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**Group:** Product Integrity

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**SUMMARY OF LABSTAT PROJECT M125/RJRT PROJECT MBIV-014:  
SISTER CHROMATID EXCHANGE ASSAYS OF SMOKELESS TOBACCO  
SAMPLES**

**OBJECTIVE**

To summarize data and conclusions from sister chromatid exchange (SCE) 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 SCE 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 cigarettes 2R4F were also tested. Smokeless tobacco samples were extracted in dimethyl sulfoxide (DMSO) for 21 hours at 37 °C. 2R4F cigarettes were smoked using Cambridge pad 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 both with and without 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 also compared to 2R4F on an "extracted nicotine" basis. Results were summarized in Labstat Report "Toxicology of Smokeless Tobacco Products: Sister Chromatid Exchange Genotoxicity, Project Code M125" Revision 2.

Nicotine extraction efficiency was close to 100% for all smokeless tobacco samples with the exception of Camel Mellow Sticks in which the extraction efficiency was variable and trended low (~ 60%). This variation in nicotine extraction efficiency for Camel Mellow Sticks should be taken into account when making sample comparisons as it may affect comparison conclusions involving this brand.

All smokeless tobacco samples tested in the SCE assay were genotoxic (i.e. statistically significant response) both with and without metabolic activation with the exception of Camel Mellow Sticks on a nicotine comparison basis in the plus metabolic activation condition. This result was due to especially large variation among slope estimates for the three replicates of Camel Mellow Sticks (which may be related to the variable nicotine extraction efficiency).

The following statistically significant differences were observed between smokeless tobacco samples:

Comparison basis	-S9 Metabolic Activation	+S9 Metabolic Activation
DMSO-extracted smokeless tobacco	Camel Snus Frost < all other smokeless samples  2S3 > Ariva Wintergreen, Copenhagen Long Cut, Camel Fresh Strips and Camel Fresh Orbs  Camel Mellow Sticks > Camel Fresh Strips	No statistically significant differences
DMSO-extracted moisture-corrected smokeless tobacco	2S3 > Copenhagen Long Cut > all other smokeless samples  Camel Mellow Sticks > Camel Fresh Strips	2S3 > Copenhagen Long Cut > all other smokeless samples  Camel Snus Frost > Camel Fresh Strips
DMSO-extracted nicotine	Camel Fresh Orbs, Camel Fresh Strips, and Camel Mellow Sticks > Ariva Wintergreen > Copenhagen Long Cut, and Camel Snus Frost  Camel Fresh Orbs, Camel Fresh Strips, and Camel Mellow Sticks > 2S3	Camel Fresh Orbs and Camel Fresh Strips > Ariva Wintergreen, Camel Snus Frost, 2S3 and Copenhagen Long Cut  Camel Mellow Sticks > Camel Snus Frost, 2S3 and Copenhagen Long Cut  Ariva Wintergreen > Copenhagen Long

Since Camel Fresh Orbs, Camel Fresh Strips, Camel Mellow Sticks and Ariva Wintergreen have lower nicotine content (mg/g) than the other smokeless samples, adjustment to a nicotine basis increases the slope of these samples relative to the other samples.

The following statistically significant differences were observed upon comparison of each smokeless tobacco sample to 2R4F:

Comparison basis	-S9 metabolic activation	+S9 metabolic activation
DMSO-extracted nicotine	2R4F > Ariva Wintergreen, Copenhagen Long Cut, 2S3 and Camel Snus Frost	2R4F > Ariva Wintergreen, Copenhagen Long Cut, 2S3 and Camel Snus Frost

## STATUS

This work is complete.

## KEYWORDS

Sister chromatid exchange, SCE, snuff, smokeless tobacco, dissolvable, Ariva Wintergreen, Copenhagen Long Cut, 2S3, Camel Snus Frost, Camel Fresh Orbs, Camel Mellow Sticks, Camel Fresh Strips, Labstat Project M125, RJRT MBIV-014, Labstat Project M97, Labstat Project M100

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**SUMMARY OF LABSTAT PROJECT M125:**  
**SISTER CHROMATID EXCHANGE ASSAYS OF SMOKELESS TOBACCO**  
**SAMPLES**

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

**Labstat project:** M125  
**Study initiated:** Labstat received samples on December 16, 2009  
**Study completed:** January 13, 2011 - date of Labstat final report Revision 2  
**Study monitor:** Betsy Bombick (RJRT)  
**Study reviewers:** Ryan Potts (RJRT), Betsy Bombick, (RJRT), Kathy Fowler (RJRT),  
Walter Morgan (RJRT)  
**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 sister chromatid exchange (SCE) assays of smokeless tobacco samples and Kentucky reference cigarette 2R4F conducted at Labstat International ULC.

