## Routine Analytical Chemistry Sub-Group
## Smokeless Tobacco Sub-Group

# Technical Report

# 2016 Collaborative Study on Nicotine in Tobacco Products

February 2017


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# 1. Summary

CORESTA Scientific Commission requested the CORESTA Routine Analytical Chemistry Sub-Group (RAC) to develop a new CORESTA Recommended Method (CRM), without the need for standard addition studies, for analysis of nicotine in all tobacco products. From 2013 to 2016, RAC and the Smokeless Tobacco Sub-Group (STS) conducted a series of collaborative studies for the determination of nicotine in tobacco and tobacco products including cigarette and cigar filler, and smokeless tobacco products. The RAC and STS eventually selected a gas chromatography mass spectrometry (GC-MS) method using methanol as the extract solvent and quinoline as the internal standard. Nineteen laboratories participated in the final collaborative study conducted in the Spring of 2016. The purpose of this study was to evaluate repeatability and reproducibility (r & R) values of the methodology and draft a new CRM for the determination of nicotine in tobacco and tobacco products. The study results confirm that the method is appropriate for the purpose and the results are suitable for inclusion into a CRM.

# 2. Introduction

In late 2015 to early 2016, the CORESTA RAC and STS conducted a collaborative study that included CORESTA Reference Products (CRPs) 1-3, three cigarette fillers, two ground cigar fillers and one mint flavoured moist smokeless tobacco (MST) product. The purpose of this study was to evaluate the repeatability and reproducibility for the stated GC-MS method.

The nicotine content of tobacco was determined by pre-treating a tobacco sample with 2N sodium hydroxide and then extracting it in methanol with quinoline as the internal standard. The resulting sample extract was analysed by GC-MS in the selected ion monitoring (SIM) mode with electron-impact (EI) ionization. Data analysis was in basic conformance with the recommendations of ISO 5725-2:1994 and ISO/TR 22971:2005. The raw data were obtained from 19 laboratories. Analyte levels were reported in units µg/g of tobacco on an as-is basis and converted to mg/g on an as-is basis for statistical analysis.

# 3. Organization

## 3.1 Participants

A list of the participating laboratories is provided in Table 1. The laboratories are listed in alphabetical order. The numerical laboratory codes used in this report do not correspond to the same order as shown in the table below. The lab codes used in this study start at number 2. This study was run concurrently with another study and laboratory 1 did not take part in this study. For consistency, the same laboratory codes were used for both the nicotine and minor alkaloids studies[1].

[1] CORESTA Technical Report: 2016 Collaborative Study on Minor Alkaloids in Tobacco Products – February 2017 [RAC-STS-055-CTR]

**Table 1: List of Participating Laboratories**

| Participants |
|---|
| Altria Client Services LLC (ACE), United States |
| Altria Client Services LLC (LPSS), United States |
| C.I.T.Montepaz S.A., Uruguay |
| China National Tobacco Quality Supervision and Test Center, China |
| Enthalpy Analytical Durham, United States |
| Enthalpy Analytical Richmond, United States |
| Global Laboratory Services, United States |
| ITG Brands, United States |
| Japan Tobacco LTRC, Japan |
| KT&G Research Institute, Korea |
| Labstat International ULC, Canada |
| Liggett Group, United States |
| Philip Morris International, Brazil |
| Philip Morris International, Switzerland |
| PT HM Sampoerna Tbk, Indonesia |
| RJ Reynolds Tobacco Company, United States |
| Swedish Match, North America, United States |
| Swedish Match, Northern Europe, Sweden |
| University of Kentucky, United States |

## 3.2 Protocol

### 3.2.1 Study Samples

Laboratories were responsible for procuring all reference and monitor samples prior to starting the study. Laboratories were requested to store the samples at approximately 4 °C upon receipt if the analyses would be conducted within one week or to store the samples at approximately -20 °C if the analyses would be delayed. The study was to be conducted from October 2015 through February 2016. Laboratories were requested to submit data by February 10, 2016. The final data, including re-checks, were received by April 2016. The samples are identified in Table 2. The two cigar filler samples were provided by Altria Client Services LLC while the mint moist smokeless tobacco sample was provided by the American Snuff Company.

**Table 2: Sample Identification**

| Sample Description |
|---|
| CRP1 - Swedish style snus pouch (2009) |
| CRP2 - American-style loose moist snuff (2009) |
| CRP3 - American-style loose dry snuff powder (2009) |
| Cigar filler #1 - Flavoured Ground Cigar Filler |
| Cigar filler #2 - Dark Air-Cured Ground Cigar (Wrapper, Binder and Filler) |
| CORESTA Monitor 8 (CM8) test piece |
| 1R6F - participants will remove the filler from the cigarettes |
| 1R5F filler - filler was pre-ground and homogenized by the University of Kentucky |
| (Mint MST) - American-style loose moist snuff with Mint |

### 3.2.2 Within Laboratory Sample Preparation

The laboratories were directed to remove the samples from cold storage at least 24 hours prior to testing and to not open the samples until equilibrated to ambient temperature. The following sample preparation procedures were to be followed:

- CRP1: Remove unit pouches from a single can. Cut the pouches in half and add the tobacco from the pouch to the extraction vessel and then add the pouch material.
- CRP2, CRP3: Samples should be analysed without further sample grinding. Aliquots may be removed from a single can after mixing the contents of a can.
- 1R6F: The filler from 20 cigarettes (1 pack) should be removed from the paper and filter materials, ground, and mixed before aliquoting.
- 1R5F filler and cigar fillers: The fillers were pre-ground and homogenized.

### 3.2.3 Sample Analysis and Data Reporting

Laboratories were requested to conduct three (3) replicate analyses for each sample. The replicates should be determined from independent tobacco extractions. Data were reported in units of µg/g, on an as-is basis and were converted to mg/g for analysis purposes.

### 3.2.4 Deviations

Laboratory Lab 6 and Lab 20 analysed only the tobacco for CRP1. The intact pouch was not analysed as requested in the protocol.

# 4. Data – Summary Descriptive Statistics

The full data set is listed in Table 3. The results are presented on an as-is basis, without correction for moisture. Each analysis includes three replicates. Raw data plots that include all replicates, without removal of outliers, are given in Appendix A. Outliers are discussed in 5.1. Data eventually dropped as outliers are included in Table 3, but were eliminated prior to r&R calculation.

