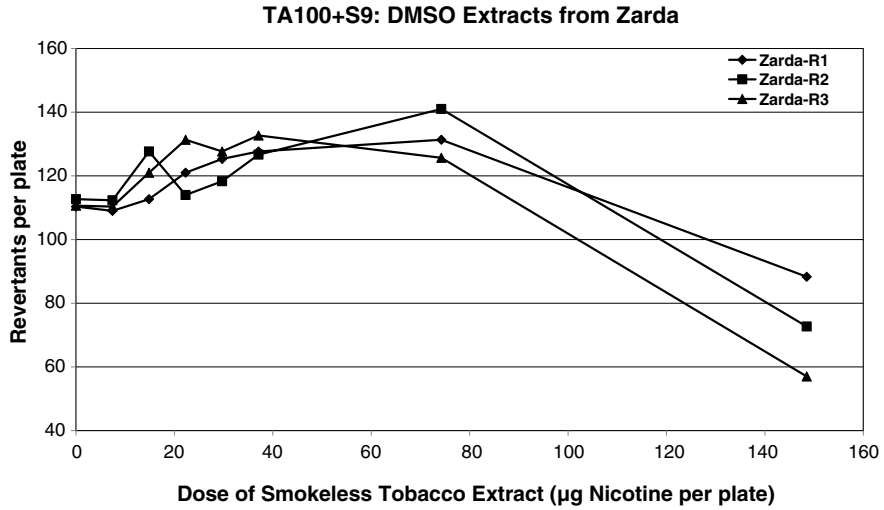

Fig. 4. Dose–response curve for TA100+S9: DMSO extracts from zarda.

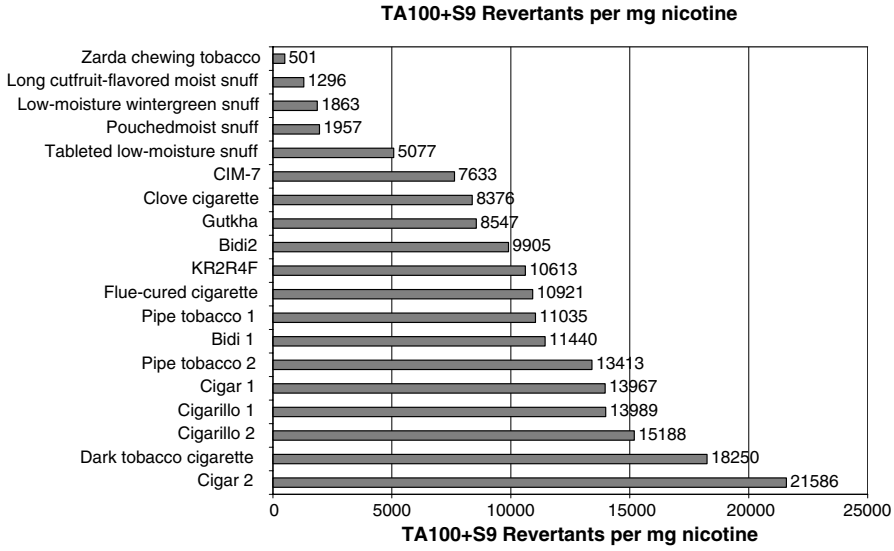

Fig. 5. Comparison of TA100+S9 Revertants per milligram nicotine for various types of tobacco products.

nicity with TA100+S9 but not TA98+S9 (Powrie et al., 1981; Pool et al., 1984).

Our work, along with the work of others as cited, already has shown that most western-style smokeless tobacco products are not mutagenic with TA98+S9. Numerous studies as cited herein have shown that the TPM from conventional cigarettes is mutagenic with TA98+S9. Furthermore, the epidemiology of cigarettes with higher TPM mutagenicity with TA98+S9 shows more evidence of smoking-related diseases than cigarettes with lower TPM mutagenicity under the same condition (Lee, 2001; Malaveille et al., 1989; Curvall et al., 1987). Thus, this provides additional evidence for the use of some smokeless tobacco products in place of cigarettes for those who chose not to refrain from tobacco use (Levy et al., 2004; Nilsson, 2006).

We do not know if the compounds in TPM after activation with S9 that are active with TA100 are the same as those in smokeless tobacco samples that are active with TA100 after the S9 metabolic activation. We do not know if revertants caused by TPM represent more or less toxicity than do revertants caused by smokeless tobacco. However, in order to estimate the possible differences in doses of mutagens received from use of smoking products with those received from use of smokeless tobacco products, we have calculated mutagenic activity on the basis of nicotine delivery for smoking products and nicotine content for smokeless products.

We are not alone in the use of adjusting measure of potential harm to nicotine delivery and a similar approach was just used by Laugesen and Fowles to compare the toxicities of mainstream cigarette smoke from cigarettes with differing nicotine deliveries (Laugesen and Fowles, 2006). We realize that this approach to comparing mutagenicities of products may be subject to criticism because of differences in intake, uptake, and metabolism among users of

Table 9
Ratios of TA98+S9/TA100+S9 for smoking products

Sample	TA98+S9 revertants per milligram Nicotine	TA100+S9 revertants per milligram Nicotine	Ratio TA98+S9/ TA100+S9
Flue-cured cigarette	19,265	10,921	1.764
Clove cigarette	19,619	8376	2.342
Dark tobacco cigarette	70,024	18,250	3.837
CIM-7	15,940	7633	2.088
KR2R4F	33,681	10,613	3.174
Cigarillo 1	51,771	13,989	3.701
Cigarillo 2	53,810	15,188	3.543
Bidi 1	22,343	11,440	1.953
Bidi 2	19,346	9905	1.953
Cigar 1	42,061	13,967	3.011
Cigar 2	64,867	21,586	3.005
Pipe tobacco 1	10,807	11,035	0.979
Pipe tobacco 2	8509	13,413	0.634

such products, and such differences are noted in many journal articles and governmental reports that are too numerous to cite. Our results for all samples assayed with TA100+S9 are shown graphically in Fig. 5. These results also are reflective of current thinking that use of some smokeless tobacco products is likely to present less risk of cancer than use of cigarettes and other smoking products (Nilsson, 2006; Rodu and Jansson, 2004).

5. Conclusions

Our initial objective for this work was to characterize the mutagenic response for a range of smoked and smokeless tobacco products. We found, although it is far from a perfect solution, that we could compare the mutagenic potency of mainstream smoke condensate from smoking articles with that of smokeless tobacco products if we based our comparisons on mutagenic potency expressed on a nicotine basis. Thus, we were able to compare the mutagenicity of TPM, measured in terms of revertants per milligram nicotine with the mutagenicity of extracts of smokeless tobacco products measured in term of revertants per milligram of nicotine in the product. This approach showed that some of the smokeless products assayed would result in less mutagenicity transmitted to the user than would occur with smoking products. Furthermore, we were not able to detect significant mutagenicity when the extracts of the smokeless tobacco products were tested with TA98+S9. This provides support to a current public health discussion that it may be better for smokers who cannot stop their need for nicotine to switch to smokeless tobacco products.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yrtph.2007.05.003.

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