**EXPERIMENTAL DESIGN**

This study was conducted to evaluate the potential of seven smokeless tobacco samples and one cigarette to induce sister chromatid exchanges.

Seven smokeless tobacco samples and one cigarette were submitted to Labstat International ULC, Kitchener, ON Canada for sister chromatid exchange (SCE) testing. The Labstat project was identified as Project M125. The samples tested were coded as follows:

Sample	Labstat Code	Sample	Labstat Code
Ariva Wintergreen	1002241	Camel Snus Frost	1002245
Copenhagen Long Cut	1002242	Camel Mellow Sticks	1002246
Camel Fresh Strips	1002243	Camel Fresh Orbs	1002247
2S3 Research moist smokeless tobacco	1002244	Kentucky Reference 2R4F Cigarettes	1002248

2R4F cigarettes were smoked using the Cambridge pad 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

The nicotine content of the smokeless tobacco samples and of the smokeless tobacco extracts was determined to allow for the calculation of nicotine extraction efficiencies.

Assays were conducted on a “DMSO-extracted smokeless tobacco” basis. All smokeless samples were tested up to 0.83 mg smokeless tobacco/mL without S9 metabolic activation and 3.3 mg smokeless tobacco/mL with S9 metabolic activation. Results from moisture and nicotine determinations were then used to calculate response on a “DMSO-extracted moisture-corrected smokeless tobacco” and “DMSO-extracted nicotine” basis. Kentucky Reference 2R4F was tested up to 75 µg TPM/mL without S9 metabolic activation and 300 µg TPM/mL with S9 metabolic activation and nicotine was used as above for further analysis.

SCE assays were conducted in triplicate using CHO-WBL cells. A long term exposure (30 hours) was conducted in the absence of metabolic activation and a short term exposure (3 hours followed by a 30 hour recovery period) was performed with metabolic activation.

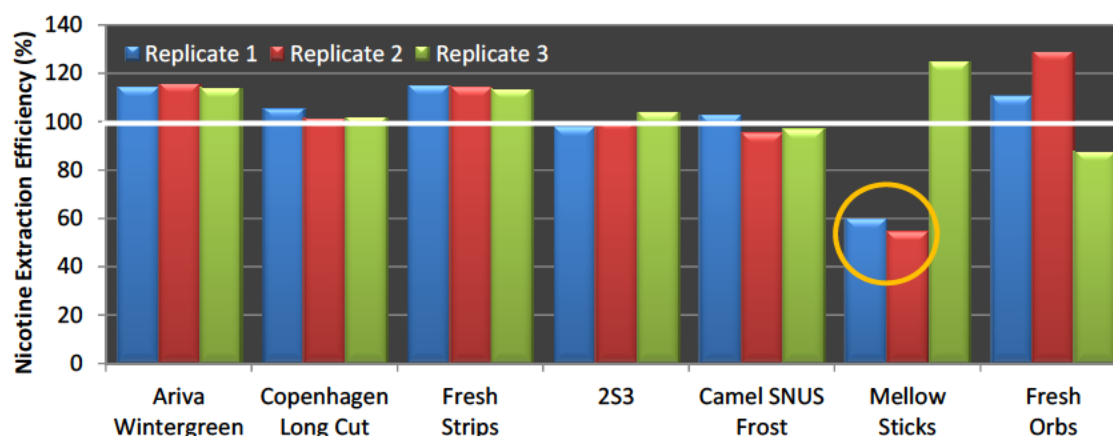
Labstat issued its first report April 21, 2010 and revised reports on December 22, 2010 and January 13, 2011. Revision was required due to request for corrections or additional statistical analysis procedures. This RDM is based on results provided in Labstat's final report, Revision 2, dated January 13, 2011.

## RESULTS

Key results are summarized below. Detailed results and data are available in the Labstat M125 report, Revision 2.

Nicotine extraction efficiency was close to 100% for all smokeless tobacco samples with the exception of Camel Mellow Sticks (See [Figure 1](#) below). Two of the three replicates for Camel Mellow Sticks had low extraction efficiencies (~ 60%). This variation in extraction efficiency was confirmed by repeating extraction of Mellow Sticks in triplicate; the repeat nicotine extraction efficiencies ranged from 50-70%. The variation in nicotine extraction efficiency for Camel Mellow Sticks should be taken into account when making sample comparisons as it may affect comparison conclusions.