**Table 3: Full Data Set (results are presented on an as-is basis)**

**The nicotine values are provided on an as-is mg/g basis without correction for moisture.**

| Lab | Rep | 1R5F | 1R6F | CM8 | CRP1 | CRP2 | CRP3 | Cigar Filler #1 | Cigar Filler #2 | Mint MST |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 1 | 17.49 | 23.26 | 33.61 | 15.29 | 14.68 | 25.45 | 9.73 | 8.90 | 13.47 |
| | 2 | 17.61 | 22.86 | 33.91 | 17.28 | 14.82 | 25.42 | 9.46 | 8.77 | 13.81 |
| | 3 | 17.32 | 22.62 | 33.43 | 15.80 | 14.59 | 25.55 | 9.54 | 8.80 | 13.81 |
| 3 | 1 | 15.88 | 18.99 | 24.76 | 10.09 | 14.36 | 21.96 | 8.91 | 8.31 | 12.48 |
| | 2 | 16.14 | 19.85 | 27.79 | 10.29 | 13.33 | 22.16 | 8.98 | 8.10 | 12.58 |
| | 3 | 15.00 | 19.81 | 27.99 | 10.48 | 13.29 | 21.49 | 8.71 | 8.15 | 13.13 |
| 4 | 1 | 15.83 | 18.42 | 27.30 | 10.72 | 13.07 | 21.92 | 8.63 | 7.56 | 12.57 |
| | 2 | 15.60 | 18.70 | 27.64 | 10.86 | 13.05 | 22.02 | 8.63 | 7.79 | 12.47 |
| | 3 | 15.60 | 18.85 | 27.82 | 10.97 | 12.97 | 22.24 | 8.50 | 7.73 | 12.56 |
| 5 | 1 | 16.27 | 20.40 | 30.04 | 10.75 | 13.91 | 20.57 | 8.77 | 8.14 | 12.67 |
| | 2 | 16.15 | 18.78 | 29.00 | 12.81 | 13.40 | 23.33 | 8.97 | 8.34 | 12.38 |
| | 3 | 16.16 | 18.95 | 28.70 | 9.94 | 13.82 | 23.55 | 8.96 | 8.48 | 12.96 |
| 6 | 1 | 16.80 | 18.09 | 27.21 | 9.58 | NA | NA | 8.14 | 7.84 | 12.12 |
| | 2 | 16.85 | 17.89 | 26.78 | 9.61 | NA | NA | 8.46 | 7.92 | 12.14 |
| | 3 | 16.91 | 17.82 | 27.19 | 9.76 | NA | NA | 8.38 | 7.88 | 12.19 |
| 7 | 1 | 15.97 | 19.33 | 27.67 | 10.26 | 13.23 | 22.93 | 9.12 | 8.11 | 13.15 |
| | 2 | 16.16 | 19.21 | 27.61 | 10.22 | 13.21 | 22.63 | 8.92 | 8.17 | 13.21 |
| | 3 | 16.01 | 19.16 | 27.88 | 10.52 | 13.63 | 22.58 | 8.80 | 8.26 | 13.10 |
| 8 | 1 | 15.50 | 18.10 | 26.97 | 10.37 | 13.40 | 23.11 | 8.67 | 7.82 | 12.69 |
| | 2 | 15.73 | 18.28 | 26.57 | 10.97 | 13.24 | 22.91 | 8.00 | 8.27 | 12.69 |
| | 3 | 15.92 | 18.49 | 26.72 | 9.87 | 13.45 | 22.54 | 8.38 | 7.99 | 12.58 |
| 9 | 1 | 15.96 | NA | 26.02 | NA | NA | NA | NA | NA | NA |
| | 2 | 16.27 | NA | 25.41 | NA | NA | NA | NA | NA | NA |
| | 3 | 16.21 | NA | 25.96 | NA | NA | NA | NA | NA | NA |
| 10 | 1 | 14.91 | 17.75 | 26.88 | 9.54 | 11.90 | 20.79 | 8.17 | 6.82 | 12.02 |
| | 2 | 14.67 | 17.48 | 27.45 | 9.87 | 12.02 | 21.46 | 7.90 | 7.18 | 11.74 |
| | 3 | 14.66 | 17.62 | 26.31 | 9.70 | 12.35 | 20.72 | 7.81 | 6.96 | 11.95 |
| 11 | 1 | 15.81 | 18.60 | 28.23 | 10.08 | 12.72 | 22.64 | 8.91 | 7.92 | 12.08 |
| | 2 | 15.76 | 19.04 | 27.97 | 9.87 | 12.63 | 22.55 | 8.99 | 8.15 | 12.17 |
| | 3 | 15.75 | 18.71 | 28.17 | 9.85 | 12.60 | 22.56 | 8.70 | 8.02 | 12.22 |
| 12 | 1 | 14.39 | 16.50 | 26.14 | 10.42 | 12.43 | 21.63 | 7.92 | 7.18 | 11.65 |
| | 2 | 14.61 | 16.45 | 25.69 | 10.55 | 12.17 | 21.78 | 7.85 | 7.42 | 11.56 |
| | 3 | 14.35 | 16.75 | 25.71 | 9.81 | 13.10 | 21.49 | 8.03 | 7.10 | 11.33 |

| Lab | Rep | 1R5F | 1R6F | CM8 | CRP1 | CRP2 | CRP3 | Cigar Filler #1 | Cigar Filler #2 | Mint MST |
|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 1 | 15.95 | 19.37 | 27.94 | 10.18 | 13.04 | 22.47 | 9.01 | 8.20 | 13.49 |
| | 2 | 15.96 | 19.39 | 28.54 | 10.37 | 12.83 | 22.82 | 9.00 | 8.30 | 13.08 |
| | 3 | 16.14 | 19.70 | 28.14 | 10.43 | 13.25 | 22.92 | 8.88 | 8.44 | 13.07 |
| 14 | 1 | 14.67 | 17.99 | 28.43 | 11.62 | 13.15 | 22.11 | 8.63 | 7.47 | 11.96 |
| | 2 | 14.69 | 17.95 | 27.51 | 11.11 | 13.76 | 21.78 | 8.52 | 7.48 | 12.32 |
| | 3 | 15.21 | 17.99 | 30.08 | 11.62 | 13.42 | 21.89 | 8.26 | 7.74 | 12.32 |
| 15 | 1 | NA | 20.06 | 26.77 | NA | NA | NA | 8.38 | 7.16 | NA |
| | 2 | NA | 19.78 | 26.57 | NA | NA | NA | 8.32 | 6.79 | NA |
| | 3 | NA | 19.98 | 27.03 | NA | NA | NA | 8.47 | 6.91 | NA |
| 16 | 1 | 16.88 | 17.56 | 27.55 | 9.44 | 12.74 | 22.09 | 8.35 | 7.71 | 12.25 |
| | 2 | 17.05 | 17.75 | 27.85 | 9.29 | 12.66 | 22.24 | 8.19 | 7.95 | 12.33 |
| | 3 | 17.03 | 17.59 | 27.44 | 9.39 | 12.69 | 22.11 | 8.19 | 7.80 | 12.40 |
| 17 | 1 | 17.15 | NA | 28.14 | 11.49 | 13.36 | 23.25 | NA | NA | NA |
| | 2 | 17.66 | NA | 27.69 | 12.17 | 14.22 | 22.95 | NA | NA | NA |
| | 3 | 17.71 | NA | 27.98 | 11.00 | 14.86 | 23.05 | NA | NA | NA |
| 18 | 1 | 15.76 | 19.00 | 32.10 | 13.17 | 13.07 | 22.53 | 8.72 | 7.51 | 12.54 |
| | 2 | 16.05 | 18.39 | 29.47 | 13.51 | 13.35 | 24.46 | 8.57 | 7.59 | 12.41 |
| | 3 | 15.59 | 19.02 | 31.15 | 11.87 | 13.06 | 21.89 | 8.76 | 8.55 | 12.89 |
| 19 | 1 | 16.22 | NA | 26.77 | 8.24 | 11.66 | 20.65 | NA | NA | NA |
| | 2 | 17.34 | NA | 26.91 | 8.60 | 11.62 | 20.82 | NA | NA | NA |
| | 3 | 19.86 | NA | 27.49 | 8.43 | 11.05 | 20.61 | NA | NA | NA |
| 20 | 1 | 16.74 | 13.82 | 26.62 | 9.22 | 12.51 | 21.17 | 7.46 | 6.99 | 11.23 |
| | 2 | 17.24 | 12.88 | 26.14 | 9.29 | 12.35 | 20.64 | 7.37 | 7.04 | 11.42 |
| | 3 | 17.21 | 12.90 | 26.24 | 9.23 | 12.28 | 20.41 | 7.42 | 6.76 | 11.30 |

NA indicate s that the laboratory did not provide data for that sample.

# 5. Data – Statistical Analysis

A statistical analysis was conducted in basic conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. A summary of the results from outlier detection and the calculated results for repeatability (r) and reproducibility (R) are given below in sections 5.1 and 5.2, respectively. Raw data plots that include all replicates shown in Table 3, prior to removal of outliers, are given in Appendix A.

## 5.1 Exclusion of Outliers

Procedures outlined in ISO 5725-2:1994 and ISO/TR 22971:2005 were generally used for the exclusion of outliers. An adaptation of Levene's Test was used for eliminating laboratories with overly large repeatability standard deviations and Grubbs' Test was used to eliminate laboratories with outlying mean values.

ISO 5725(2) also recommends the use of Mandel's h and k plots. Mandel's h statistic is the same as the statistic used in Grubbs' Test. Similarly Mandel's k statistic, associated with within lab standard deviation, is statistically equivalent to the c-value calculated in Cochran's Test ( $k = \sqrt{n_{labs}}c$ ). However, the critical values associated with Mandel's h and k statistics do not make allowance for multiple testing and can therefore, give a false impression of statistical significance. Thus, Mandel's h and k statistics do not add fundamentally new information and as typically employed may lead to incorrect conclusions. For those reasons, we do not include Mandel's h and k plots.

The intent of ISO 5725-2:1994 is to eliminate outliers that exceed a 1 % critical value. This was accomplished by an adaptation of Levene's Test. Levene's Test is preferable to Cochran's Test, which is recommended in ISO 5725-2:1994, because of Cochran's Test's extreme sensitivity to deviations from normality. Grubbs' Test and an adaptation of Levene's Test were applied at the standard nominal 1 % significance level to determine outliers and the results are shown in Table 4. Levene's Test is mentioned in ISO/TR 22971:2005 as an alternative to Cochran's Test. However, Levene's Test does not directly apply without adaptation. For more details, see the footnote below[2].

