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Date: February 3, 2011

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R018005



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Report #: RDM KWF 2011, 006

of Pages: 17

**SUMMARY OF LABSTAT PROJECT M97:
BACTERIAL MUTAGENICITY (AMES) ASSAYS OF SMOKELESS TOBACCO
SAMPLES**

OBJECTIVE

To summarize data and conclusions from Ames bacterial mutagenicity assays of smokeless tobacco samples and Kentucky reference cigarette 2R4F, conducted at Labstat International ULC.

SUMMARY

Seven smokeless tobacco samples were submitted to Labstat International ULC (Kitchener, Canada) for Ames mutagenicity testing: 2S3 Research Moist Smokeless Tobacco, Camel Snus Frost, Camel Fresh Orbs, Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut, and Ariva Wintergreen. Kentucky Reference 2R4F cigarettes were also tested. Smokeless tobacco samples were extracted in dimethyl sulfoxide (DMSO) for 21 hours at 37°C. 2R4F cigarettes were smoked using ISO smoking regimen 35/60/2 with no vent blocking and total particulate matter (TPM) was extracted from Cambridge filter pads with DMSO. Assays were conducted in triplicate with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 in the presence and absence of S9 metabolic activation. Smokeless samples were compared on "DMSO-extracted smokeless tobacco" basis, "DMSO-extracted moisture-corrected smokeless tobacco" basis, and "DMSO-extracted nicotine" basis. The seven smokeless samples were compared to 2R4F on "extracted nicotine" basis. Results were summarized in Labstat Report "Toxicology of Smokeless Tobacco Products: Bacterial Reverse Mutagenicity, Project Code M97", Revision 5.

In general, the responses for all smokeless tobacco samples were weak or non-existent depending on the strain/S9 combination, indicating low levels of mutagenic activity for these extracts as compared to the mutagenic activity of 2R4F tobacco smoke TPM assayed under similar conditions. The following table identifies those strains/S9 conditions in which statistically significant responses for smokeless samples were observed (i.e. average slope greater than zero):

Basis	Smokeless samples with statistically significant response (i.e. average slope>0)	Conditions
DMSO-extracted smokeless tobacco	Camel Mellow Sticks	TA98 –S9
	Copenhagen Long Cut	TA98 +S9
	2S3	TA100 –S9
	Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut	TA100 +S9
	Camel Snus Frost, Camel Mellow Sticks	TA102 +S9
	Copenhagen Long Cut	TA1537 +S9
DMSO-extracted moisture-corrected smokeless tobacco	Camel Mellow Sticks	TA98 –S9
	Copenhagen Long Cut	TA98 +S9
	2S3	TA100 –S9
	Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut	TA100 +S9
	Camel Snus Frost, Camel Mellow Sticks	TA102 +S9
	Copenhagen Long Cut	TA1537 +S9
DMSO-extracted nicotine	Camel Mellow Sticks	TA98 –S9
	Copenhagen Long Cut	TA98 +S9
	2S3	TA100 –S9
	Camel Fresh Strips, Copenhagen Long Cut	TA100 +S9
	Camel Snus Frost, Camel Mellow Sticks	TA102 +S9

Statistically significant differences between smokeless samples with significant mean slopes are reported below:

Comparison Basis	Results	Conditions
DMSO-extracted smokeless tobacco	Camel Snus Frost > Camel Mellow Sticks	TA102 +S9
DMSO-extracted moisture-corrected smokeless tobacco	Copenhagen Long Cut > Camel Fresh Strips and Camel Mellow Sticks	TA100 +S9
	Camel Snus Frost > Camel Mellow Sticks	TA102 +S9
DMSO-extracted nicotine	Camel Snus Frost > Camel Mellow Sticks	TA102 +S9

Camel Snus Frost and Copenhagen Long Cut appear to be the most mutagenic among the smokeless tobacco samples evaluated across the few responsive strains where differences were found to be significant.

Statistically significant differences between K2R4F and smokeless samples with significant mean slopes are reported below:

Comparison Basis	Results	Conditions
DMSO-extracted nicotine	K2R4F > Camel Mellow Sticks	TA98 –S9
	K2R4F > Copenhagen Long Cut	TA98 +S9
	K2R4F > 2S3	TA100 –S9
	K2R4F > Copenhagen Long Cut and Camel Fresh Strips	TA100 +S9

STATUS

This work is complete.