Figure 1: Nicotine extraction efficiency



### **Calculation of SCE Response**

Data for all concentrations were included in the analysis, except for the DMSO control SCE counts for the plus S9 condition to preserve linearity of effect. Linear regression models were fit to mean SCE counts using method of ordinary least squares.

All smokeless tobacco samples tested in the SCE assay were genotoxic (i.e. statistically significant response) both with and without metabolic activation with the exception of Camel Mellow Sticks on a nicotine comparison basis in the plus metabolic activation condition. This result was due to especially large variation among slope estimates for the three replicates of Camel Mellow Sticks (which may be related to the variable nicotine extraction efficiency).

### **Comparisons**

Test samples were compared using analysis of variance on log-transformed slope estimates using the Bonferroni adjustment for multiple comparisons with  $p < 0.05$  after adjustment considered statistically significant. If variation of slope estimates among replicate assays was grossly inconsistent among the samples (within sample standard deviations different by more than a factor of 15), pairwise t-test comparisons of the samples with Bonferroni adjusted p-values were performed and reported instead of the ANOVA-comparisons.

The smokeless tobacco samples were compared to each other (21 comparisons) on the following basis:

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

The seven smokeless tobacco samples were also compared to 2R4F on extracted nicotine basis.

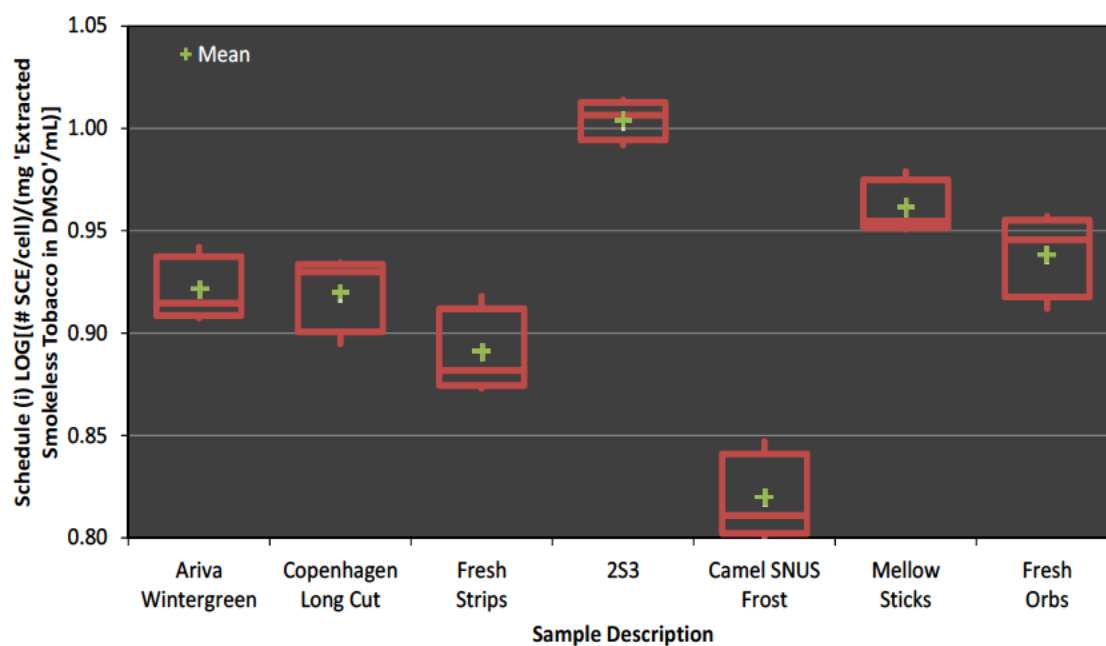
### A. Results of comparisons on “DMSO-extracted smokeless tobacco” (as-is) basis