The initial examination for outliers indicated that Lab 2 tended to give outlying results. For that reason Lab 2 was dropped from the analysis and the data were again examined for outliers.

**Table 4: Outliers**

| Sample | Levene's Outliers Lab | Grubbs' Outliers Lab |
|---|---|---|
| 1R5F | 19 | – |
| 1R6F | – | 20 |

The (–) symbol indicates an outlier was not detected.

## 5.2 Calculation of Repeatability and Reproducibility

After removal of outlying data based on numerical data consistency methods discussed above (Grubbs' Test and Levene's Test), the final repeatability and reproducibility (r & R) results were calculated and are shown in Table 5. It should be understood that the r & R results reflect both laboratory variability and product consistency.

[2] Levene's Test is commonly used to determine if each of several subpopulations have the same variance. Since it was designed to test for overall differences, not to determine if the largest variance is significantly greater than the others, some adaptation is necessary to use the approach to eliminate laboratories whose within lab variation is too large. Levene's Test was adapted to this purpose by Morton, who presented the approach utilized in this report at the 2014 CORESTA Congress (Quebec, Canada, presentation ST28, October 14, 2014). Specifically, the approach taken here is a two-step process with a lab being eliminated as an outlier if both steps are statistically significant. First, Levene's Test was run at a nominal $\alpha$-level of 0.02. Second a comparison of the largest variance to the remaining variances is carried out at a one-sided nominal level of $\alpha$=0.01/number of labs. Dividing by the number of labs is to account for multiple testing, since it is not known *a priori* which lab will have the largest variance. Simulation studies were carried out by Morton and presented at the 2014 CORESTA Congress and these results demonstrated that this process has an overall $\alpha$-level near 0.01 and is robust to deviations from normality.

**Table 5: Repeatability (r) and Reproducibility (R) Limits for as-is Nicotine (mg/g)**

| Product | No. of Labs * | Mean | Repeatability | | Reproducibility | |
|---|---|---|---|---|---|---|
| | | | r | r (%) | R | R (%) |
| 1R5F - Ground Cigarette Filler | 16 | 15,97 | 0,65 | 4,0% | 2,44 | 15,3% |
| 1R6F - Ground Cigarette Filler | 14 | 18,56 | 0,90 | 4,8% | 2,74 | 14,7% |
| CM8 - Ground Cigarette Filler | 18 | 27,52 | 1,91 | 7,0% | 3,83 | 13,9% |
| CRP1 - Pouched Snus | 16 | 10,36 | 1,40 | 13,5% | 3,18 | 30,7% |
| CRP2 - Loose Moist Snuff | 15 | 12,98 | 0,93 | 7,2% | 2,09 | 16,1% |
| CRP3 - Loose Dry Snuff Powder | 15 | 22,10 | 1,66 | 7,5% | 2,60 | 11,8% |
| Cigar Filler #1 - Flavored Ground Cigar Filler | 15 | 8,46 | 0,42 | 5,0% | 1,29 | 15,3% |
| Cigar Filler #2 - Dark Air-Cured Ground Cigar (Wrapper, Binder, and Filler) | 15 | 7,73 | 0,58 | 7,4% | 1,46 | 18,9% |
| Mint MST | 14 | 12,37 | 0,50 | 4,1% | 1,59 | 12,8% |

* The number of laboratory data sets after removal of outliers.

# 6. Data Interpretation

The GC/MS method described herein was compared to the CRM 62 [3] methyl tert-butyl ether (MTBE) and hexane methods. The R (%) values are statistically significantly higher for the GC/MS method than for the CRM 62 MTBE method (p=0.002) but similar to the hexane method (p=0.14). A graph of the three methods' relative reproducibilities is given in Figure 1.

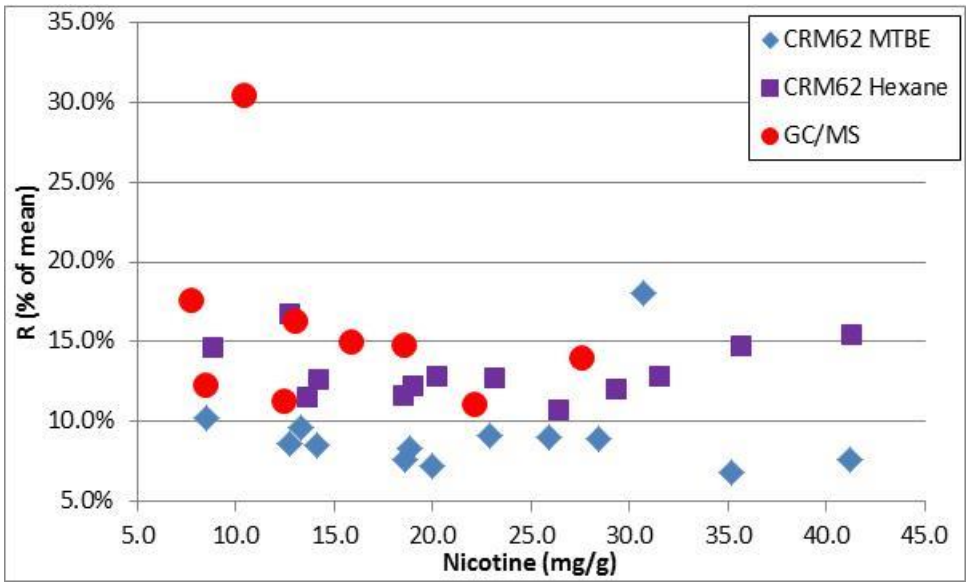



**Figure 1: %R values, versus nicotine content, for this study compared to CRM 62.**

[3] CORESTA Recommended Method No. 62 – Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis – February 2005 (Routine Analytical Chemistry Sub-Group).

The values for repeatability (r) and reproducibility (R) are shown in Table 5. The values for r & R demonstrate similar repeatability and reproducibility in both tobacco and smokeless tobacco. This table also includes values for % R for each sample, which range from approximately 12 % to over 30 %. These results indicate a similar level of variability for the determination of nicotine in both tobacco and smokeless tobacco, with the possible exception of CRP1, the only pouched product in this study. It was reported that the CRP-1 sample exceeded the recommended sample mass given in the protocol. In order to fall within the linear calibration range of the method, this sample required either dilution of the original sample or preparation of a new sample using less tobacco mass, which may explain the higher level of variability seen with this sample. The overall level of variability seen in this study was slightly higher than the CRM 62[4] collaborative study on nicotine in tobacco (Figure 1).

# 7. Recommendations

In 2015, RAC and STS conducted an inter-laboratory study for the determination of nicotine in tobacco and smokeless tobacco products. The overall level of variability seen in this study was slightly higher when compared to values reported in CRM 62 but this may be related to sample homogeneity or lack of experience with the method compared to CRM 62 and not the analytical method used for the current study. However, the study results for the pouched product, CPR1, showed higher variability as compared to the loose tobacco products in this study. In order to better accommodate pouched products, it is recommended that the method be modified to either extend the calibration range or to provide an alternative sample preparation procedure for pouched products. The results for this study showed similar r & R values for both tobacco and smokeless tobacco products which indicates that this method is appropriate for the analysis of both tobacco and smokeless tobacco products. No evidence was found for smokeless tobacco related method interferences such as interferences with nicotine or the internal standard and with flavour compounds present in these samples. The lack of interferences from flavour compounds eliminated the need for the standard addition experiments required in some other methods for the analysis of nicotine in smokeless tobacco. The results of this study were discussed during the CORESTA RAC and STS meeting held in April 2016, in Lausanne, Switzerland. The RAC and STS agreed that this method is fit for use for the determination of nicotine in tobacco and smokeless tobacco products.

[4] CORESTA Recommended Method No. 62 – Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis – February 2005 (Routine Analytical Chemistry Sub-Group).