KEYWORDS

Ames, mutagenicity, snuff, smokeless tobacco, dissolvable, Ariva, Wintergreen, Copenhagen Long Cut, 2S3, Camel Snus Frost, Camel Fresh Orbs, Camel Mellow Sticks, Camel Fresh Strips, 2R4F, Labstat Project M97, Labstat Project M100, Labstat Project M125

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SUMMARY OF LABSTAT PROJECT M97: BACTERIAL MUTAGENICITY (AMES) ASSAYS OF SMOKELESS TOBACCO SAMPLES

Test facility: Labstat International ULC
262 Manitou Drive
Kitchener, ON Canada N2C 1L3

Labstat project #: M97
Study initiated: Labstat received samples on September 16, 2008
Study completed: January 28, 2011 (date of final report, Revision 5)
Study monitor: Suzana Theophilus (RJRT)
Study reviewers: Ryan Potts (RJRT), Betsy Bombick (RJRT), Kathy Fowler (RJRT),
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Study director: Amit Trivedi (Labstat International ULC)
Study personnel: Labstat personnel
Statistician: Wendy Wagstaff (Labstat International ULC)

STUDY OBJECTIVES

To summarize data and conclusions from Labstat Project M97, Ames bacterial mutagenicity assays on smokeless tobacco samples and Kentucky reference cigarette 2R4F.

EXPERIMENTAL DESIGN

This study was conducted to evaluate the potential mutagenicity of seven smokeless samples and one cigarette using the Ames mutagenicity assay. Work was conducted at Labstat International ULC. Labstat identified the project as M97.

The samples tested were coded by Labstat as follows:

Sample	Labstat Code	Sample	Labstat Code
Camel Snus Frost	084394	2S3 research moist smokeless tobacco	084395
Camel Fresh Strips	084454	Camel Mellow Sticks	084455
Copenhagen Long Cut	084456	Ariva Wintergreen	084457
Camel Fresh Orbs	084458	Kentucky Reference 2R4F	084396

2R4F cigarettes were smoked using ISO smoking regimen 35/60/2 with no vent blocking. Total particulate matter (TPM) was extracted from Cambridge filter pads with DMSO.

Smokeless tobacco samples were extracted with DMSO using the following methodology:

- dispersion in DMSO (1:9 w/v) using an ultrasonic homogenizer
- incubation at 37°C for 21 hours followed by centrifugation & ultra-filtration
- storage at -80°C prior to assay

Assays were conducted on a “DMSO-extracted smokeless tobacco” basis and all smokeless samples were tested up to 5.55 mg extracted smokeless tobacco/plate on this basis. Results from moisture and nicotine determinations were then used to calculate response on “DMSO-extracted moisture-corrected smokeless tobacco” basis and “DMSO-extracted nicotine” basis. Triplicate Ames assays were conducted using the preincubation modification, \pm S9 metabolic activation, with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537. Averages were used for statistical analyses.

Labstat issued its first report October 9, 2008. Subsequent revisions were issued October 17, 2008, October 26, 2009, December 15, 2009, December 22, 2010, and January 28, 2011. Revisions were required due to requests for additional or revised procedures for statistical analysis of the data. This RDM is based on results provided in Labstat’s final report, Revision 5, dated January 28, 2011.

RESULTS

Key results are summarized below. Detailed results and data are available in the Labstat M97 report, Revision 5.

There was evidence of toxicity at the highest concentration for each assay (as evidenced by a thinning of the background lawn of bacteria) with exception of replicate one for both Camel Fresh Strips and Camel Mellow Sticks.

A. Overall Mutagenic Response

1. Determination of mutagenic response

Slope values, i.e., revertants/concentration unit (e.g., per mg DMSO-extracted smokeless tobacco, etc.), were calculated for each smokeless sample under each assay condition (strain \pm S9 activation) on the following basis:

- DMSO-extracted smokeless tobacco (as-is)
- DMSO-extracted moisture-corrected smokeless tobacco (dry weight)
- DMSO-extracted nicotine

2R4F TPM was analyzed only on a revertants/ μ g nicotine basis.

Specific activity (response) was determined by fitting a quadratic model to the sample dose and revertant colony count for each individual replicate assay, and removing the highest dose data until the quadratic term was no longer significant. Only doses contributing to downward curvature were considered for exclusion.

Since low slope values were observed for most of the test samples, statistical significance for a mean slope depends as much on the variation among the replicates as on their mean. Thus, comparisons of the means of these samples may be based on a pooled standard deviation estimate that under-estimates the true standard deviation, resulting in over-sensitivity of comparisons.

2. Comparison of assay results

ANOVA-based comparisons with Bonferroni adjustment for multiple comparisons were conducted unless the variation of the slope estimates among replicate assays was grossly inconsistent among the samples (within sample standard deviations different by more than a factor of 15). In those cases unequal variance pairwise t-test comparisons with Bonferroni-adjusted p-values were performed. P-values < 0.05 after adjustment were considered statistically significant.