#### Statistically significant results of ANOVA-based comparisons

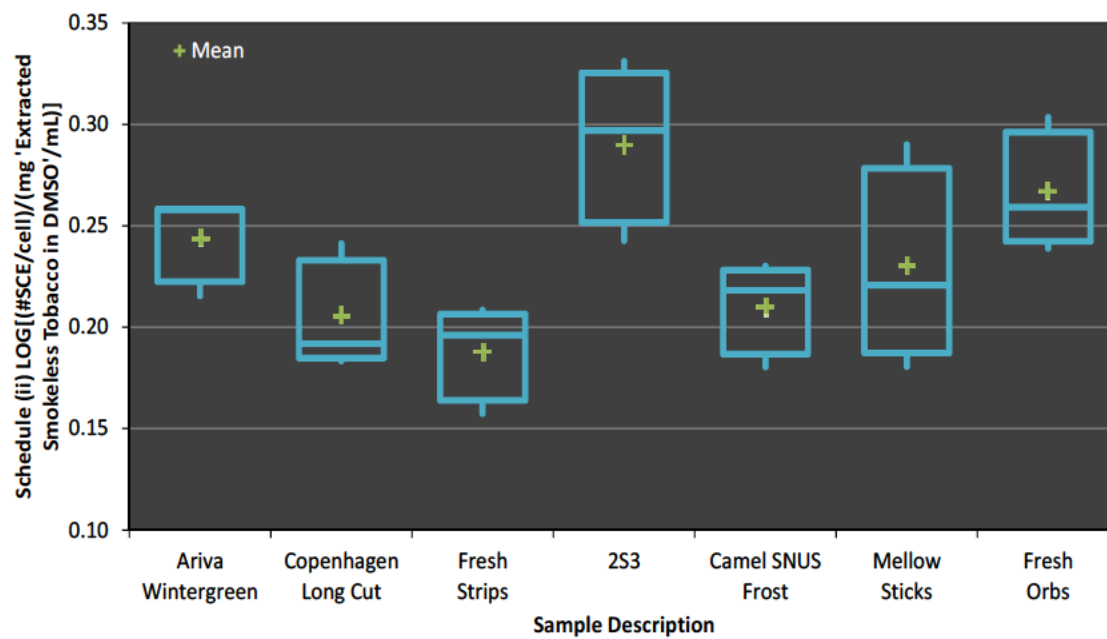
-S9 Metabolic Activation	+S9 Metabolic Activation
Camel Snus Frost < all other smokeless samples 2S3 > Ariva Wintergreen, Copenhagen Long Cut, Camel Fresh Strips and Camel Fresh Orbs Camel Mellow Sticks* > Camel Fresh Strips	No statistically significant differences

\*Note: The nicotine extraction efficiency for the three replicates of Camel Mellow Sticks was quite variable; this may affect all slope comparison conclusions involving this brand.

#### Box and Whisker Plot: –S9 Metabolic Activation





**Box and Whisker Plot: +S9 Metabolic Activation**

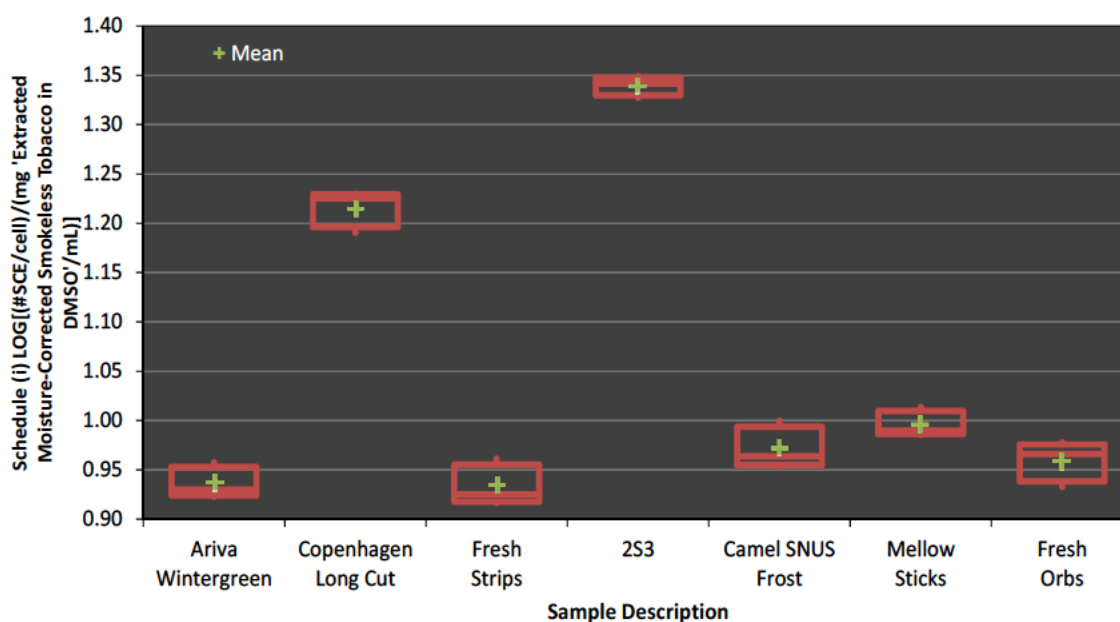
## B. Results of comparisons on “DMSO-extracted moisture-corrected smokeless tobacco” (dry weight) basis