# APPENDIX A: Study Protocol

CORESTA ROUTINE ANALYTICAL CHEMISTRY SUB-GROUP
CORESTA SMOKELESS TOBACCO SUB-GROUP

Project Title: The Determination of Nicotine in Tobacco Products by GC-MS

Type of Document: Collaborative Study Protocol

Date: November 19th, 2015

Written by: Gene Gillman, Study Coordinator

**Confidentiality Notice: All data submitted by participating laboratories will be coded and kept confidential.**

## 1. Introduction

The overall objective of this project is to develop a CORESTA Recommended Method (CRM) for the determination of nicotine in tobacco and tobacco products. Tobacco products include cigarette and cigar filler, and smokeless tobacco products

## 2. Objective

The objective of this study is to calculate repeatability (r) and reproducibility (R) for the GC-MS method that is provided.

**Note: Use of any method other than that specified will not support the study objectives and the data cannot be included.**

## 3. Time schedule

| Date | Activity |
|---|---|
| October 20, 2015 | Laboratories state their intention to participate **and order study materials** |
| November 6, 2015 | Finalize protocol and distribute |
| February 10, 2016 | Laboratories submit results by this date |
| Spring 2016 | Discuss results at RAC and STS meetings |

**Note: Although each participant should read the applicable methods to determine what supplies are needed in order to participate in the study, the following supplies may need to be ordered:**

1. ISO Guide 34 certified reference standard Nicotine is recommended.
2. GC column: CAM column (30m x 0.25mm id x 0.25µm df) or equivalent polar, base-deactivated PEG column (e.g. Stabilwax-DB or Carbowax Amine).

## 4. Participating Laboratories

Following receipt of this protocol, the participating laboratories will confirm or notify the study coordinator of their intent to participate. Please include your complete company name and location.

## 5. Samples

The samples listed in Table 2 will be analyzed. Samples should be ordered from:

- CORESTA Reference Products (CRPs) - North Carolina State University
- 1R6F cigarettes, 3R4F cigarettes, and 1R5F filler - University of Kentucky 1R6F cigarettes, 3R4F cigarettes, and 1R5F filler - University of Kentucky can be ordered from **https://refcig.uky.edu/client/index.html**
- Mint Flavored US Moist Snuff – American Snuff Company
  Contact Dr. John Bunch to request samples.
  [redacted]
- Two processed Cigar Filler Samples – Altria
  Contact Dr. Karl Wagner to request samples.
  [redacted]

| | |
|---|---|
| Cerulean<br>Rockingham Drive<br>Linford Wood East<br>Milton Keynes MK14 6LY<br>United Kingdom<br>Tel: +44 1908 23 38 33<br>Fax: +44 1908 23 53 33<br>e-mail: sales@cerulean.com | Borgwaldt KC GmbH<br>Spare Parts Department,<br>Schnackenburgallee 15,<br>D-22525 Hamburg<br>Germany,<br>Tel: +49 40 85 31 380<br>Fax: +49 40 850 56 00<br>e-mail: BKC@Borgwaldt.com |

Participants may use an internal supply of these products assuming the samples have been stored unopened and under suitable conditions. It is critical that the CRPs have been stored at the recommended temperature of −20°C, or they should not be used.

**Processed Cigar Filler samples**

The samples should be stored at -20ºC for long term storage. At a minimum, the following equilibration procedure must be followed to ensure water re-equilibrates throughout the samples

1. Remove the samples from -20ºC.
2. Allow the unopened samples to equilibrate in the refrigerator for a minimum of 24 hours.
3. Allow the unopened samples to equilibrate at ambient conditions for a minimum of 1hour prior to opening.

**Shake the ground filler samples vigorously prior to opening to break clumps and re-homogenize the samples.**

**All remaining samples should be retained in sealed containers at −20°C as they may be used for future collaborative studies for other analytes.**

**Table 2: Samples**

| | |
|---|---|
| CRP1 - Swedish style snus pouch | 3 cans |
| CRP2 - American-style loose moist snuff | 3 cans |
| CRP3 - American-style loose dry snuff powder | 3 cans |
| Cigar filler #1- Pre-ground and homogenized by ALCS | 1 container (~20g) |
| Cigar filler #2- Pre-ground and homogenized by ALCS | 1 container (~20g) |
| CORESTA Monitor 8 (CM8) test piece | 1 carton |
| 1R6F filler - participants will remove the filler from the cigarettes | 1 carton |
| 1R5F filler - filler will be pre-ground and homogenized by the University of Kentucky | 1 container |
| American-style loose moist snuff with Mint (Mint US MST) | 4 cans |

## 6. Analysis

6.1. Analytes: Nicotine will be determined in each sample. Use of ISO Guide 34 certified reference standards is recommended for this study. Laboratories may use their own source or purchase the suggested material listed above.

6.2. Methods: Participating laboratories should use the supplied GC-MS method for the determination of the analytes. Please keep in mind that data generated from methods

other than specified in this protocol do not support the study objectives and cannot be included in the study results.

Replicates and Sample Handling: Conduct three (3) independent replicate analyses for each sample. The replicates should be determined from independent tobacco extractions**.**

6.3. Sample preparation: Additional sample preparation requirements are listed in Table 3

**Table 3: Sample Preparation Requirements**

| Product | Sample Preparation |
|---|---|
| CRP1 | Remove unit pouches from a single can. Cut the pouches in half and add the tobacco from the pouch to the extraction vessel and then add the pouch material. |
| CRP2, CRP3, Mint US MST | Samples should be analysed without further sample grinding. Aliquots may be removed from a single can after mixing the contents of a can. |
| 1R6F, Cigar Filler, CM8 | The filler from 20 cigarettes (1 pack) should be removed from the paper and filter materials, ground, and mixed before aliquoting. |
| 1R5F filler and Cigar fillers | The tobacco from these products has been pre-ground and homogenized. Therefore, these filler samples should be mixed in the container and used as-is. |

## 7. Data Reporting:

Participating laboratories should use the embedded Excel document for data reporting. The analytes should be reported on an as-is and a dry weight basis. Other requested methodological details should also be reported in the data reporting sheet. The completed data sheet should be sent to the following:

Gene Gillman: [redacted]

| | |
|---|---|
| Nicotine Analytical Method | Removed |
| Final Data Reporting Worksheet | Removed |

## 8. Statistical Analysis

A statistical analysis in general conformance with ISO 5725-2:1994 and ISO/TR 22971:2005 will be conducted.

## 9. Presentation of the Results

The final output will be a presentation for discussion at the Spring 2016 RAC and STS meetings.

# APPENDIX B: Analytical Method

## DETERMINATION OF NICOTINE IN TOBACCO AND TOBACCO PRODUCTS BY GC-MS – METHOD FOR COLLABORATIVE TEST

### 1. INTRODUCTION

This document has been prepared for use by CORESTA RAC and STS members participating in the 2015 collaborative test for nicotine. The purpose of this method is to quantitatively measure the amount of nicotine in tobacco products by GC-MS using electron-impact (EI) ionization.

### 2. SCOPE

This method is applicable to the determination of the nicotine in a wide range of smokeless tobacco products, cigarette tobacco, and cigar tobacco.

### 3. PRINCIPLE

Nicotine is extracted from tobacco with aqueous sodium hydroxide (2N) and methanol using quinoline as an internal standard. After shaking for 30 minutes, the sample is filtered then analyzed by GC-MS using electron-impact (EI) ionization.

### 4. CHEMICALS

| *Standards* | | *CAS No.* |
|---|---|---|
| 4.1 | Quinoline | [91-22-5] |
| 4.2 | (-)-Nicotine (NIC) | [54-11-5] |
| *Ancillary Chemicals* | | |
| 4.3 | Methanol (HPLC/ACS grade) | [67-56-1] |
| 4.4 | Sodium Hydroxide (NaOH) – 2N solution | [1310-73-2] |
| 4.5 | Water (Type 1 or HPLC grade) | [7732-18-5] |

### 5. EQUIPMENT

In addition to the general glassware and apparatus found within a typical analytical laboratory, the following is required when performing this analytical method:

5.1 Capillary GC with MS detector and split inlet.

5.2 Analytical balance (with 0.1 mg accuracy)

5.3 Orbital Shaker (or equivalent)

5.4 Dispensette capable of delivering 40 mL (or equivalent)

5.5 Eppendorf repeater with disposable tips, or equivalent

*Per sample replicate:*

5.6 50-mL polypropylene centrifuge tube with screw-cap (or equivalent)

5.7 Syringe and syringe filter (0.45 µm, nylon)

5.8 Amber autosampler vial (2 mL) with PTFE screw-cap

## 6. STANDARDS PREPARATION

The following is an ***example*** of how to prepare stock standards and calibration standards. Different amounts and volumes can be used, if necessary, to prepare the standards, provided the concentration of the calibration standards prepared covers the anticipated concentration range of the samples.