Comparisons were first conducted between all smokeless samples even if the mean slopes were not statistically significant (21 comparisons). Comparisons were then conducted only between smokeless samples for which the mean slopes were statistically significant (i.e. mean slopes greater than zero). Likewise, comparisons were conducted between each smokeless sample and K2R4F even if the mean slopes were not statistically significant. Comparisons were then conducted between K2R4F and the smokeless samples only in the instances where the mean slope for both K2R4F and the smokeless sample was statistically significant. All comparisons are reported in the Labstat report. This RDM reports only those comparisons conducted between statistically significant slopes.

Overall Results

Ariva Wintergreen and Camel Fresh Orbs did not induce statistically significant mutagenic responses with any strain on any basis examined. All other samples induced statistically significant responses under at least one strain and S9 condition.

In general, the responses for all smokeless tobacco samples were weak or non-existent (depending on the strain/S9 condition evaluated), indicating low levels of mutagenic activity for these extracts as compared to 2R4F cigarette smoke TPM using similar assay conditions. Only TA1537, which has a low background of spontaneous revertants, provided any response more than 2X background.

B. Results on DMSO-extracted Smokeless Tobacco (as-is) Basis

1. Determination of Positive Response

DMSO-extracted Smokeless Tobacco (as-is) Mutagenicity Assessment

Strain	TA98		TA100		TA102		TA1535		TA1537	
S9	-	+	-	+	-	+	-	+	-	+
Camel Snus Frost	ns	ns	ns	ns	ns	Sig	ns	ns	ns	ns
2S3	ns	ns	Sig	ns	ns	ns	ns	ns	ns	ns
Camel Fresh Strips	ns	ns	ns	Sig	ns	ns	ns	ns	ns	ns
Camel Mellow Sticks	Sig	ns	ns	Sig	ns	Sig	ns	ns	ns	ns
Copenhagen Long Cut	ns	Sig	ns	Sig	ns	ns	ns	ns	ns	Sig
Ariva Wintergreen	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Camel Fresh Orbs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Sig: statistically significant response, i.e. average of 3 replicate slopes significantly > 0

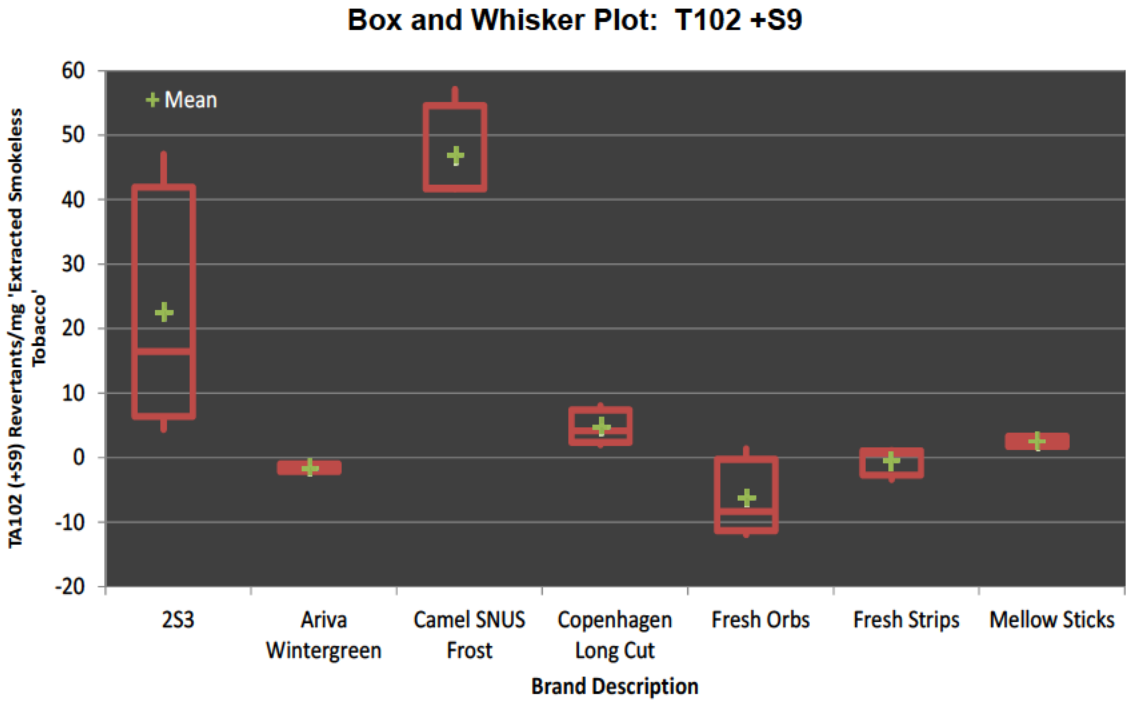
ns: response not statistically significant

2. Results of Statistical Comparisons

Statistically significant differences between smokeless samples with significant mean slopes are reported below:

Statistically significant differences for DMSO-extracted smokeless tobacco basis	Strain/condition
Camel Snus Frost > Camel Mellow Sticks	TA102 +S9

Box and whisker plots are shown only for those strains/conditions in which a statistically significant difference between samples was observed.