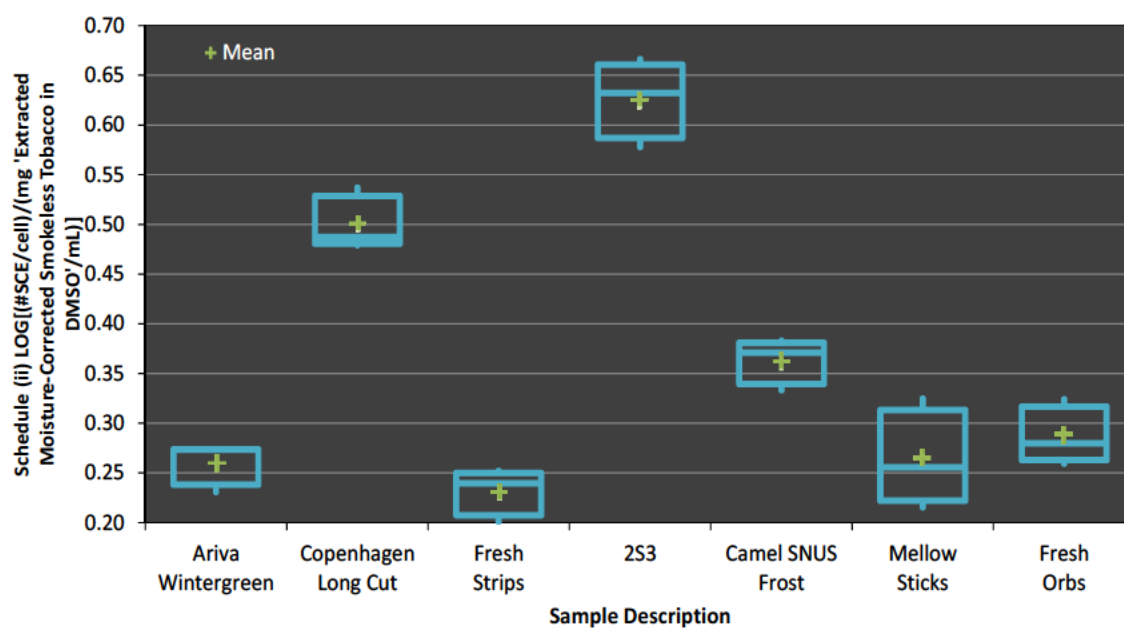
### Statistically significant results of ANOVA-based comparisons

-S9 Metabolic Activation	+S9 Metabolic Activation
2S3 > Copenhagen Long Cut > all other smokeless samples	2S3 > Copenhagen Long Cut > all other smokeless samples
Camel Mellow Sticks* > Camel Fresh Strips	Camel Snus Frost > Camel Fresh Strips

\*Note: The nicotine extraction efficiency for the three replicates of Camel Mellow Sticks was quite variable; this may affect all slope comparison conclusions involving this brand.