### 6.1 Internal Standard

#### 6.1.1 Quinoline Stock Solution (~ 50 mg/mL)

Accurately weigh approximately 1.25 g of quinoline into a 25-mL volumetric flask. Add a small amount of MeOH to dissolve then make to volume with MeOH. Mix well.

#### 6.1.2 Internal Standard Spiking Solution (ISSS)

Accurately add 2.0 mL of the stock solution prepared in 6.1.1 to a 25-mL volumetric flask. Make to volume with MeOH and mix well.

### 6.2 Calibration Blank (Standard 0)

Accurately pipette 0.25 mL of the ISS solution (prepared in 6.1.2) into a 25-mL volumetric flask.

Make to volume with MeOH and mix well.

### 6.3 Calibration Standards – Nicotine

#### 6.3.1 1° Stock in MeOH (approx. 50 mg/mL)

Accurately weigh approximately 1.0 g nicotine, into a 20-mL volumetric flask.

Dissolve in MeOH then bring to volume and mix well.

#### 6.3.2 2° Stock in MeOH

Transfer 1.0 mL of the nicotine 1° (*prepared in 6.3.1*) to a single 50-mL volumetric flask.

Dilute to volume with MeOH and mix well.

#### 6.3.3 Calibration Standards

Take appropriate aliquots of the 2° stock *(prepared in 6.3.2)* and transfer to separate 25-mL volumetric flasks (Table 1).

Add 250 µL ISTD working solution (*prepared in 6.1.2*) to each.

Make to volume with MeOH and mix well.

**Table 1. Nicotine calibration standards – Nominal Concentrations (actual concentrations will vary depending upon the amount weighed and the purity of the analyte)**

| Standard ID | Stock Vol. (mL) | Final Vol. (mL) | Nominal NIC Conc. (µg/mL) |
|---|---|---|---|
| 1 | 0.1 | 25 | 4 |
| 2 | 0.2 | 25 | 8 |
| 3 | 0.5 | 25 | 20 |
| 4 | 1 | 25 | 40 |
| 5 | 3 | 25 | 120 |
| 6 | 5 | 25 | 200 |
| 7 | 10 | 25 | 400 |

## 7. PROCEDURE

### 7.1 Sample Extraction

7.1.1 Accurately weigh 0.25 ± 0.05 g of tobacco into a 50-mL PP centrifuge tube, or equivalent.

7.1.2 Add 4 mL NaOH pre-treatment solution.

7.1.3 Let samples sit for approximately 30 minutes.

7.1.4 Accurately add 400 µL ISSS and 40 mL MeOH.

7.1.5 Shake or stir for approximately 30 minutes.

7.1.6 Filter an aliquot into each of two autosampler vials.

**Note: Adjust volumes as needed for portioned tobacco products. The ratio of ISSS to MeOH must be constant in all samples.**

### 7.2 Sample Run Order

#### 7.2.1 Priming Sample/System Suitability

A priming sample (matrix) must be injected from 2 to 3 times to ensure that active sites in the system are minimized.

#### 7.2.2 Calibration Blank

At least one reagent blank should be injected to ensure no carryover from the sample.

#### 7.2.3 Calibration Standards

Arranged in increasing levels of concentration, beginning and ending with a reagent blank.

#### 7.2.4 Samples

Samples (including any blanks, recoveries and/or reference samples) are typically analyzed in batches of ten to twelve or less.

#### 7.2.5 Continuing Calibration (CC)

CC standards are analyzed to verify that the calibration is still valid. A reagent blank should be analyzed after every CC to monitor the carryover.

## 7.3 GC-MS Apparatus and Operation Parameters

The MS is operated in EI/SIM mode. The retention times and ions monitored are listed in Table 3.

**Table 2. GC-MS condition and parameters.**

| | |
|---|---|
| Analytical Column | CAM column (30m x 0.25mm id x 0.25µm df) or equivalent polar, base-deactivated PEG column (e.g. Stabilwax-DB or Carbowax Amine) |
| Column Flow | 1.0 mL/min (UHP Helium) |
| Injection Port Temperature | 230°C |
| Injection Port Liner | Ultra Inert split, straight with glass wool, or equivalent |
| Injection Volume | 1 µL |
| Injection Mode | Split, 60:1 |
| Initial Oven temperature | 110 °C hold for 1 minute |
| Temperature ramp | 10 °C/min to 235°C, hold 4.5 minutes or more |
| Transfer Line temperature | 230°C |
| MS Source temperature | 230°C |
| MS Quad temperature | 150°C |
| Solvent Delay | Approx. 5 minutes |

**Table 3. Analyte retention times and ions monitored.**

| Name | MW | Ret Time (min.) | Quantifier Ion (m/z) | Qualifier Ion (m/z) | Internal Standard |
|---|---|---|---|---|---|
| Quinoline | 129.2 | 8.9 | 129 | N/A | N/A |
| (-)-Nicotine | 162.2 | 8.2 | 84 or 162 | 162 or 84 | Quinoline |

## 7.4 Integration/Quantitation Parameters

The relative areas of the analytes and the internal standard in each calibration standard are used to create a calibration curve. The curves are linear with 1/x weighting.

## 8. CHROMATOGRAMS

Figures 1-2 illustrate typical chromatograms that can be expected to be obtained with this method.

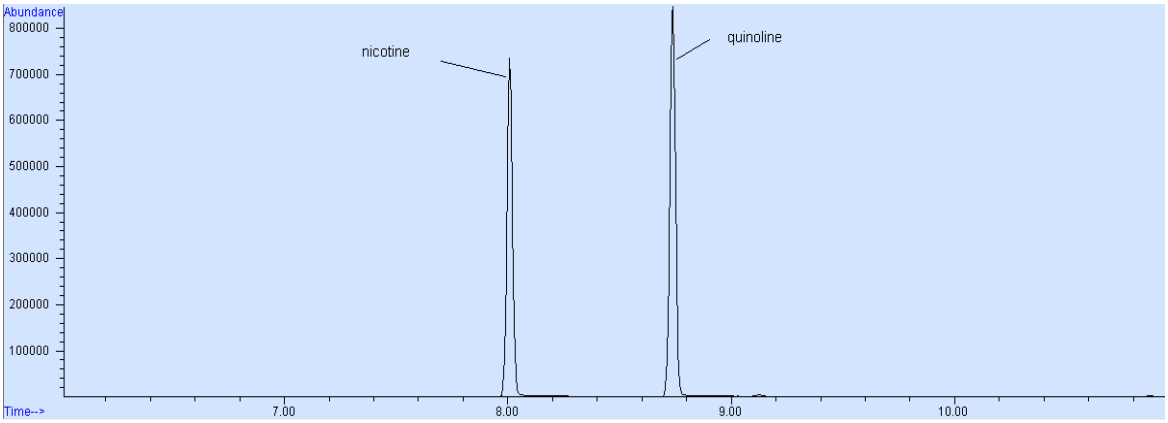



**Figure 1. Total Ion Chromatogram for nicotine and quinoline in calibration standard (Nic ~ 100 µg/mL)**

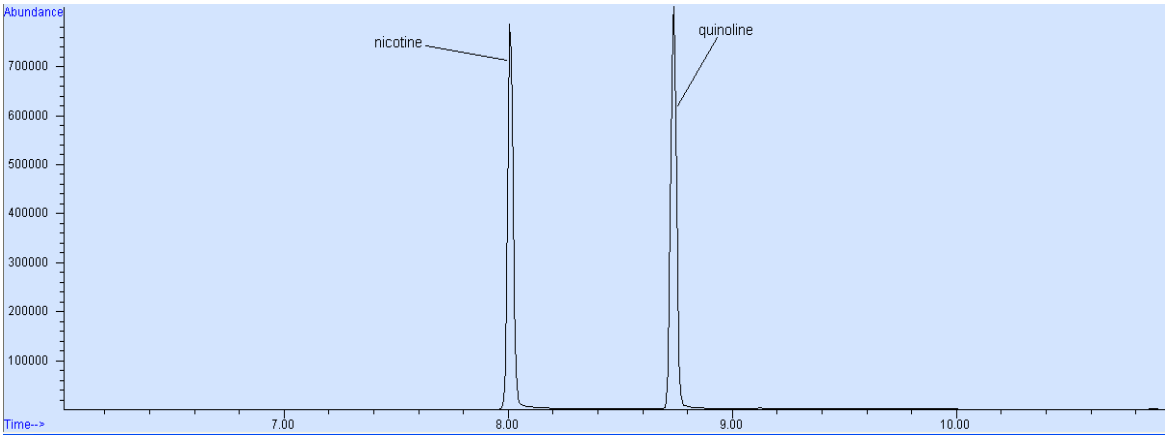



**Figure 2. Total Ion Chromatogram for nicotine and quinoline in 3R4F sample extract.**

## 9. CALCULATIONS

The analyte concentration (in µg/mL) is determined by the internal standard calibration method using the regression equation derived from the calibration curve. Results are then converted and reported on a per weight basis, typically µg/g.