C. Results on DMSO-extracted Moisture-Corrected (Dry Weight) Basis

1. Determination of Positive Response

DMSO-extracted Moisture-Corrected Smokeless Tobacco (Dry Weight) Mutagenicity Assessment

Strain	TA98		TA100		TA102		TA1535		TA1537	
S9	-	+	-	+	-	+	-	+	-	+
Camel Snus Frost	ns	ns	ns	ns	ns	Sig	ns	ns	ns	ns
2S3	ns	ns	Sig	ns	ns	ns	ns	ns	ns	ns
Camel Fresh Strips	ns	ns	ns	Sig	ns	ns	ns	ns	ns	ns
Camel Mellow Sticks	Sig	ns	ns	Sig	ns	Sig	ns	ns	ns	ns
Copenhagen Long Cut	ns	Sig	ns	Sig	ns	ns	ns	ns	ns	Sig
Ariva Wintergreen	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Camel Fresh Orbs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Sig: statistically significant response, i.e. average of 3 replicate slopes significantly > 0

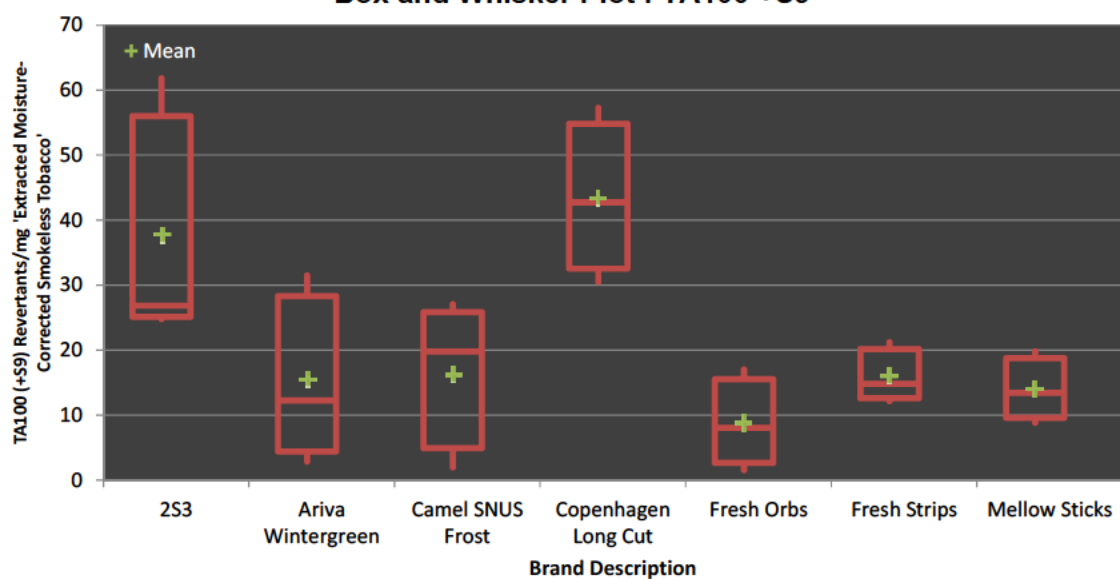
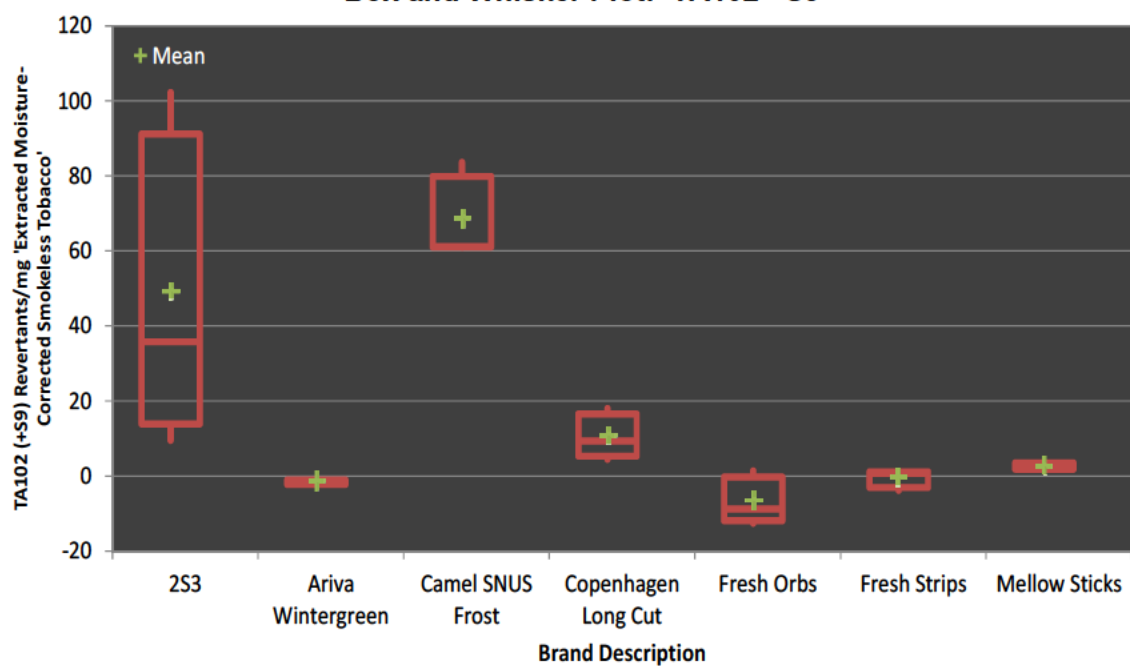
ns: response not statistically significant