### Box and Whisker Plot: -S9 Metabolic Activation



**Box and Whisker Plot: +S9 Metabolic Activation**

### C. Results of comparisons on “DMSO-extracted nicotine” basis for smokeless samples

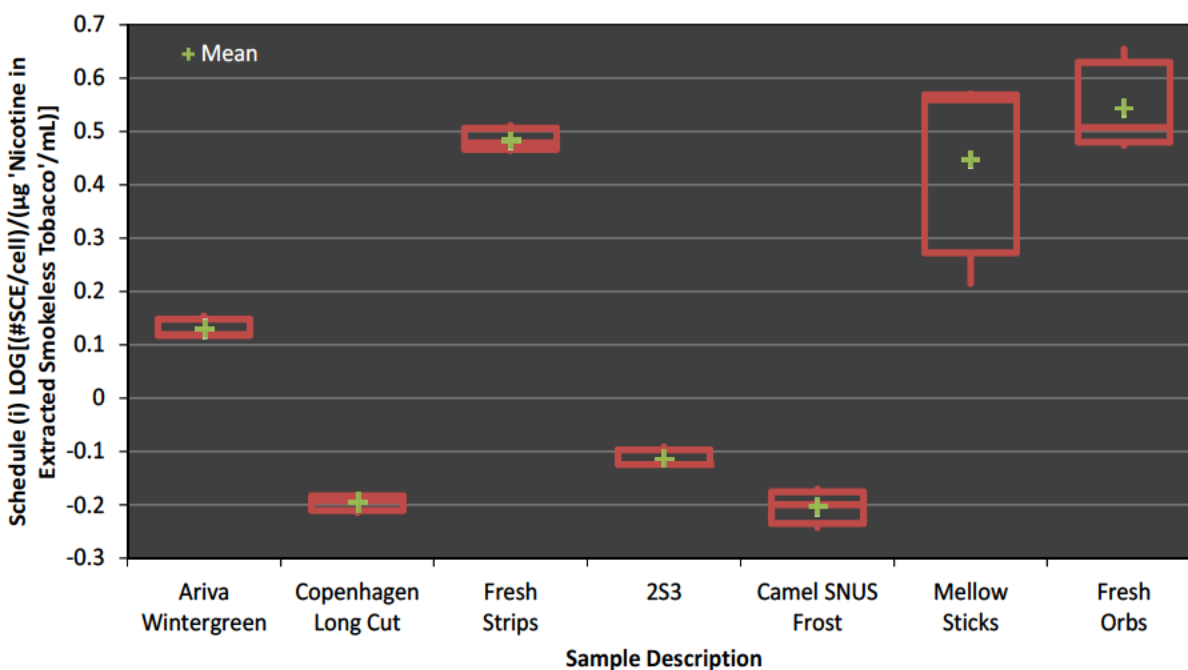
#### Statistically significant results of ANOVA-based comparisons

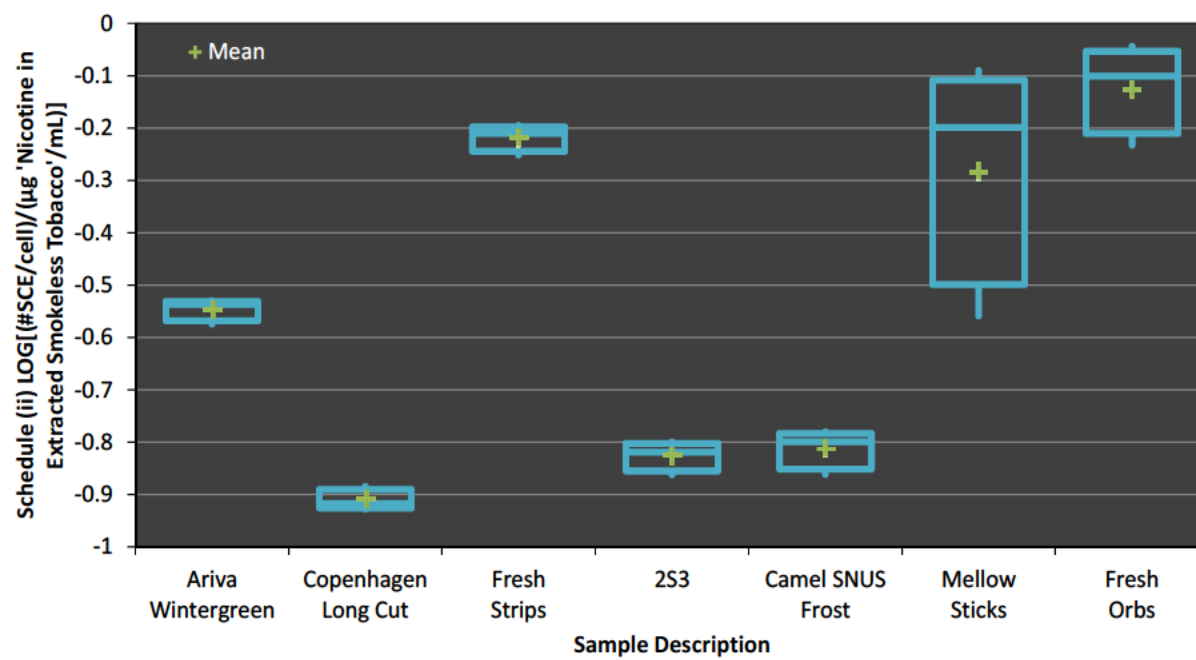
-S9 Metabolic Activation	+S9 Metabolic Activation
Camel Fresh Orbs, Camel Fresh Strips and Camel Mellow Sticks* > Ariva Wintergreen > Copenhagen Long Cut and Camel Snus Frost	Camel Fresh Orbs and Camel Fresh Strips > Ariva Wintergreen, Camel Snus Frost, 2S3 and Copenhagen Long Cut
Camel Fresh Orbs, Camel Fresh Strips and Camel Mellow Sticks* > 2S3	Camel Mellow Sticks* > Camel Snus Frost, 2S3 and Copenhagen Long Cut
	Ariva Wintergreen > Copenhagen Long Cut

\*Note: The nicotine extraction efficiency for the three replicates of Camel Mellow Sticks was quite variable; this may affect all slope comparison conclusions involving this brand.

Since Camel Fresh Orbs, Camel Fresh Strips, Camel Mellow Sticks and Ariva Wintergreen have lower nicotine content (mg/g) than the other smokeless samples, adjustment to a nicotine basis increases the slope of these samples relative to the other samples.

#### Box and Whisker Plot: -S9 Metabolic Activation



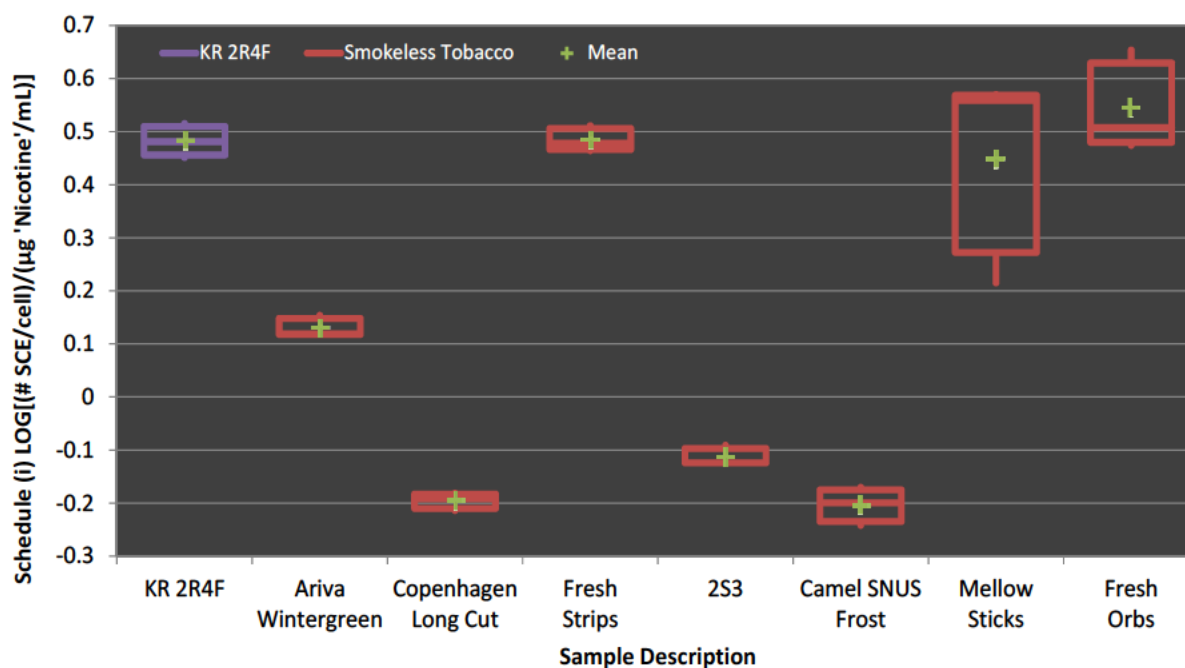
**Box and Whisker Plot: +S9 Metabolic Activation**