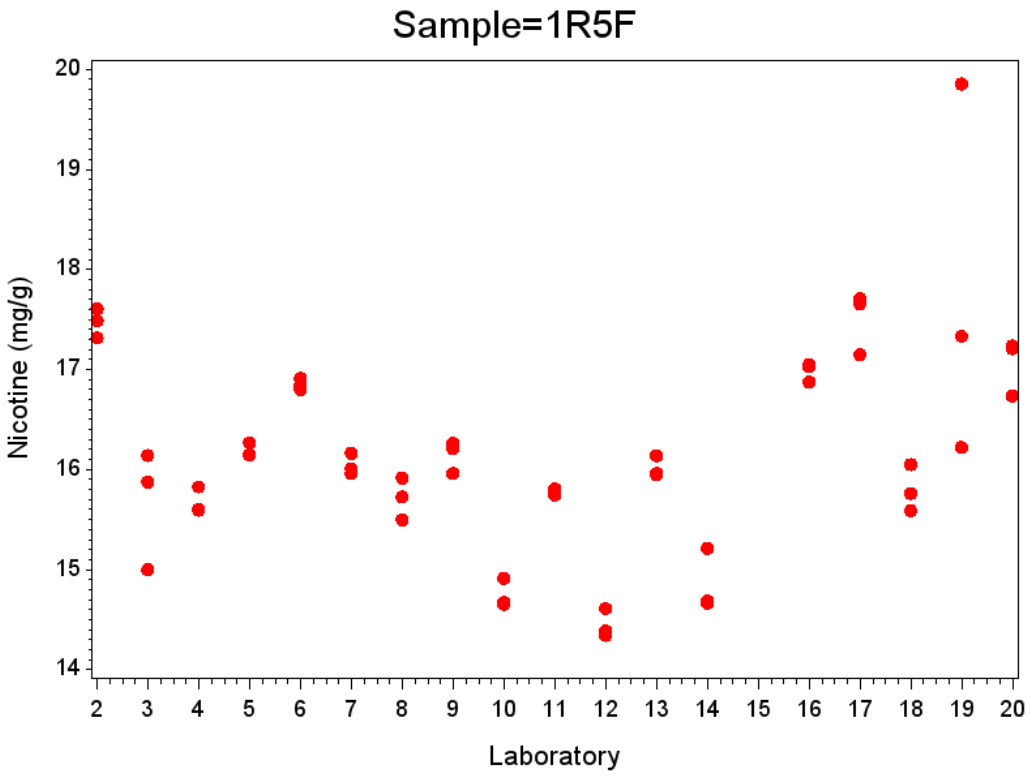
$$Analyte\ conc.\,(\mu g/g) = \frac{Analyte\ conc.\,(\mu g/mL) \times Sample\ Vol.\,(40\ mL) \times MF}{Sample\ Weight\ (g)}$$

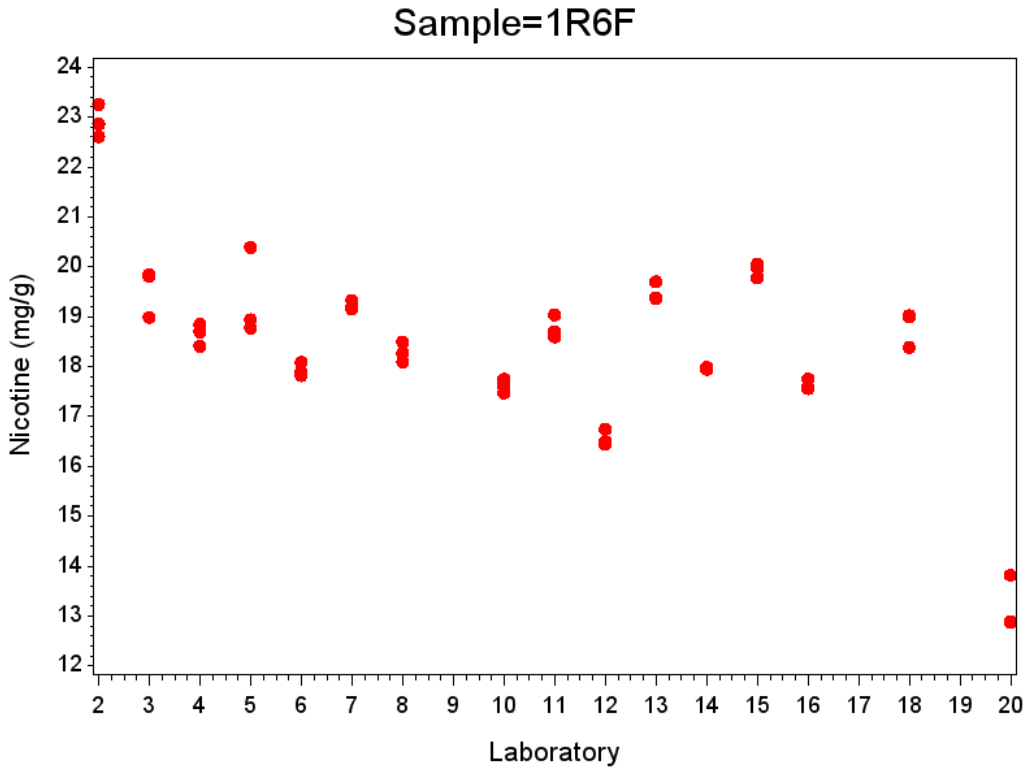
**Note: there is no volume correction required for the addition of the pre-treatment solution as it dilutes all analytes and internal standards equally.**

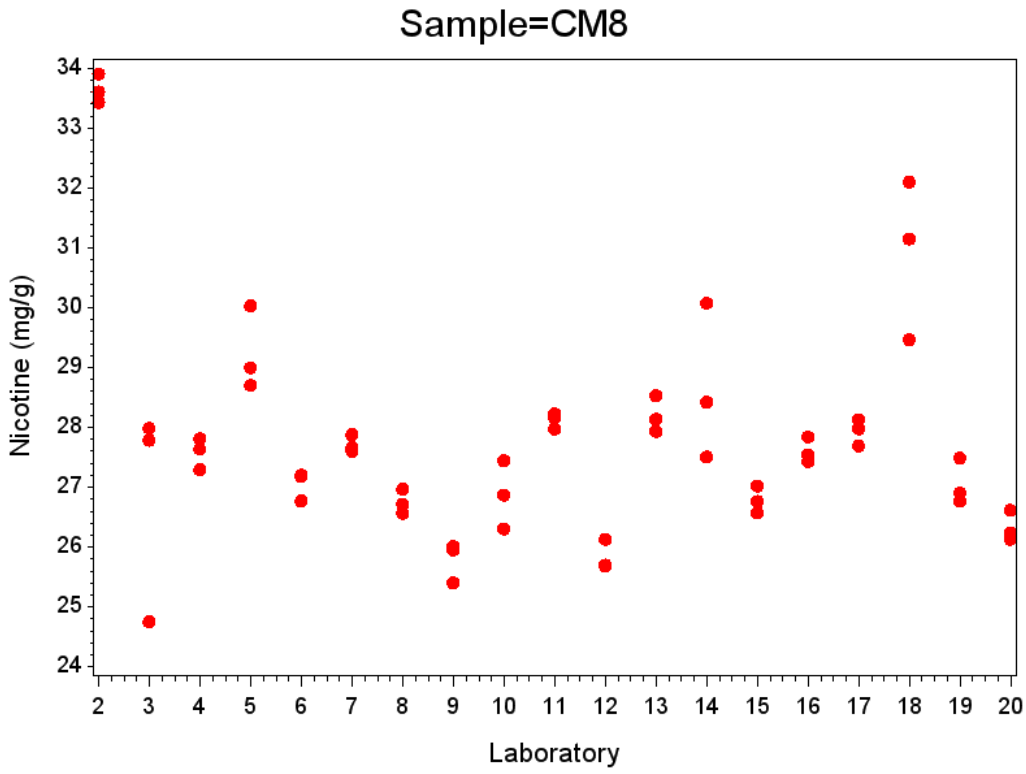
When reporting results on an 'as is' basis (i.e. not corrected for moisture content), the moisture factor (MF) value is 1. In instances where results are required to be reported on a dry weight basis (dwb), the following calculation is required to obtain MF for a sample:

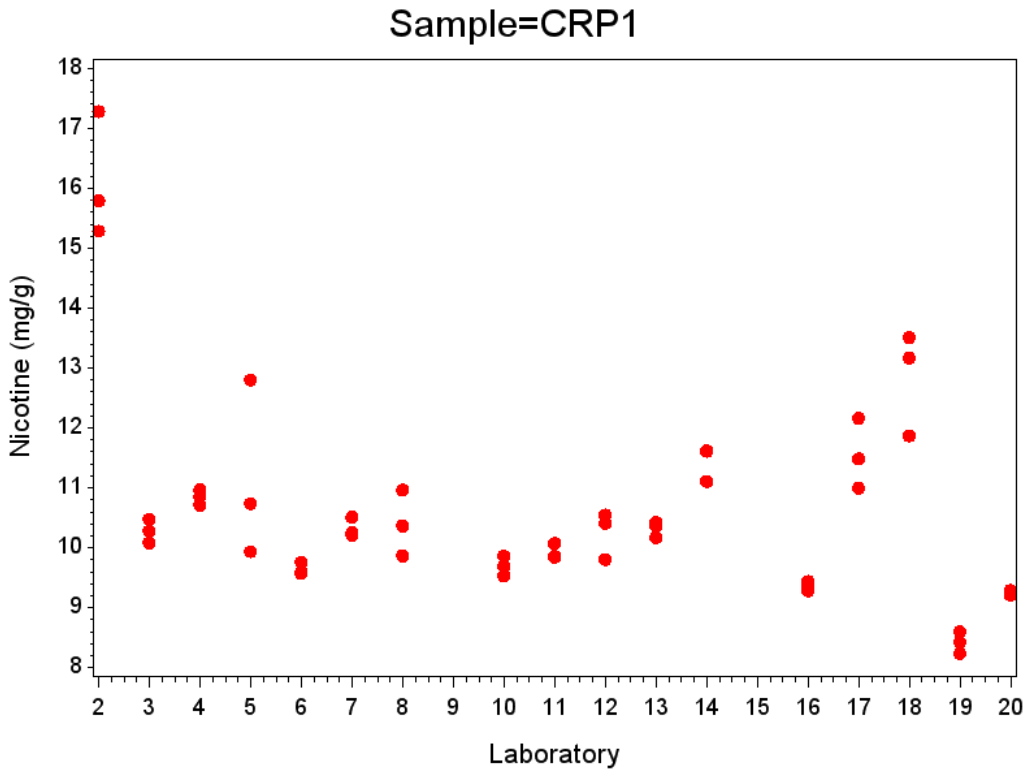
$$Moisture\ Factor\ (MF) = \frac{100}{100 - Moisture(\%)}$$