2. Results of Statistical Comparisons

Statistically significant differences between smokeless samples with significant mean slopes are reported below:

Statistically significant differences for DMSO-extracted moisture-corrected smokeless tobacco basis	Strain/condition
Copenhagen Long Cut > Camel Fresh Strips and Camel Mellow Sticks	TA100 +S9
Camel Snus Frost > Camel Mellow Sticks	TA102 +S9

Box and whisker plots are shown only for those strains/conditions in which a statistically significant sample difference was observed.

Box and Whisker Plot : TA100 +S9**Box and Whisker Plot: TA102 +S9**

D. Results on a DMSO-extracted Nicotine Basis for Smokeless Samples

1. Determination of Positive Response

DMSO-extracted Nicotine in Smokeless Tobacco Mutagenicity Assessment

Strain	TA98		TA100		TA102		TA1535		TA1537	
S9	-	+	-	+	-	+	-	+	-	+
Camel Snus Frost	ns	ns	ns	ns	ns	Sig	ns	ns	ns	ns
2S3	ns	ns	Sig	ns	ns	ns	ns	ns	ns	ns
Camel Fresh Strips	ns	ns	ns	Sig	ns	ns	ns	ns	ns	ns
Camel Mellow Sticks	Sig	ns	ns	ns	ns	Sig	ns	ns	ns	ns
Copenhagen Long Cut	ns	Sig	ns	Sig	ns	ns	ns	ns	ns	ns
Ariva Wintergreen	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Camel Fresh Orbs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

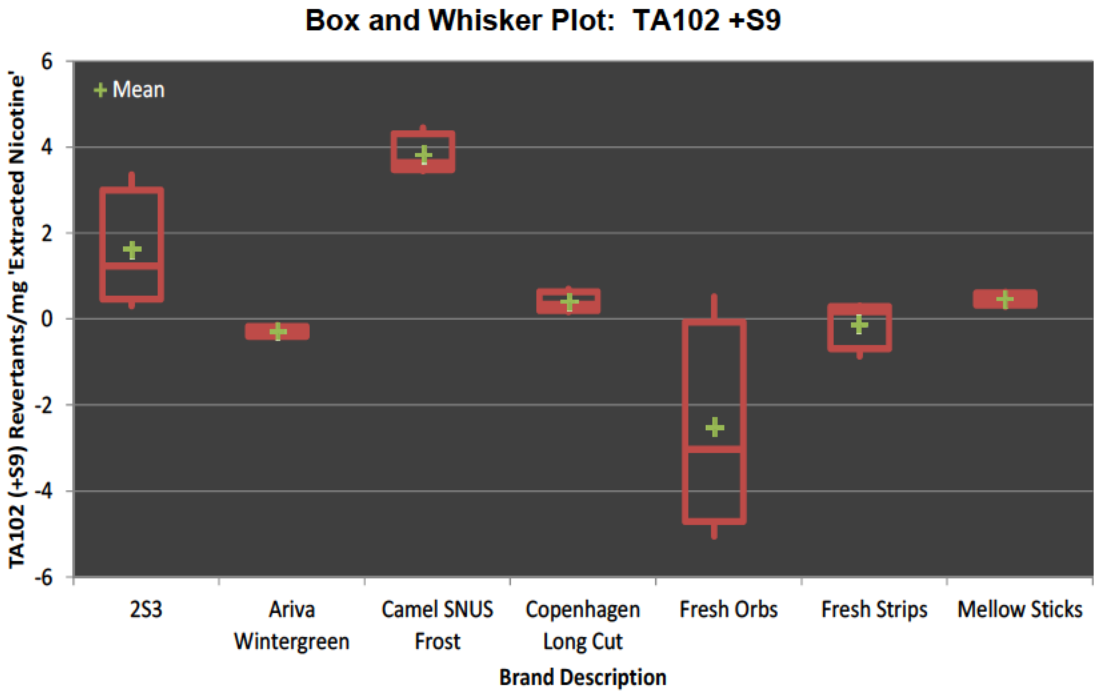
Sig: statistically significant response, i.e. average of 3 replicate slopes significantly > 0
 ns: response not statistically significant

2. Results of Statistical Comparisons

Statistically significant differences between smokeless samples with significant mean slopes are reported below:

Statistically significant differences for DMSO-extracted nicotine basis for smokeless samples	Strain/condition
Camel Snus Frost > Camel Mellow Sticks	TA102 +S9

Box and whisker plots are shown only for those strains/conditions in which a statistically significant sample difference was observed.



F. K2R4F Mutagenicity Responses

K2R4F slopes were evaluated on a TPM, dry particulate matter (DPM) and μg nicotine basis, with statistically significant responses under all three evaluation criteria for certain strains/S9 conditions:

Strain	TA98		TA100		TA102		TA1535		TA1537	
S9	-	+	-	+	-	+	-	+	-	+
K2R4F	Sig	Sig	Sig	Sig	ns	ns	ns	ns	ns	Sig

Sig: statistically significant response, i.e. average of 3 replicate slopes significantly > 0

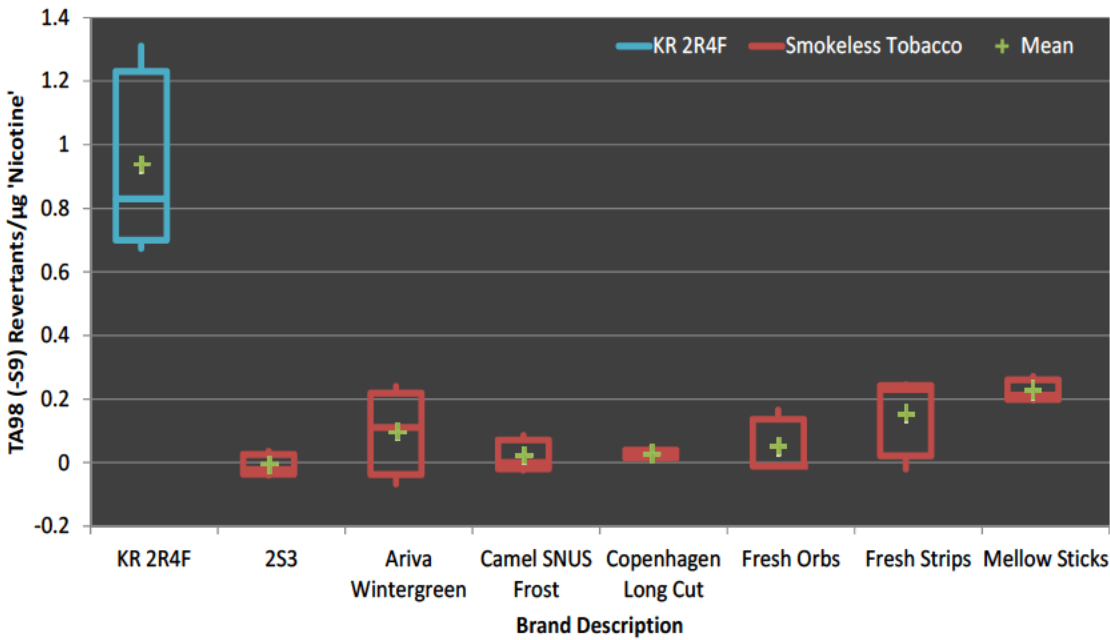
ns: response not statistically significant

K2R4F was compared to the smokeless samples on DMSO-extracted nicotine basis. Statistically significant differences between K2R4F and smokeless samples for only those instances where the mean slopes for both K2R4F and the smokeless sample were significant (i.e. mean slope > 0) are reported below:

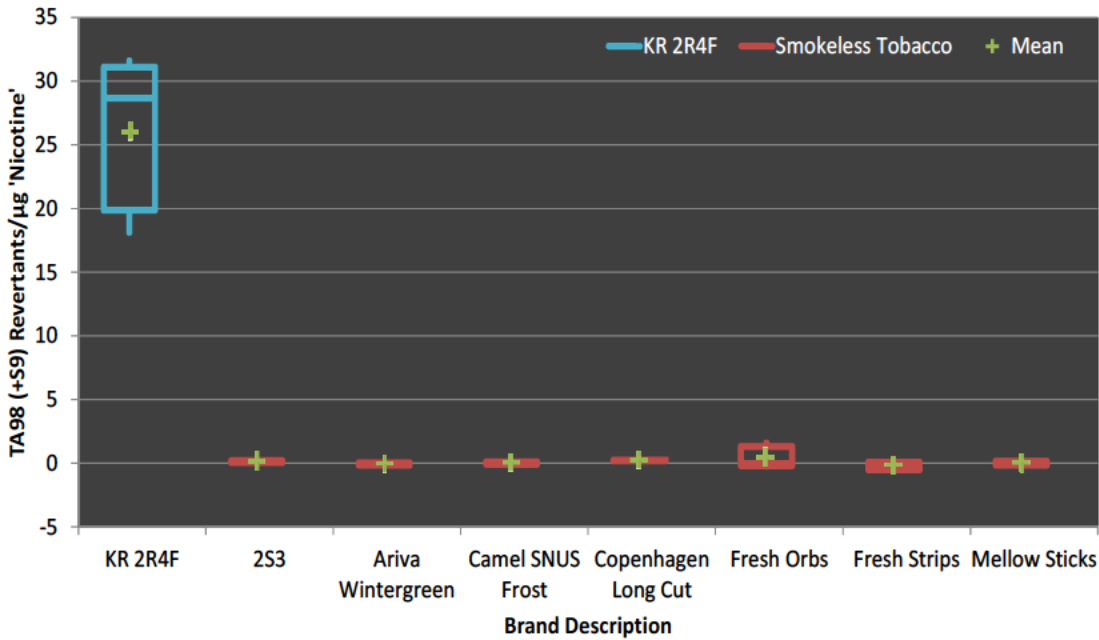
Statistically significant differences for DMSO-extracted nicotine basis for smokeless samples and 2R4F	Strain/condition
K2R4F > Camel Mellow Sticks	TA98 –S9
K2R4F > Copenhagen Long Cut	TA98 +S9
K2R4F > 2S3	TA100 –S9
K2R4F > Copenhagen Long Cut and Camel Fresh Strips	TA100 +S9

Box and whisker plots are shown only for those strains/conditions in which a statistically significant sample difference was observed. Note that smokeless responses were close to zero in most cases.

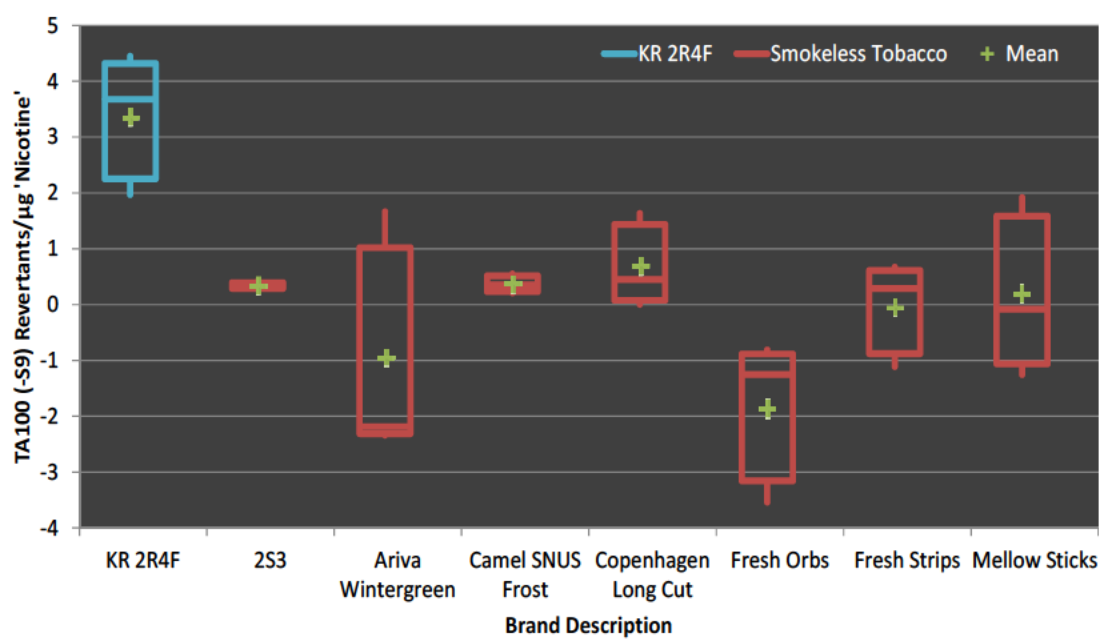
Box and Whisker Plot TA98 –S9



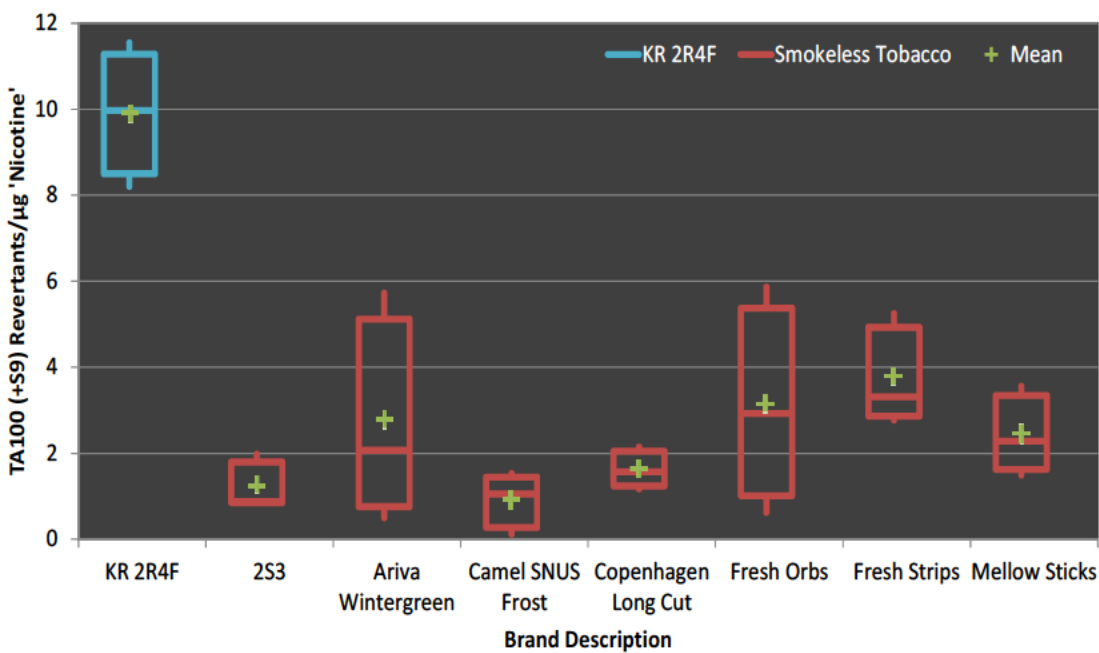
Box and Whisker Plot: TA98 +S9



Box and Whisker Plot TA100 –S9



Box and Whisker Plot: TA100 +S9



SUMMARY AND CONCLUSIONS

In general, the responses for all smokeless tobacco test brands were weak or non-existent depending on the strain/S9 combination, indicating low levels of mutagenic activity for these extracts as compared to the mutagenic activity of 2R4F tobacco smoke TPM assayed under similar conditions. For example, only TA1537, which has a low background of spontaneous revertants, provided any response more than 2X background (specifically, Camel Snus Frost, 2S3 and Copenhagen Long Cut elicited 2X response in at least one replicate). Camel Snus Frost and Copenhagen Long Cut appear to be the most mutagenic among the smokeless tobacco samples evaluated across the few responsive strains where differences were found to be significant.

Statistically significant differences between smokeless samples with significant mean slopes are reported below:

Comparison Basis	Results	Conditions
DMSO-extracted smokeless tobacco	Camel Snus Frost > Camel Mellow sticks	TA102 +S9
DMSO-extracted moisture-corrected smokeless tobacco	Copenhagen Long Cut > Camel Fresh Strips and Camel Mellow Sticks	TA100 +S9
	Camel Snus Frost > Camel Mellow Sticks	TA102 +S9
DMSO-extracted nicotine	Camel Snus Frost > Camel Mellow Sticks	TA102 +S9

Statistically significant differences between K2R4F and smokeless samples with significant mean slopes for each are reported below:

Comparison Basis	Results	Conditions
DMSO-extracted nicotine	K2R4F > Camel Mellow Sticks	TA98 –S9
	K2R4F > Copenhagen Long Cut	TA98 +S9
	K2R4F > 2S3	TA100 –S9
	K2R4F > Copenhagen Long Cut and Camel Fresh Strips	TA100 +S9