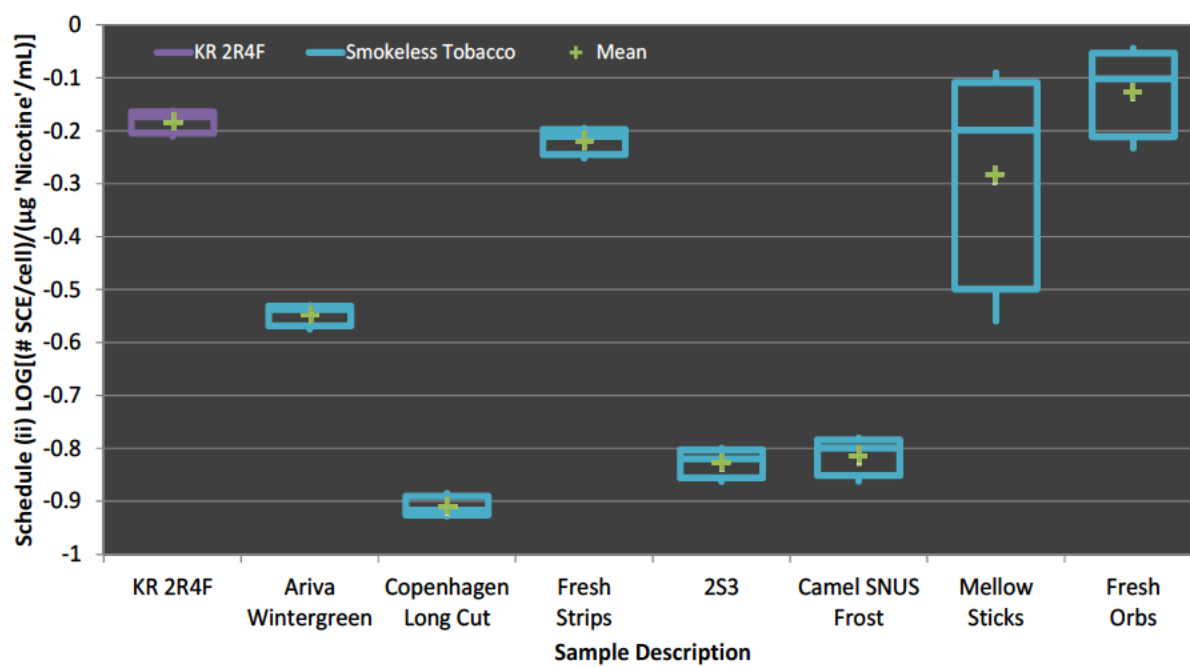
# D. Results of comparisons on “DMSO-extracted nicotine” basis between smokeless samples and 2R4F

Statistically significant results of ANOVA-based comparisons  
between 2R4F and each smokeless sample

-S9 Metabolic Activation	+S9 Metabolic Activation
2R4F > Ariva Wintergreen, Copenhagen Long Cut, 2S3 and Camel Snus Frost	2R4F > Ariva Wintergreen, Copenhagen Long Cut, 2S3 and Camel Snus Frost

Box and Whisker Plot: -S9 Metabolic Activation



**Box and Whisker Plot: +S9 Metabolic Activation**

## SUMMARY AND CONCLUSIONS

Nicotine extraction efficiency was close to 100% for all smokeless tobacco samples with the exception of Camel Mellow Sticks in which the extraction efficiency was variable and trended low (~ 60%). This variation in nicotine extraction efficiency for Camel Mellow Sticks should be taken into account when making sample comparisons as it may affect comparison conclusions involving this brand.

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The following statistically significant differences were observed between smokeless tobacco samples:

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DMSO-extracted smokeless tobacco	Camel Snus Frost < all other smokeless samples  2S3 > Ariva Wintergreen, Copenhagen Long Cut, Camel Fresh Strips and Camel Fresh Orbs  Camel Mellow Sticks > Camel Fresh Strips	No statistically significant differences
DMSO-extracted moisture-corrected smokeless tobacco	2S3 > Copenhagen Long Cut > all other smokeless samples  Camel Mellow Sticks > Camel Fresh Strips	2S3 > Copenhagen Long Cut > all other smokeless samples  Camel Snus Frost > Camel Fresh Strips
DMSO-extracted nicotine	Camel Fresh Orbs, Camel Fresh Strips, and Camel Mellow Sticks > Ariva Wintergreen > Copenhagen Long Cut, and Camel Snus Frost  Camel Fresh Orbs, Camel Fresh Strips, and Camel Mellow Sticks > 2S3	Camel Fresh Orbs and Camel Fresh Strips > Ariva Wintergreen, Camel Snus Frost, 2S3 and Copenhagen Long Cut  Camel Mellow Sticks > Camel Snus Frost, 2S3 and Copenhagen Long Cut  Ariva Wintergreen > Copenhagen Long

Since Camel Fresh Orbs, Camel Fresh Strips, Camel Mellow Sticks and Ariva Wintergreen have lower nicotine content (mg/g) than the other smokeless samples, adjustment to a nicotine basis increases the slope of these samples relative to the other samples.



The following statistically significant differences were observed upon comparison of each smokeless tobacco sample to 2R4F:

Comparison basis	-S9 metabolic activation	+S9 metabolic activation
DMSO-extracted nicotine	2R4F > Ariva Wintergreen, Copenhagen Long Cut, 2S3 and Camel Snus Frost	2R4F > Ariva Wintergreen, Copenhagen Long Cut, 2S3 and Camel Snus Frost