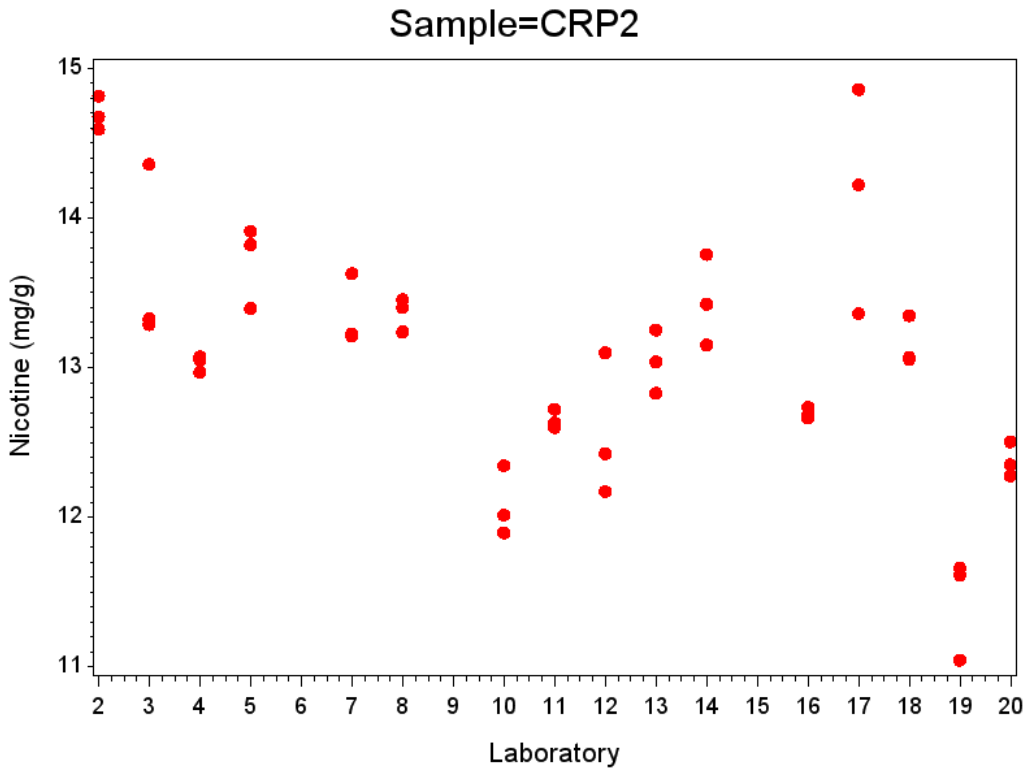
# APPENDIX C: Raw Data Plots

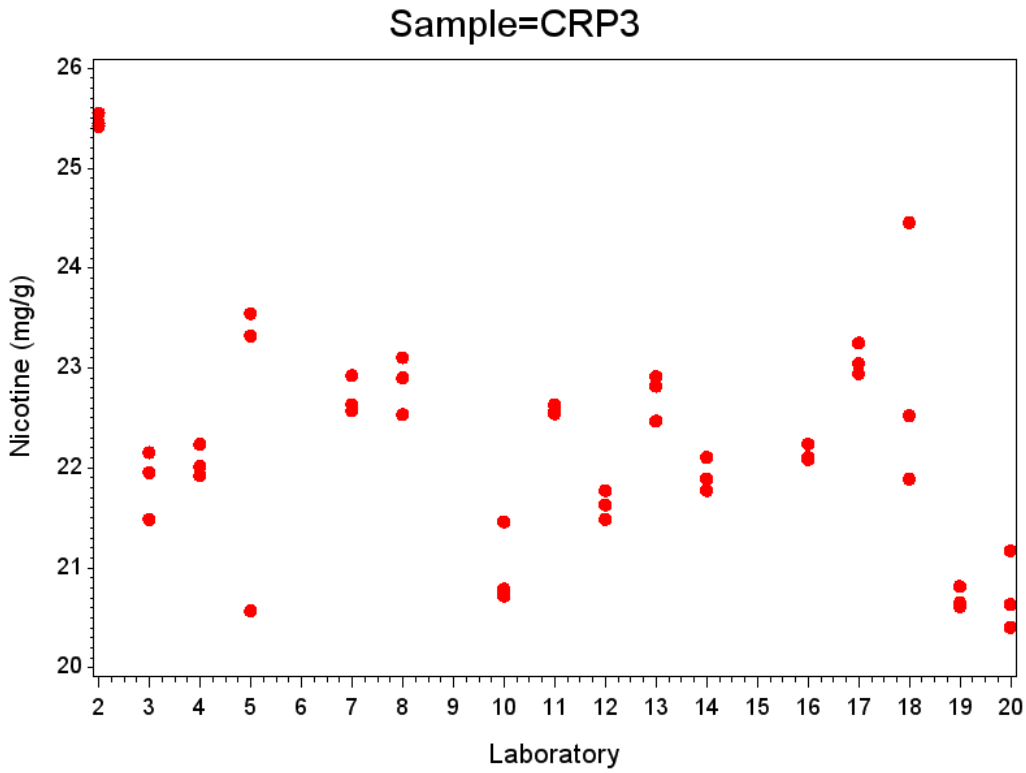


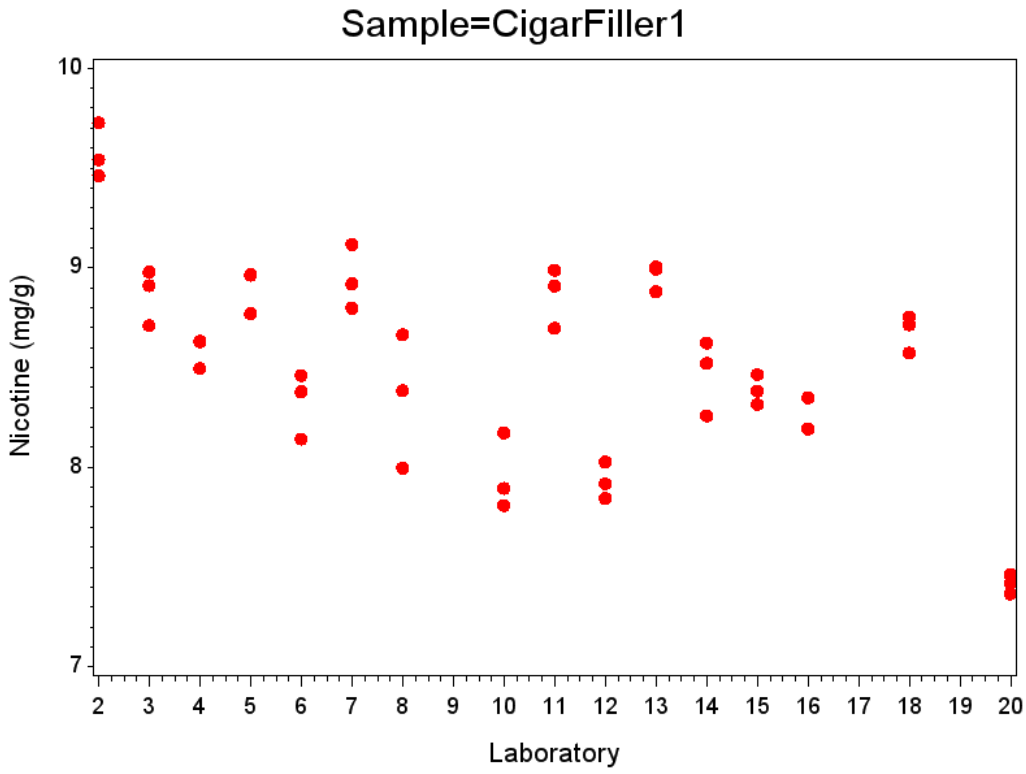



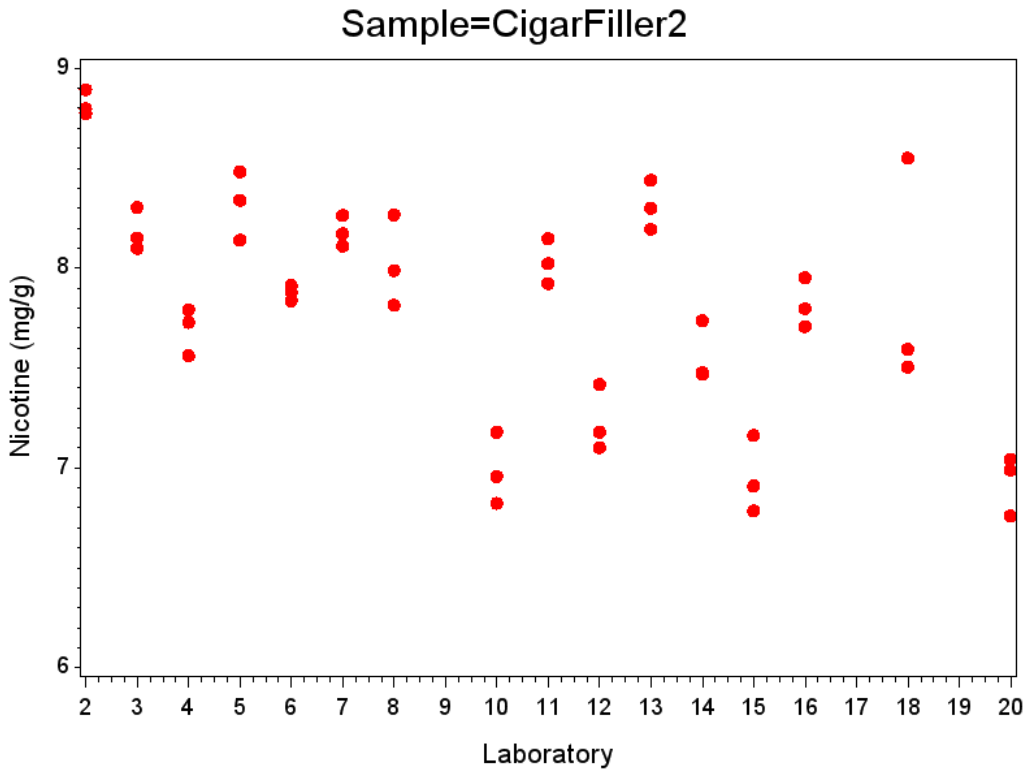

Sample=CM8
Nicotine (mg/g)
Laboratory

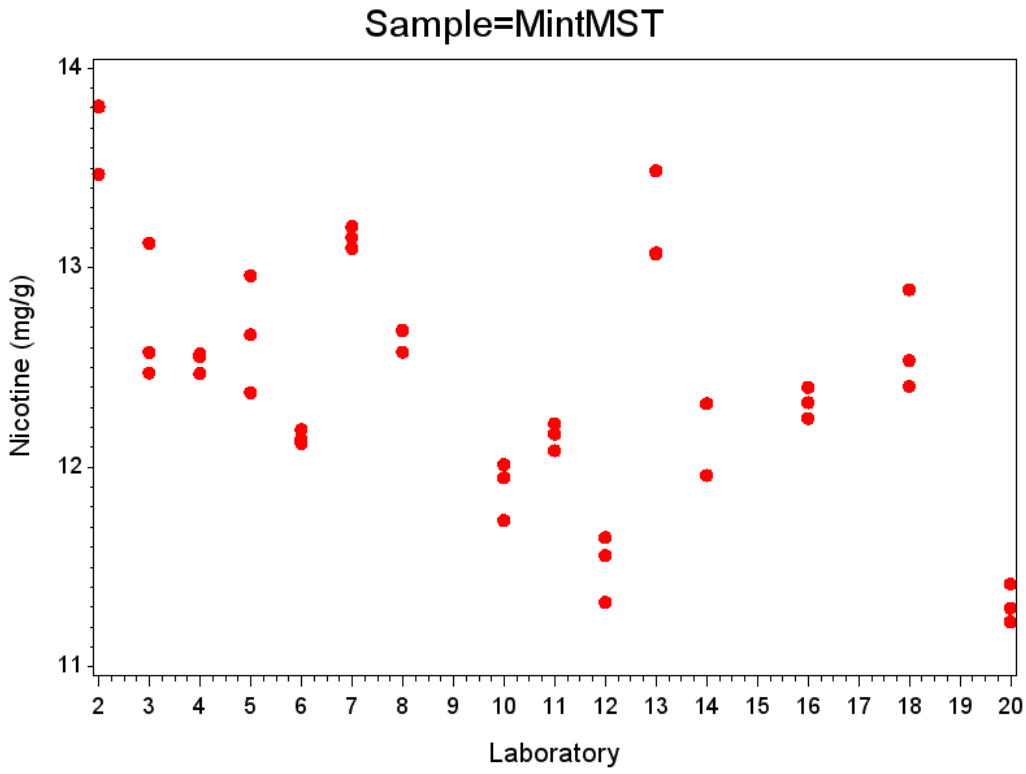

Sample=CRP1
Nicotine (mg/g)
Laboratory


Sample=CRP2
Nicotine (mg/g)
15
14
13
12
11
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=CRP3
Nicotine (mg/g)
26
25
24
23
22
21
20
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=CigarFiller1
Nicotine (mg/g)
10
9
8
7
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=CigarFiller2
Nicotine (mg/g)
9
8
7
6
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=MintMST
Nicotine (mg/g)
14
13
12
11
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory