Routine Analytical Chemistry Sub-Group
Smokeless Tobacco Sub-Group

Technical Report

2016 Collaborative Study on Minor Alkaloids in Tobacco Products

February 2017

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1. Summary

From 2013 to 2016, the CORESTA Routine Analytical Sub-Group (RAC) and Smokeless Tobacco Sub-Group (STS) conducted a series of collaborative studies for the determination of nornicotine and anabasine in tobacco and tobacco products. The RAC and STS eventually selected a gas chromatography mass spectrometry (GC-MS) method using methanol as the extract solvent and 6-methylquinoline and d4-nornicotine as the internal standards. Fifteen laboratories participated in the final collaborative study that was conducted in the spring of 2016. The purpose of this study was to calculate repeatability and reproducibility (r & R) values and determine if the methodology was suitable for the determination of nornicotine and anabasine in tobacco and tobacco products. The study results showed high variation, particularly for nornicotine. Further work on minor alkaloid analysis is required.

2. Introduction

In 2013 the RAC began discussion regarding the development of a simple, robust and efficient method to analyze nicotine and the minor alkaloids in a wide range of tobacco products/matrices without the need for standard addition studies. Although both CRM 62 and the Center for Disease Control (CDC)[1] method have been shown to be capable of extracting nicotine from tobacco products, both methods were shown to be not suitable for the extraction of the minor alkaloids of regulatory interest (nornicotine, anabasine, myosmine and anatabine). From 2013 to 2015, preliminary studies were done using an early version of the method described in this report. A number of issues hindered the progress:

1. Initially, more concentrated sodium hydroxide (5N) was recommended and it was added to the calibration standards as well as the samples. The carryover of the alkaloids in the inlet of the GC was unacceptably high and deterioration of chromatography was seen after only a handful of injections. The method was re-validated with a lower level of sodium hydroxide and the removal of it from the calibration standard preparation.
2. The first series of experiments gave generally unsatisfactory results for all of the minor alkaloids. An investigation into the possible cause revealed that the lack of pure neat materials and/or certified custom mixes was likely the main contributing factor. More recently, Guide 34 reference standards[2] became available for nornicotine and anabasine and it was hoped that the use of these and/or the use of higher purity neat materials would improve the results of the study.

In late 2015 through early 2016, the CORESTA RAC and STS conducted a collaborative study that included three 2009 CORESTA Reference Products (CRPs), three cigarette fillers, two flavored cigar fillers and one mint moist smokeless tobacco (MST) product. This report presents the results from that study. The purpose of this study was to calculate the repeatability and reproducibility for the stated GC-MS method. The contents of nornicotine and anabasine in tobacco were determined by pretreating the sample with an aqueous solution of 2N sodium hydroxide and then extracting the sample with methanol and internal standards

[1] Federal Register, Vol. 74, No. 4, Wednesday, January 7, 2009 712-719.

[2] ISO Guide 34:2009: General requirements for the competence of reference material producers, International Organization for Standardization, Geneva, Switzerland.

(6- methylquinoline and d4-nornicotine). The resulting sample extract was analyzed by a GC-MS in the selected ion monitoring (SIM) mode with electron-impact (EI) ionization. Data were provided by 15 laboratories. Analyte levels were reported in units µg/g of tobacco on an as-is basis. The data were analyzed in basic conformance with the recommendations of ISO 5725-2:1994 and ISO/TR 22971:2005.

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.

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
Enthalpy Analytical Durham, United States
Enthalpy Analytical Richmond, United States
Global Laboratory Services, United States
Imperial Tobacco Group Reemtsma, Germany
ITG Brands, United States
KT&G Research Institute, Korea
Labstat International ULC, Canada
Philip Morris International, Brazil
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

The Study protocol and analytical method are provided in appendices A and B, respectively. Laboratories were responsible for procuring all samples prior to starting the study with the exception of the cigar fillers and the mint MST which were distributed by participating laboratories. 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.

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 - flavored, pre-ground and homogenized
Cigar filler #2 - flavored, pre-ground and homogenized
CORESTA Monitor 8 (CM8) test piece
1R6F - participants were required to remove the filler from the cigarettes
1R5F filler - filler was pre-ground and homogenized
(Mint MST) - Mint flavored American-style loose moist snuff

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: Extract and analyze unit pouches. Cut the pouch in half and add the tobacco from the pouch to the extraction vessel and then add the pouch material to the extraction vessel.
- CRP2, CRP3, and Mint 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: 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) independent replicate analyses for each sample. The replicates should be determined from independent tobacco extractions. Data were to be reported in units of μg/g, on an as-is basis.

4. Data – Summary Descriptive Statistics

The full data sets are provided in Table 3A and 3B for nornicotine and anabasine, respectively. 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, prior to removal of outliers, are given in Appendix C.

Table 3A: Nornicotine Data Set (μg/g, as-is basis)

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
1	1	967.2	NA	1457	293.7	375.3	763.2	NA	NA	308.3
	2	989.4	NA	1732	277.5	386.0	770.3	NA	NA	305.6
	3	993.4	NA	1541	278.7	387.6	773.2	NA	NA	317.9
2	1	684.5	807.4	1271	233.5	230.1	501.4	424.5	370.7	194.4

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
	2	636.2	742.23	1299	260.2	215.0	508.1	421.8	355.7	182.8
	3	635.6	748.7	1222	247.7	235.5	491.2	423.8	383.1	194.0
3	1	813.0	980.2	1284	189.3	315.4	628.6	520.2	479.0	249.6
	2	888.6	932.1	1208	184.2	264.8	627.0	516.4	475.2	261.2
	3	794.1	834.4	1864	199.7	281.5	581.9	536.4	477.0	259.6
4	1	842.6	754.0	1356	221.5	261.2	594.3	494.7	449.9	285.8
	2	789.4	769.3	1437	219.6	277.8	595.3	492.9	445.2	262.3
	3	835.6	756.0	1401	225.4	268.7	601.4	516.7	442.3	270.1
5	1	810.2	924.4	1188	205.3	280.7	491.0	527.6	462.1	338.7
	2	816.6	746.7	1395	247.9	260.3	617.2	500.7	469.6	292.0
	3	799.0	669.6	1362	190.5	268.2	638.9	514.4	465.5	296.8
6	1	883.1	613.6	1080	181.8	NA	NA	399.0	349.7	189.7
	2	894.3	613.4	1114	177.4	NA	NA	415.4	347.3	187.2
	3	885.0	615.1	1111	182.5	NA	NA	405.3	347.5	193.9
7	1	831.8	852.2	1334	217.7	274.5	639.3	560.6	501.2	294.0
	2	852.1	832.2	1434	213.7	280.0	647.4	560.4	489.4	292.4
	3	867.9	841.4	1431	218.7	296.5	624.1	542.6	491.4	284.1
8	1	796.3	773.5	1376	194.5	267.9	536.5	468.6	453.9	285.2
	2	786.4	735.2	1398	216.1	266.9	577.5	499.1	443.3	290.8
	3	810.5	744.0	1333	212.0	249.6	590.2	497.3	465.4	265.2
9	1	963.1	NA	1363	NA	NA	NA	NA	NA	NA
	2	989.4	NA	1362	NA	NA	NA	NA	NA	NA
	3	996.0	NA	1340	NA	NA	NA	NA	NA	NA
10	1	695.3	719.6	1288	167.2	212.6	531.5	437.6	399.6	248.7
	2	716.8	772.2	1313	183.0	224.8	537.7	454.6	413.5	246.0
	3	778.2	733.5	1314	193.6	245.7	537.3	497.9	393.0	238.5
11	1	808.6	786.8	1352	207.1	277.9	618.5	513.2	447.1	229.6
	2	786.0	744.3	1437	202.1	278.9	635.0	523.5	472.2	225.1
	3	780.7	777.8	1488	204.5	278.1	608.9	513.5	447.9	220.3
12	1	723.0	628.0	1365	220.0	236.0	546.0	470.0	399.0	227.0
	2	755.0	709.0	1262	242.0	257.0	555.0	453.0	399.0	228.0
	3	722.0	722.0	1369	221.0	257.0	570.0	493.0	406.0	224.0
13	1	833.0	766.9	1389	232.2	299.0	621.4	532.1	491.7	302.6
	2	824.6	766.4	1389	231.9	300.7	649.5	534.8	487.1	293.2
	3	862.5	764.4	1402	241.3	295.0	633.0	536.2	481.2	282.7
14	1	758.2	821.8	1287	192.3	297.6	599.1	470.8	420.9	297.2
	2	806.3	730.8	1649	241.6	285.9	567.5	462.2	430.9	252.7

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
	3	792.0	924.5	1248	234.8	269.4	565.4	470.0	394.6	298.4
15	1	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA= Not analyzed.

Table 3B: Anabasine Data Set (µg/g, as-is basis)

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
1	1	116.1	NA	271.7	49.6	70.8	115.8	NA	NA	56.9
	2	119.4	NA	278.2	46.0	73.9	114.2	NA	NA	56.6
	3	124.6	NA	280.9	51.8	70.2	115.3	NA	NA	56.6
2	1	110.9	141.0	282.4	54.6	59.0	104.7	55.3	46.2	52.2
	2	112.3	125.7	292.0	65.4	61.5	108.4	51.0	44.3	47.6
	3	108.4	125.9	286.6	53.4	58.4	107.0	51.7	43.9	52.4
3	1	105.6	126.7	232.3	37.5	58.8	107.1	55.1	38.8	48.0
	2	102.4	114.7	268.2	38.4	62.8	100.2	55.0	44.9	52.2
	3	95.0	125.7	266.1	37.1	60.2	97.6	57.0	41.4	54.0
4	1	110.8	110.2	241.5	43.5	59.1	100.3	54.4	44.9	51.9
	2	107.0	109.4	248.5	44.0	58.4	98.6	55.1	44.1	50.2
	3	104.6	111.5	251.8	44.2	58.1	100.5	50.6	43.7	53.1
5	1	111.5	132.0	245.5	40.4	70.7	90.0	62.7	50.7	65.2
	2	114.9	104.6	261.3	48.8	65.4	106.7	59.1	51.0	56.5
	3	112.6	110.0	249.8	38.3	68.2	109.9	59.3	48.8	57.4
6	1	103.5	94.5	217.0	39.1	NA	NA	44.6	39.9	42.0
	2	100.6	94.1	213.4	37.2	NA	NA	44.2	38.8	41.5
	3	102.1	95.0	218.1	37.8	NA	NA	45.2	38.8	43.5
7	1	103.8	112.7	237.5	40.7	56.1	103.3	54.1	43.9	52.0
	2	103.5	109.3	241.0	39.0	57.9	102.8	56.9	46.7	52.4
	3	103.5	107.6	243.6	40.1	60.9	101.9	54.7	47.5	51.4
8	1	102.7	109.0	244.2	37.6	60.7	99.7	48.2	44.1	51.6
	2	99.9	102.0	229.2	43.5	55.7	102.3	49.0	42.2	49.7
	3	108.5	108.4	241.0	39.9	58.4	105.3	54.8	42.0	53.4
9	1	111.5	NA	265.1	NA	NA	NA	NA	NA	NA
	2	111.9	NA	265.8	NA	NA	NA	NA	NA	NA
	3	114.8	NA	265.6	NA	NA	NA	NA	NA	NA
10	1	97.6	98.2	221.1	46.0	57.6	92.3	55.3	52.4	57.0
	2	92.9	104.9	211.7	46.4	58.8	97.7	55.1	46.7	57.1

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
	3	98.6	103.0	218.7	44.4	61.3	97.2	57.0	49.0	55.7
11	1	106.4	102.8	251.8	51.7	64.0	105.4	61.0	45.7	51.3
	2	105.0	106.8	257.0	48.0	62.5	101.2	56.7	47.8	54.4
	3	105.5	106.1	254.7	44.1	65.1	101.0	55.5	47.5	51.5
12	1	98.4	102.0	244.0	45.5	57.8	109.0	49.5	39.6	45.3
	2	105.0	99.1	232.0	46.5	52.9	98.2	50.0	45.5	47.5
	3	98.6	103.0	233.0	38.7	56.7	96.0	49.3	39.2	46.3
13	1	102.8	110.0	244.3	40.4	59.6	96.6	51.6	43.5	47.8
	2	103.7	105.2	245.5	40.8	57.8	98.8	50.7	43.2	48.6
	3	102.0	103.7	252.4	39.8	58.1	96.5	50.4	41.7	48.1
14	1	90.6	117.4	228.2	37.6	47.5	95.6	50.8	34.2	45.6
	2	82.3	97.4	251.3	34.3	51.9	84.7	48.5	35.3	41.5
	3	96.2	97.4	234.7	38.5	52.6	81.1	45.3	34.8	40.2
15	1	107.2	123.9	295.4	43.0	54.9	110.6	49.6	45.1	48.6
	2	110.5	123.5	285.8	42.6	57.3	112.3	46.1	44.9	51.3
	3	109.0	120.5	276.3	42.2	55.7	113.6	49.6	45.0	52.7

NA= Not analyzed.

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, without removal of outliers, are given in Appendix C.

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.[3]

Table 4: Outliers

Sample	Levene's Outliers Lab	Grubbs' Outliers Lab
[Nornicotine]		
1R6F	**Lab 5**	**–**
CM8	**Lab 3#**	**–**
[Anabasine]		
No outliers identified		

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

One of the replicates for Lab 3 for nornicotine was treated as a single-point outlier and only that value was omitted.

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 Tables 5A and 5B. It should be understood that the r & R results reflect both laboratory variability and product homogeneity.

Table 5A: Repeatability (r) and Reproducibility (R) Limits for as-is Nornicotine (µg/g)

Product	No. of Labs *	Mean	Repeatability		Reproducibility	
			r	r (%)	R	R (%)
1R5F	14	821	70	8.5%	259	31.5 %
1R6F	11	767	121	15.8%	242	31.5 %
CM8	14	1348	240	17.8%	358	26.6 %
CRP1	13	218	38	17.6%	84	38.6 %
CRP2	12	277	35	12.6%	117	42.1 %
CRP3	12	599	77	12.8%	198	33.0 %
Cigar Filler 1	12	489	39	8.1%	128	26.3 %
Cigar Filler 2	12	435	27	6.1%	133	30.6 %
Mint MST	13	259	35	13.3%	119	45.8 %

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

[3] 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 α-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 α=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 α-level near 0.01 and is robust to deviations from normality.

Table 5B: Repeatability (r) and Reproducibility (R) Limits for as-is Anabasine (µg/g)

Product	No. of Labs *	Mean	Repeatability		Reproducibility	
			r	r (%)	R	R (%)
1R5F	15	105	9.3	8.8%	22.0	20.9 %
1R6F	13	110	17.8	16.1%	31.9	29.0 %
CM8	15	251	22.3	8.9%	62.8	25.1 %
CRP1	14	43	8.6	20.0%	17.1	39.5 %
CRP2	13	60	5.6	9.3%	15.7	26.2 %
CRP3	13	102	12.9	12.6%	22.2	21.8 %
Cigar Filler 1	13	53	5.7	10.8%	13.1	24.9 %
Cigar Filler 2	13	44	5.0	11.4%	12.3	28.0 %
Mint MST	14	51	6.0	11.7%	14.8	29.0 %

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

6. Data Interpretation

The values for Repeatability (r) and Reproducibility (R) both on an absolute basis and as percents of the means are shown in Tables 5A and 5B. The r % & R % values demonstrate similar repeatability and reproducibility for the various types of smokeless tobacco products, cigar filler and cigarette filler and did not trend with analyte concentration. The values for % R for each sample range from approximately 26 % to over 46 % for nornicotine and 21 % to over 40 % for anabasine.

7. Recommendations

In 2015, RAC and STS conducted an interlaboratory study for the determination of anabasine and nornicotine in tobacco and smokeless tobacco products. The overall level of variability seen in this study was higher than expected. This may be related to issues with the purity of the standard materials or lack of experience with the analytical method. During prior RAC meetings, it was reported that the purity of minor alkaloids standards varied even when purchased from the same vendor. While Guide 34 standards were requested for this study, evaluation of the overall quality and consistency of those standards was outside of the scope of this collaborative study. In addition, the question was raised by the group if the 0.25 g sample size required by the method was adequate given the relatively low levels of nornicotine and anabasine in the samples. The results for this study showed similar r % & R % values for all sample matrices, which indicates that this method performs similarly in the analysis of cigarette filler, cigar filler, and smokeless tobacco products and the observed variability may be due to issues with standard purity not the analytical method. No evidence was found for matrix related interferences with the analytes or the internal standard.

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 guidance for the determination of anabasine and nornicotine in cigarette filler, cigar filler, and smokeless tobacco products; however, the method is not suitable for a recommended method at this time.

APPENDIX A: Study Protocol

CORESTA ROUTINE ANALYTICAL CHEMISTRY SUB-GROUP

CORESTA SMOKELESS TOBACCO SUB-GROUP

Project Title: The Determination of Select Minor Alkaloids in Tobacco Products by GC-MS

Type of Document: Collaborative Study Protocol

Date: November 6th, 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 nornicotine and anabasine in tobacco and tobacco products. Tobacco products include cigarette filler, 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
November 6, 2015	Finalize protocol and distribute
February 10, 2016	Laboratories submit results by this
Spring 2016	Discuss results at RAC and STS

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 Nornicotine solution is recommended. A suggested supplier is Cerilliant: item N-071, 1 mg/mL in methanol (1mL ampule). **Use of another supplier will NOT disqualify a laboratory from the study.**

2. ISO Guide 34 certified reference standard Anabasine solution is recommended. A suggested supplier is Cerilliant: item A-097, 1 mg/mL in methanol (1mL ampule). **Use of another supplier will NOT disqualify a laboratory from the study.**

3. 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 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**.** bunchj@americansnuff.com
- Two processed Cigar Filler Samples – Altria
- Contact Dr. Karl Wagner to request samples**. Karl.A.Wagner@altria.com**
- CORESTA Monitor 8 (CM8) Test Piece – Borgwaldt or Cerulean (below)

Cerulean **Rockingham Drive** **Linford Wood East** **Milton Keynes MK14 6LY** **United Kingdom** **Tel: +44 1908 23 38 33** **Fax: +44 1908 23 53 33** **e-mail: sales@cerulean.com**	**Borgwaldt KC GmbH** **Spare Parts Department,** **Schnackenburgallee 15,** **D-22525 Hamburg** **Germany,** **Tel: +49 40 85 31 380** **Fax: +49 40 850 56 00** **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 -20 ºC 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 -20 ºC.
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- Preground and homogenized by ALCS	1 container (~20g)
Cigar filler #2- Preground 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 preground 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: Nornicotine and anabasine will be determined in each sample**. Use of ISO Guide 34 certified reference standards is recommended for this study. Suggested sources are:**

Available from Cerilliant (www.sigma-aldrich.com)

Nornicotine solution N-071, 1 mg/mL in methanol (1 mL ampule)

Anabasine solution A-097, 1 mg/mL in methanol (1 mL ampule)

6.2 Laboratories may use their own source or purchase the suggested material listed above.

6.3 **Note:** Commercial sources of the minor alkaloids have been shown to vary significantly in quality and purity. Concentrated stock solutions for each compound should be checked by GC in full scan mode to estimate the amounts of impurities based on relative peak areas.

6.4 **Methods:** Participating laboratories must 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 used.

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

6.6 **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, 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 preground 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 e-mail address:

gene.gillman@enthalpy.com

Minor Alkaloids 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 SELECT MINOR ALKALOIDS 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 select minor alkaloids. The purpose of this method is to quantitatively measure the amount of selected alkaloids in tobacco products by GC-MS using electron-impact (EI) ionization.

2. Scope

This method is applicable to the determination of the following alkaloids in a wide range of smokeless tobacco products cigarette tobacco, and cigar tobacco: nornicotine and anabasine.

3. Principle

Alkaloids are extracted from tobacco with aqueous sodium hydroxide (2N) and methanol using 6-Methylquinoline and d4-nornicotine as internal standards. After shaking for 30 minutes, the sample is filtered then analyzed by GC-MS using electron-impact (EI) ionization.

4. Chemicals

Note: Commercial sources of the minor alkaloids have been shown to vary significantly in quality and purity. Concentrated stock solutions for each compound should be checked by GC in full scan mode to estimate the amounts of impurities based on relative peak areas.

ISO Guide 34 certified reference standards are recommended. As an example, Cerilliant sells the following solutions:

Nornicotine solution N-071, 1 mg/mL in methanol (1 mL ampule)

Anabasine solution A-097, 1 mg/mL in methanol (1 mL ampule)

Use of another supplier will not disqualify a laboratory from the study.

d4-Nornicotine is available from a number of sources but 100 mg is available from CDN isotopes for ~$250 USD, PN D4174

Standards		*CAS No.*
4.1	d4-Nornicotine	[66148-18-3]
4.2	6-Methylquinoline	[91-62-3]
4.3	(-)-Nornicotine (NOR)	[494-97-3]
	or (+/-)-Nornicotine	[5746-86-1]
4.4	(-)-Anabasine (ANAB)	[494-52-0]
	or (+/-)-Anabasine	[13078-04-1]

Ancillary Chemicals

4.5	Methanol (HPLC/ACS grade)	[67-56-1]
4.6	Sodium Hydroxide (NaOH) – 2N solution	[1310-73-2]
4.7	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 Standards

6.1.1 d4-Nornicotine Stock Solution (~ 4 mg/mL)

Accurately weigh approximately 100 mg of d4-nornicotine 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 6-Methylquinoline Stock Solution (~ 4 mg/mL)

Accurately weigh approximately 100 mg of 6-Methylquinoline into a 25-mL volumetric flask. Add a small amount of MeOH to dissolve then make to volume with MeOH. Mix well.

6.1.3 Internal Standard Spiking Solution (ISSS)

Accurately add 2.0 mL of the individual stock solutions prepared in 6.1.1 and 6.1.2 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 6.1.3) into a 25-mL volumetric flask.

Make to volume with MeOH and mix well.

6.3 Calibration Standards from Neat – (Nornicotine, Anabasine)

6.3.1 Individual 1° Stocks in MeOH

Accurately weigh approximately 50 mg nornicotine and 50 mg anabasine to 0.1 mg into separate 20-mL volumetric flasks.

Dissolve each in MeOH then bring to volume and mix well.

6.3.2 Combined 2° Stock in MeOH

Transfer 1.0 mL of the nornicotine 1° stock and 0.5 mL of the anabasine 1° stock (*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 6.1.3*) to each.

Make to volume with MeOH and mix well.

Table 1. Calibration standards from neat analytes – 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 NOR Conc. (µg/mL)	Nominal ANAB Conc. (µg/mL)
1	0.10	25.0	0.2	0.1
2	0.20	25.0	0.4	0.2
3	0.50	25.0	1	0.5
4	1.0	25.0	2	1
5	3.0	25.0	6	3
6	5.0	25.0	10	5
7	8.0	25.0	16	8

6.4 Calibration Standards from Guide 34 Standards – (Nornicotine, Anabasine)

6.4.1 Individual 1° Solutions

The following preparation is based upon the following certified standard solutions:

Nornicotine solution 1 mg/mL in methanol (or equivalent)

Anabasine solution 1 mg/mL in methanol (or equivalent)

6.4.2 Combined 2° Stock in MeOH

Transfer 1.0 mL of the nornicotine and 0.5 mL of the anabasine standard solutions to a single 25-mL volumetric flask.

Bring to volume with MeOH and mix well.

6.4.3 Calibration Standards

Transfer aliquots of the nornicotine and anabasine 2° stock (*prepared in 6.4.2)* to volumetric flasks according to Table 2.

Add 100 µL ISTD working solution (*prepared in 6.1.3*) to each.

Bring to volume with MeOH and mix well.

Table 2. Calibration standards from certified standard solutions - Nominal Concentrations (actual concentrations will vary depending upon the certified concentration of the primary solutions)

Standard ID	Stock Vol. (mL)	Final Vol. (mL)	Nominal NOR Conc. (µg/mL)	Nominal ANAB Conc. (µg/mL)
1	0.05	10	0.2	0.1
2	0.1	10	0.4	0.2
3	0.2	10	0.8	0.4
4	0.5	10	2	1
5	1	10	4	2
6	2	10	8	4
7	4	10	16	8

7. PROCEDURE

Note: If ground tobacco is to be used, it must be freshly ground and stored in a refrigerator until needed. Nornicotine has been found to decrease in ground 3R4F stored at room temperature.

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 to 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 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 4.

Table 3. 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, 50:1
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 4. Analyte retention times and ions monitored.

Name	MW	Ret Time (min.)	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Internal Standard
6-Methylquinoline	129.2	9.6	143	N/A	N/A
d4-Nornicotine	152.2	10.5	151	N/A	N/A
Nornicotine	148.2	10.6	148	*119	d4-NOR
Anabasine	162.2	10.9	162	*119	6-MeQ

*(*Note that these qualifier ions have limited usefulness as there are many compounds having these ions in their spectra.)*

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.

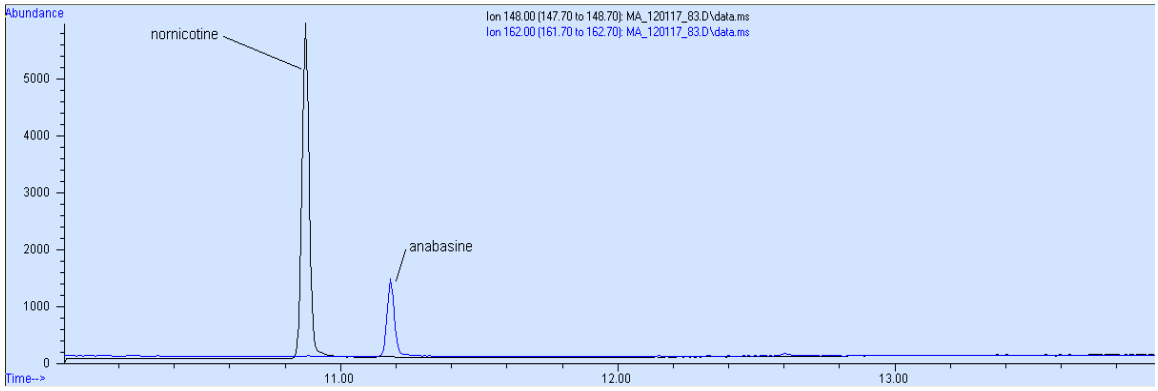


Figure 1. Ion Chromatograms for nornicotine and anabasine in a calibration standard (NOR ~ 12 µg/mL, ANAB ~ 1.9 µg/mL)

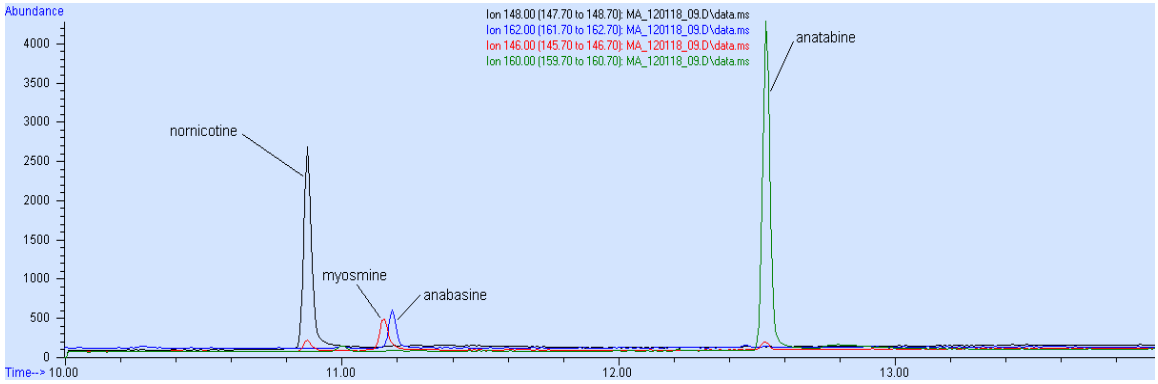


Figure 2. Ion Chromatograms for nornicotine and anabasine in a 3R4F extract.

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.

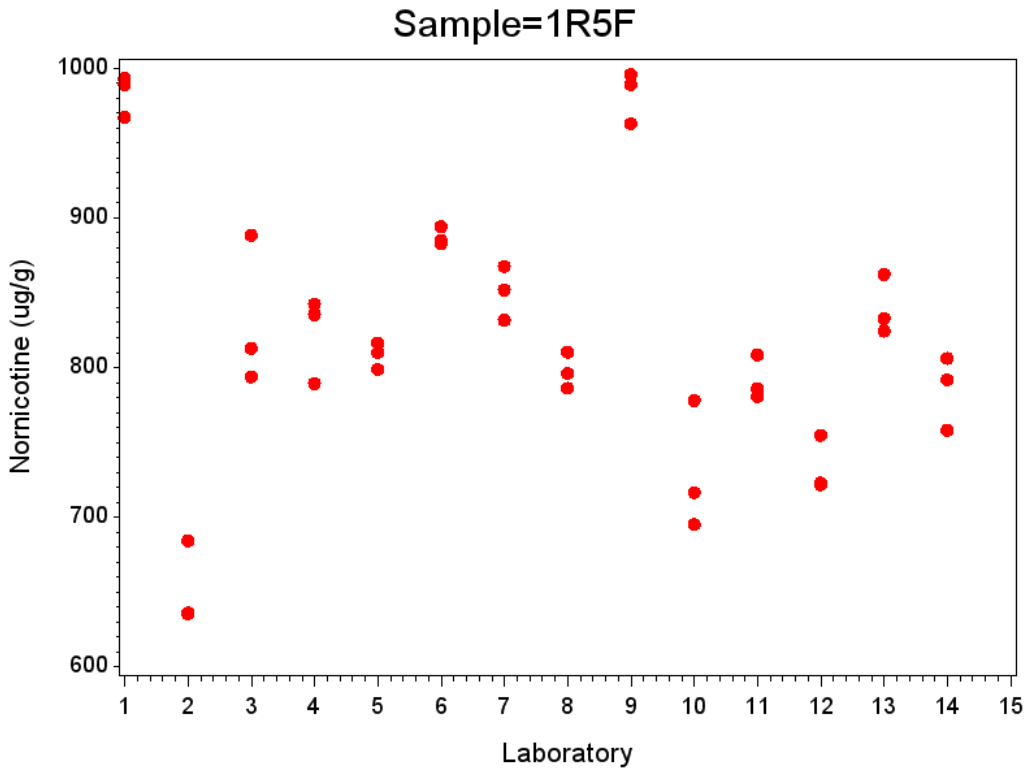
$$Analyte\ conc.\ (\mu g/g)\ = \frac{Analyte\ conc.\ (\mu g/mL)\ \times\ Sample\ Vol.\ (40\ mL)\ \times\ MF}{Sample\ Weight\ (g)}$$

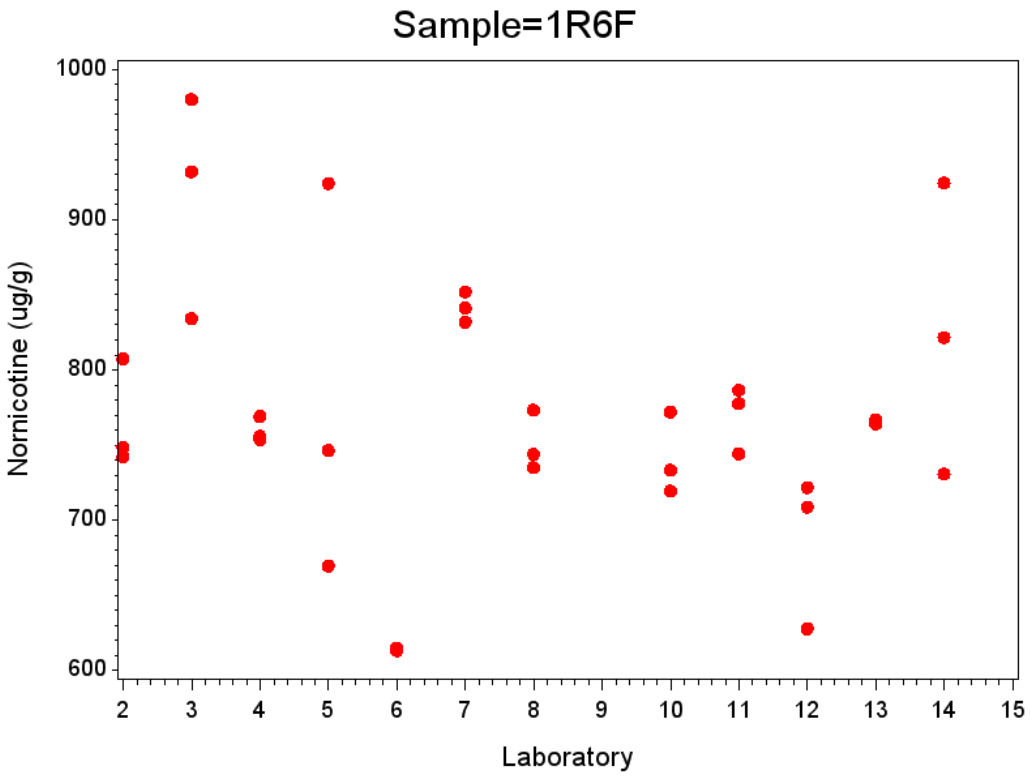
Note: there is no volume correction required for the addition of the pre-treatment solution as it dilutes all analytes and internal standards equally.

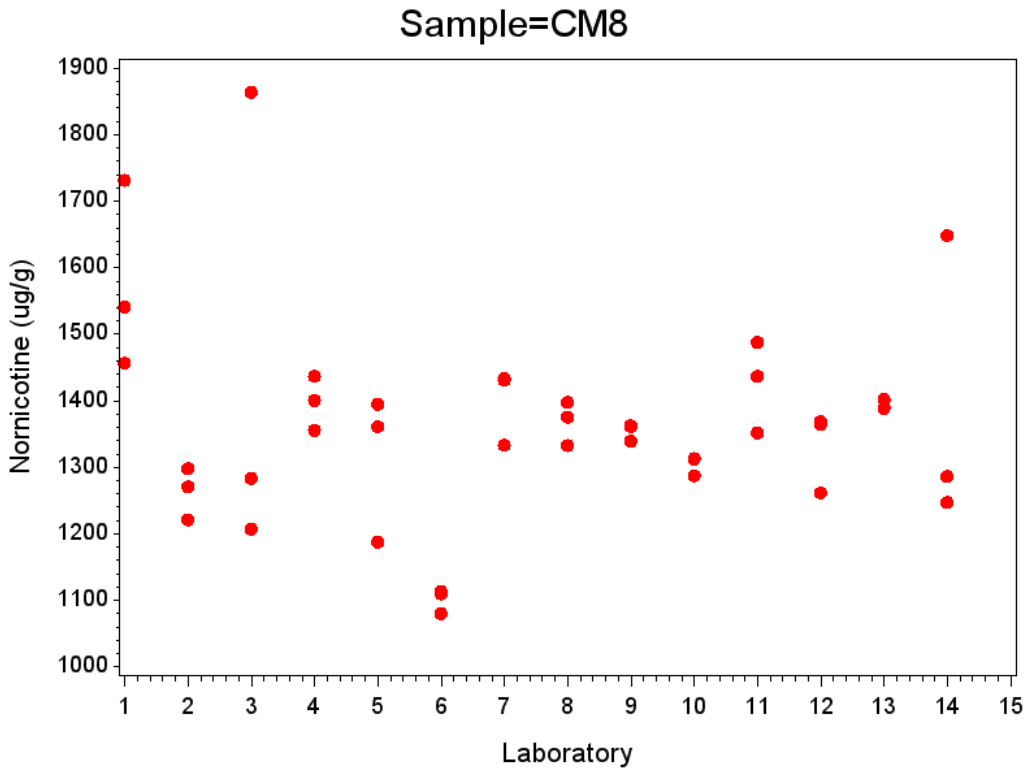
When reporting results on an 'as is' basis (i.e. not corrected for moisture content), the moisture factor (MF) value is 1. In instances where results are required to be reported on a dry weight basis (dwb), the following calculation is required to obtain MF for a sample:

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

APPENDIX C: Raw Data Plots



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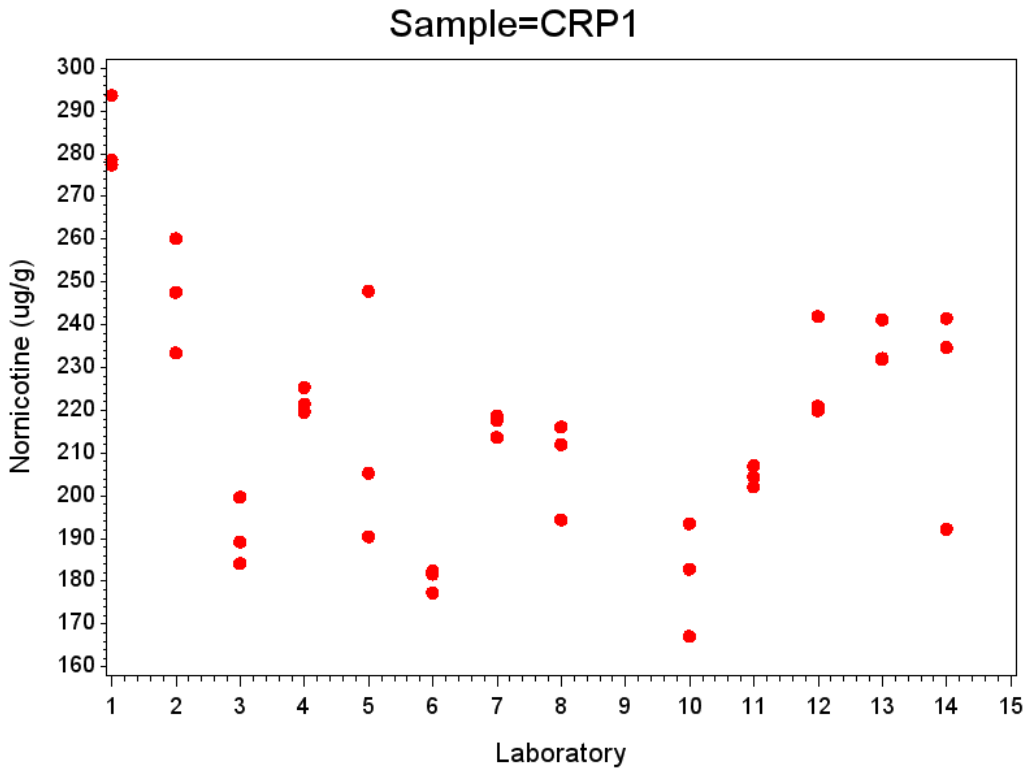
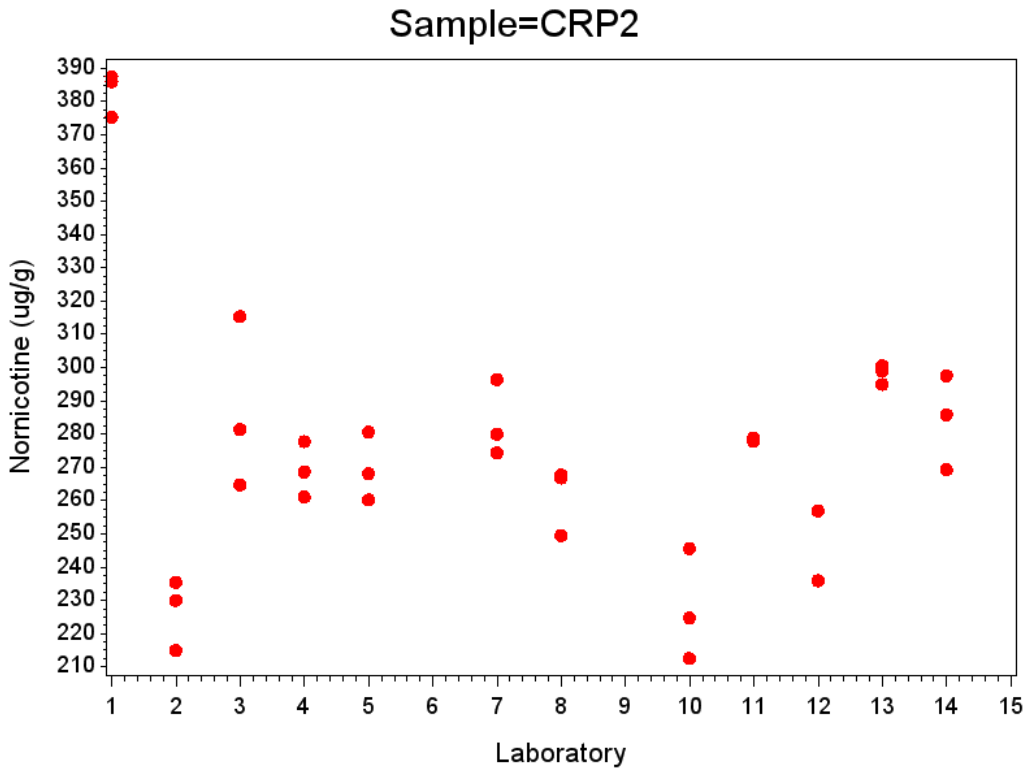
Nornicotine (ug/g)
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Laboratory


Sample=CRP1
Nornicotine (ug/g)
300
290
280
270
260
250
240
230
220
210
200
190
180
170
160
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Laboratory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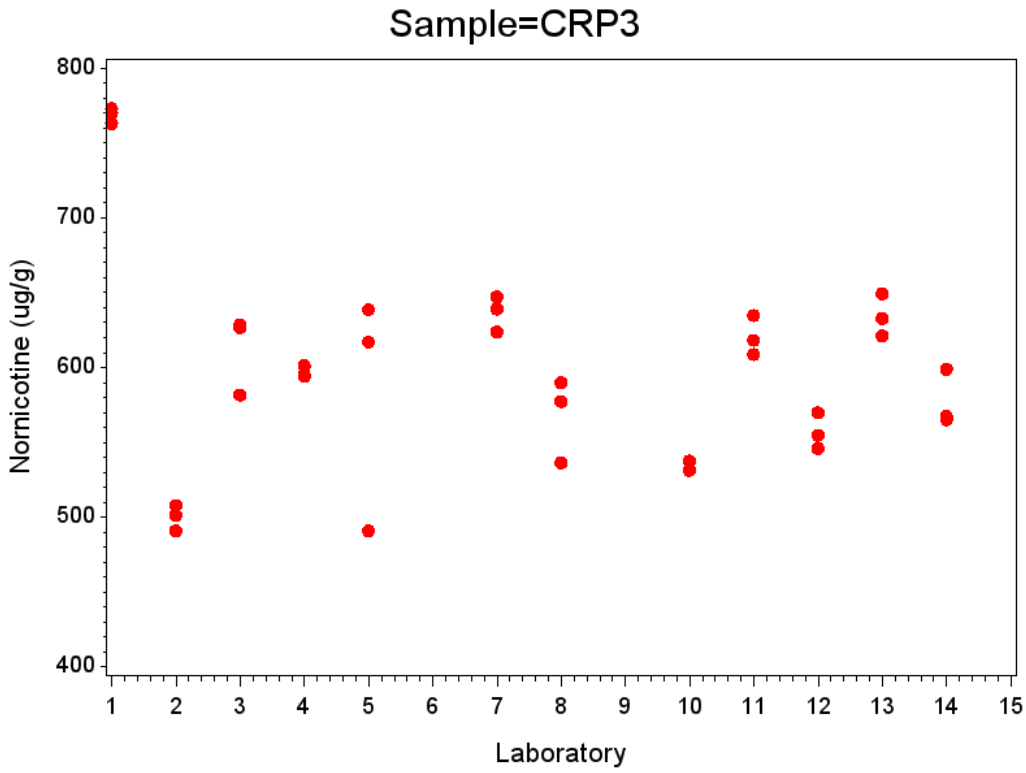
Nornicotine (ug/g)
390
380
370
360
350
340
330
320
310
300
290
280
270
260
250
240
230
220
210
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory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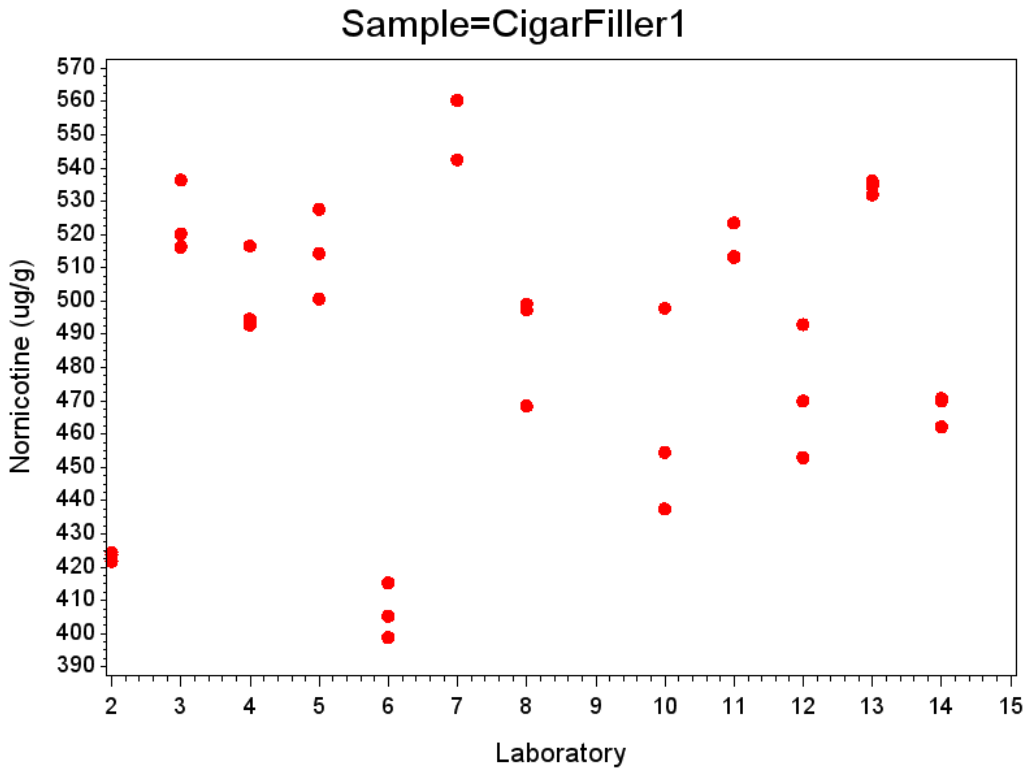
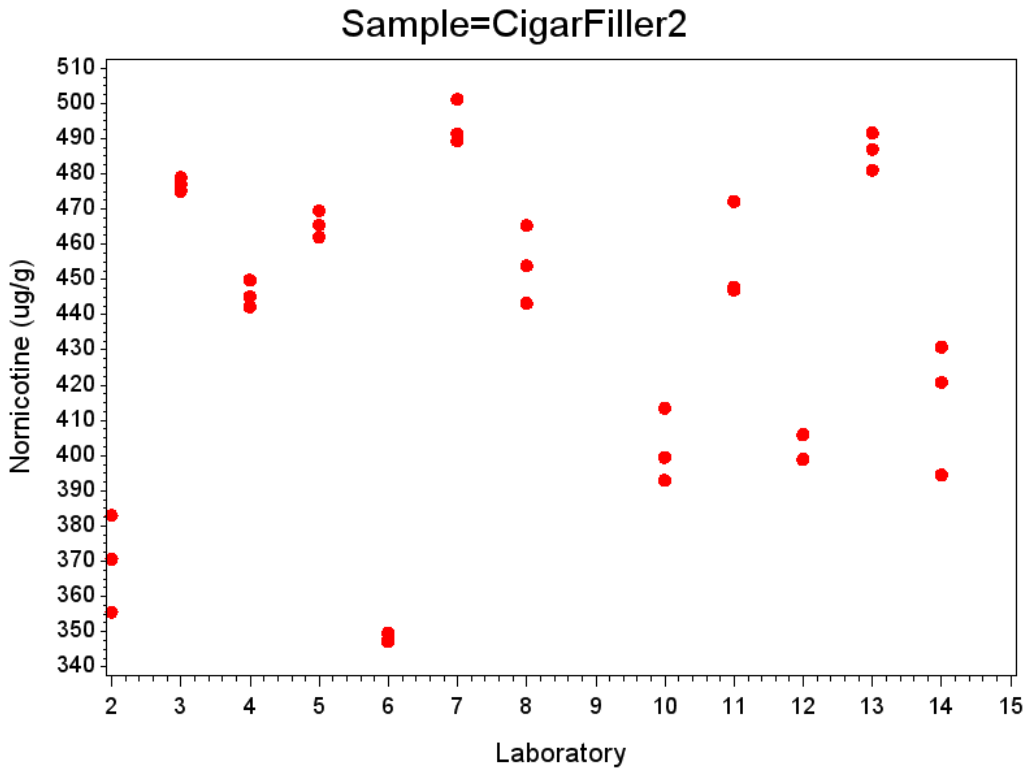
Nornicotine (ug/g)
800
700
600
500
400
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory


Sample=CigarFiller1
Nornicotine (ug/g)
570
560
550
540
530
520
510
500
490
480
470
460
450
440
430
420
410
400
390
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory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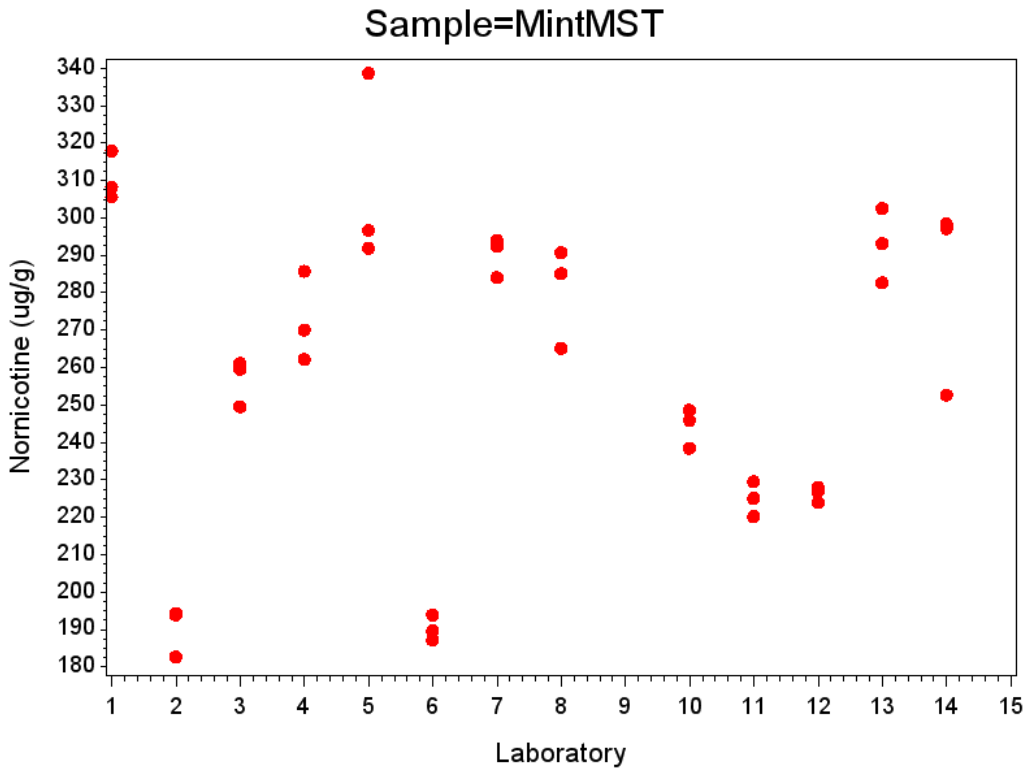
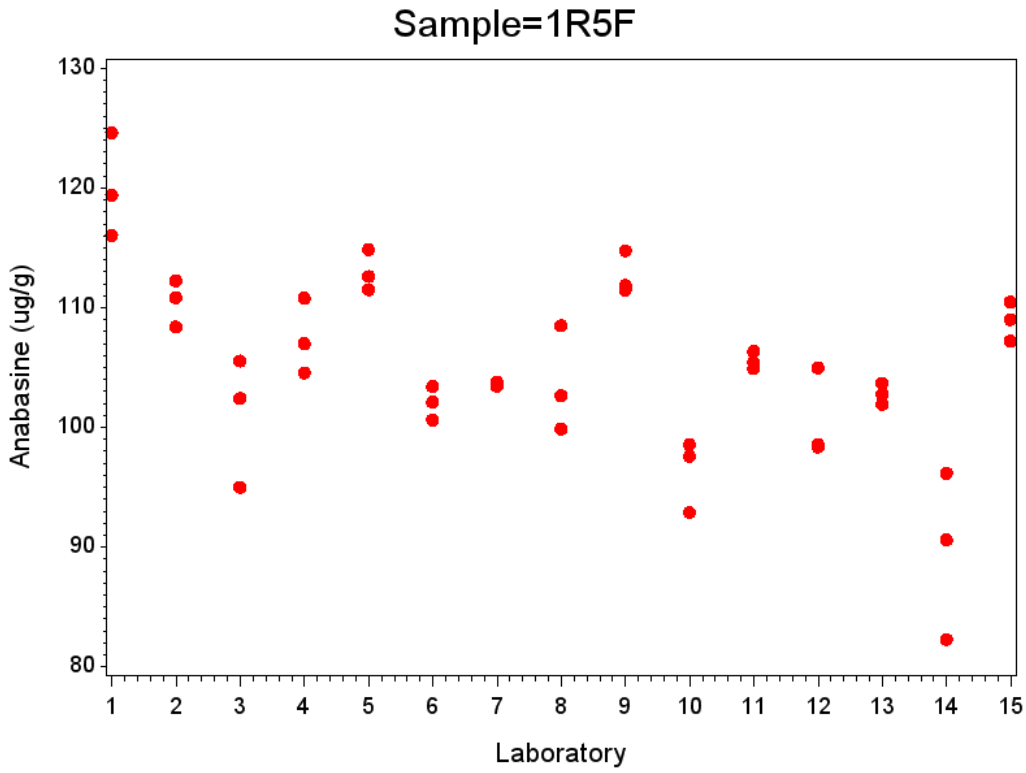
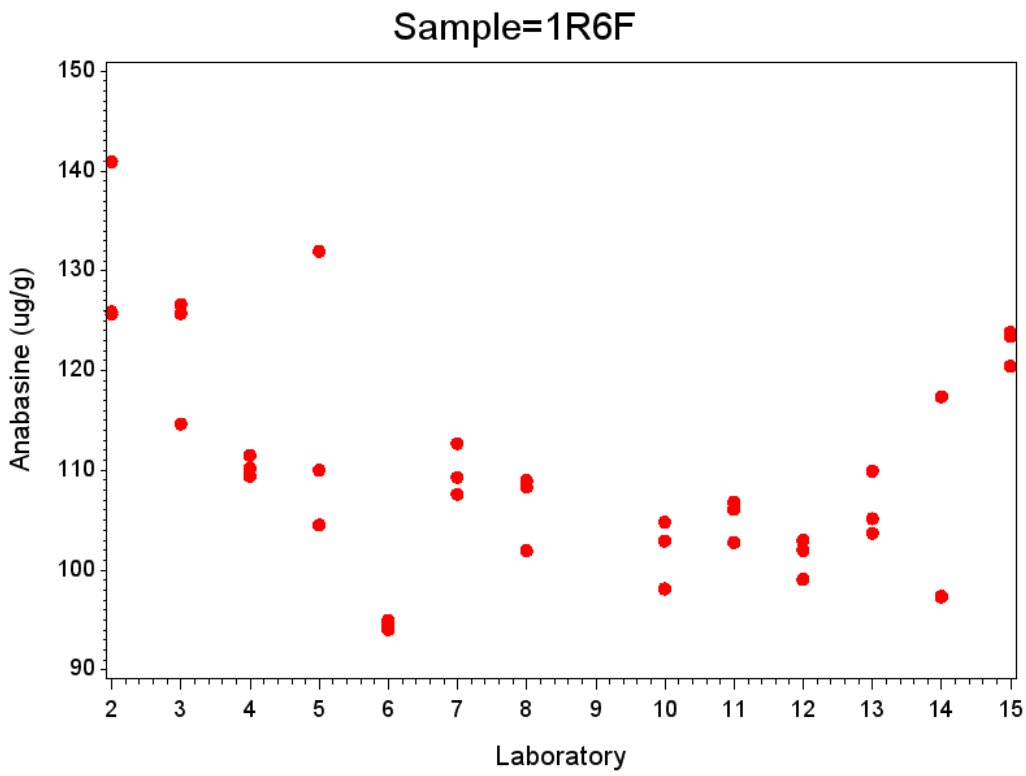
Nornicotine (ug/g)
510
500
490
480
470
460
450
440
430
420
410
400
390
380
370
360
350
340
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory


Sample=MintMST
Nornicotine (ug/g)
340
330
320
310
300
290
280
270
260
250
240
230
220
210
200
190
180
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory


Sample=1R5F
Anabasine (ug/g)
130
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory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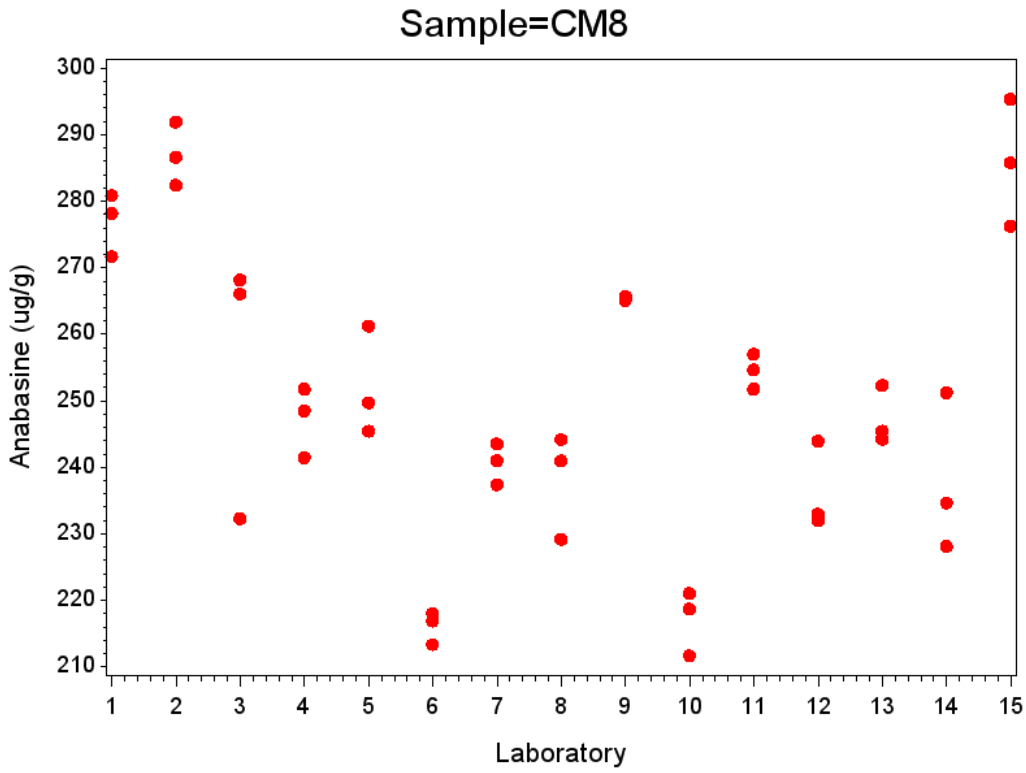
Anabasine (ug/g)
150
140
130
120
110
100
90
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory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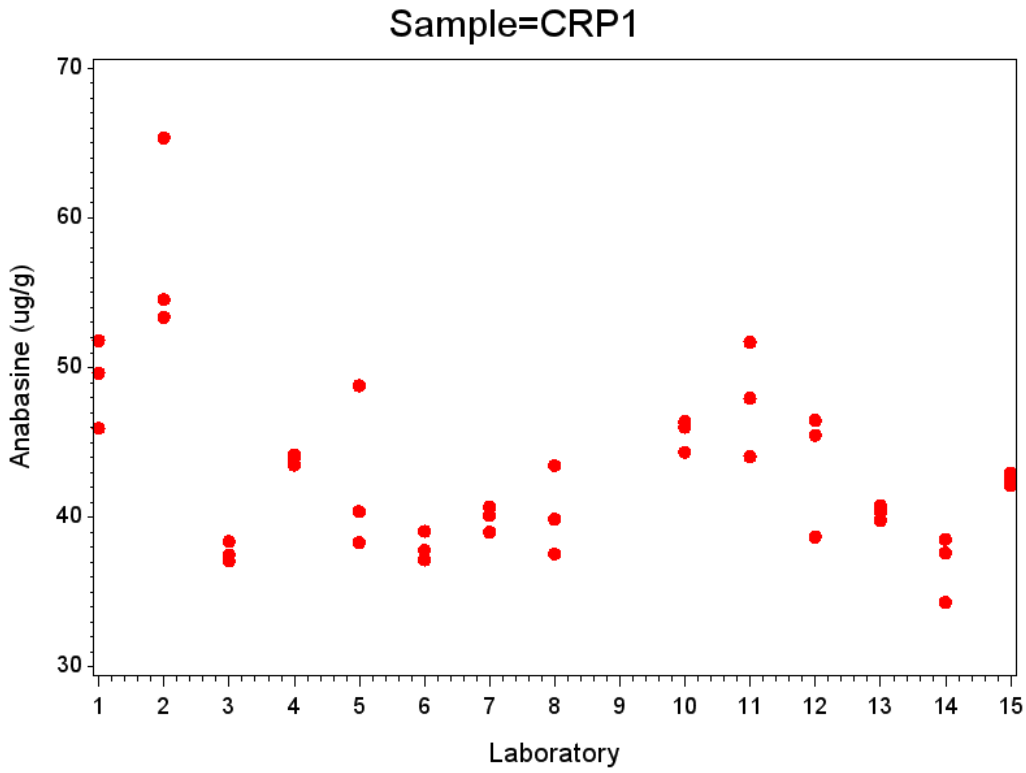
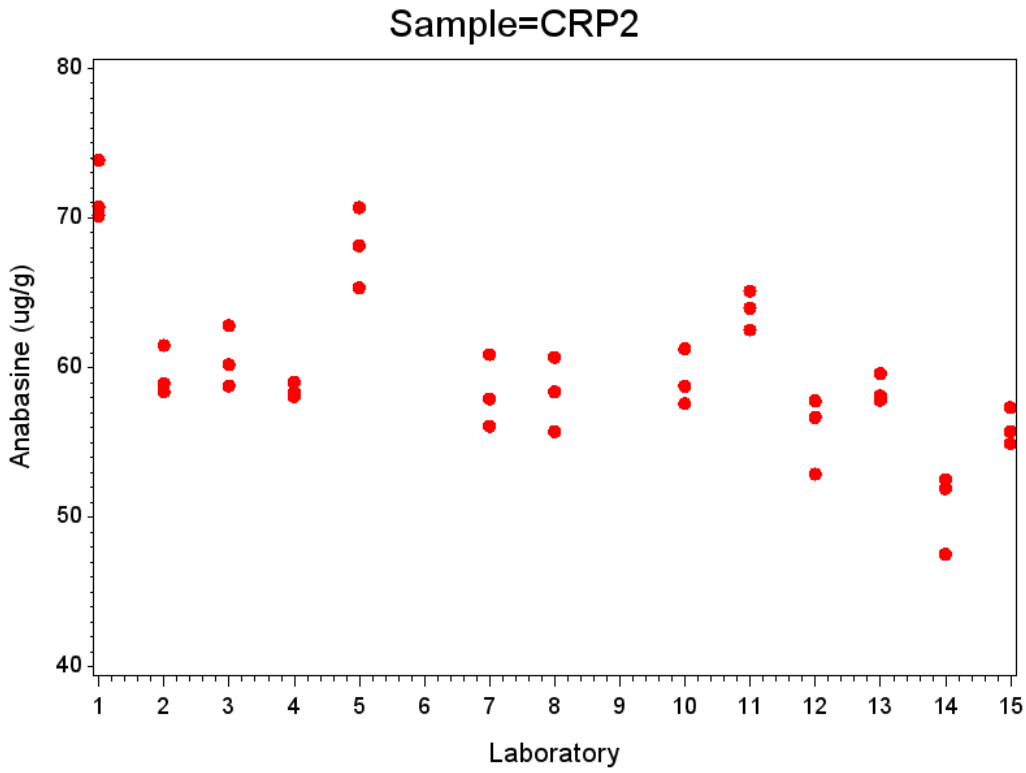
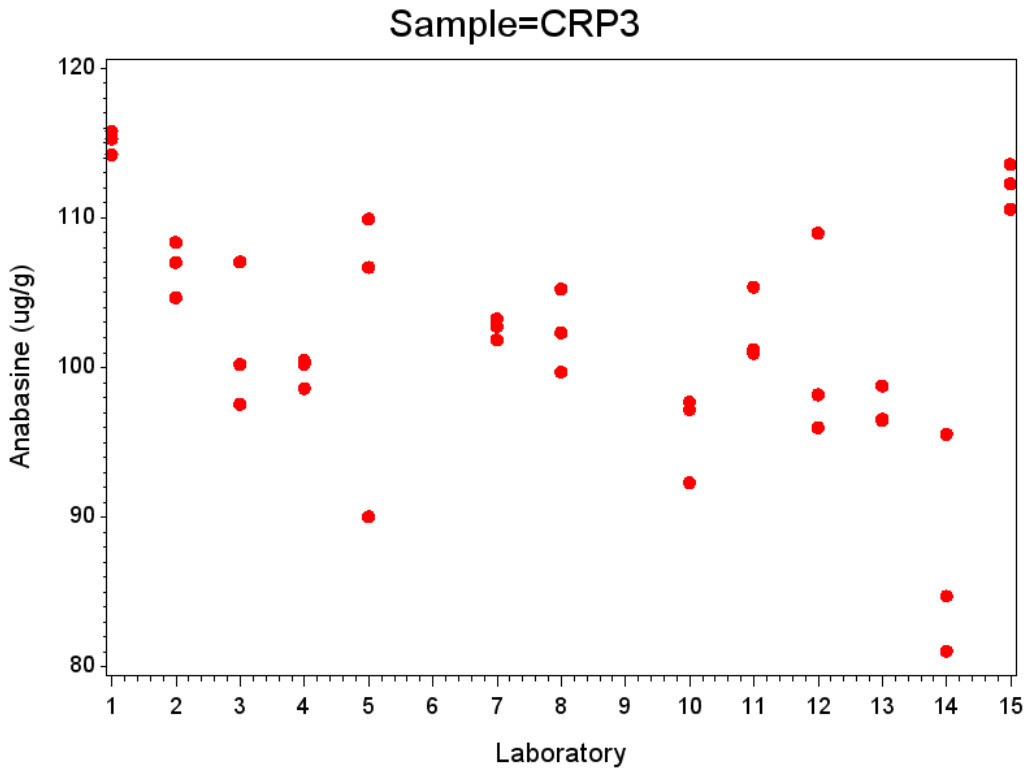
Anabasine (ug/g)
300
290
280
270
260
250
240
230
220
210
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory


Sample=CRP1
Anabasine (ug/g)
70
60
50
40
30
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory


Sample=CRP2
Anabasine (ug/g)
80
70
60
50
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory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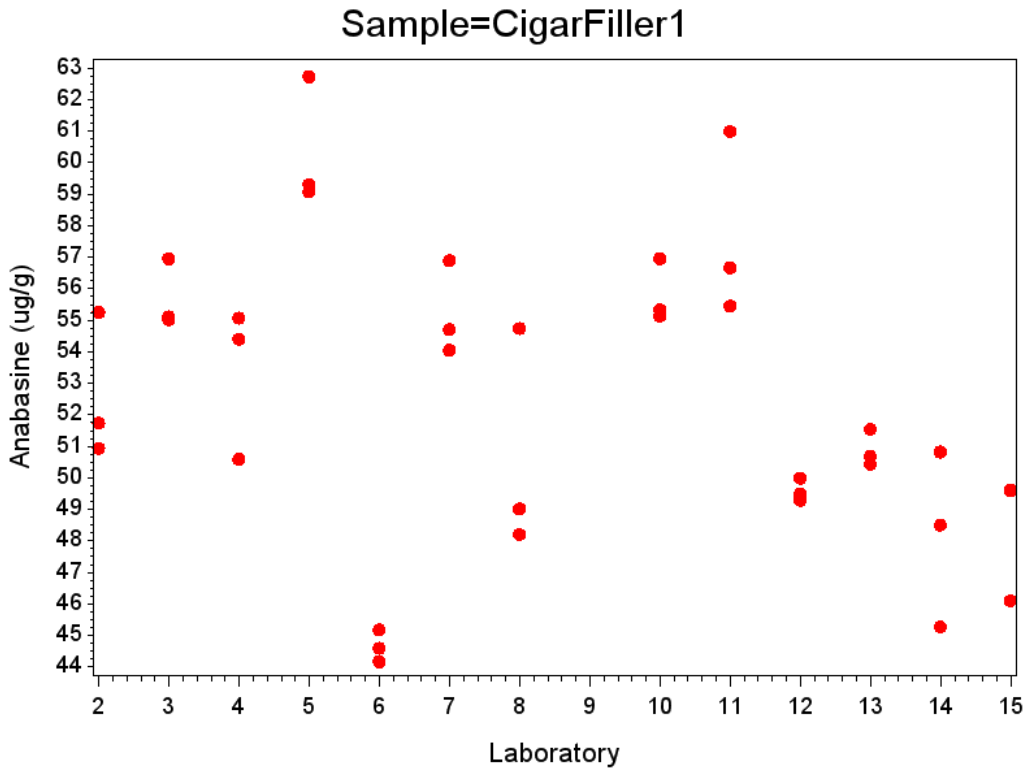
Anabasine (ug/g)
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Laboratory


Sample=CigarFiller1
Anabasine (ug/g)
63
62
61
60
59
58
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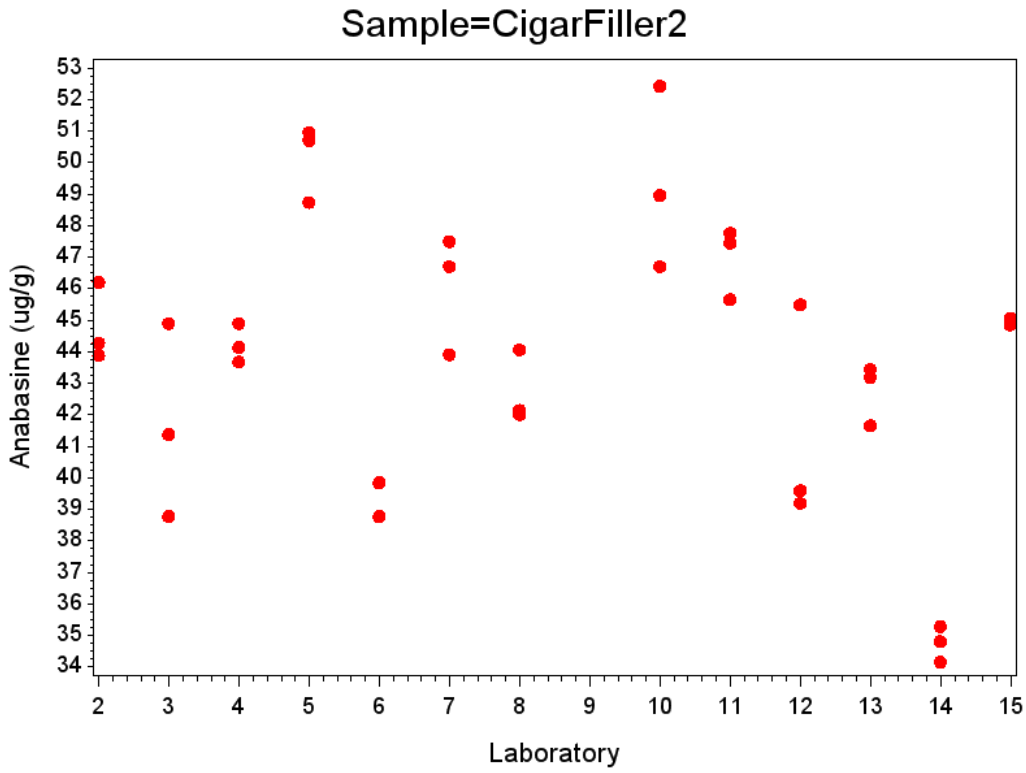
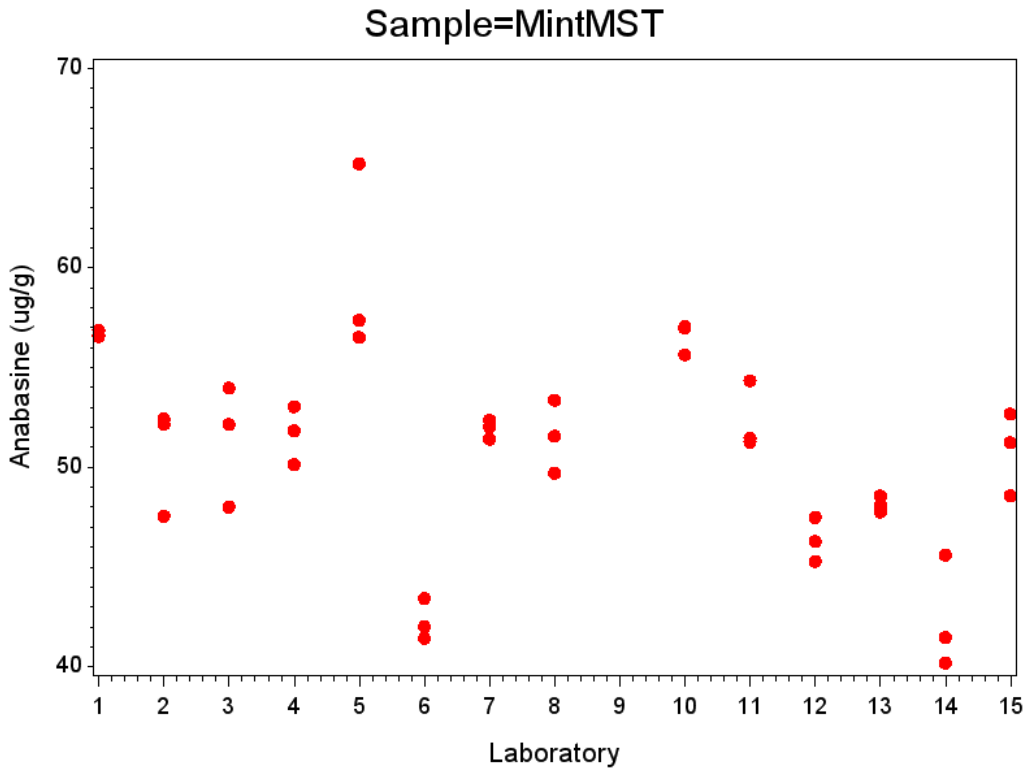
Laboratory


Sample=CigarFiller2
Anabasine (ug/g)
Laboratory


Sample=MintMST
Anabasine (ug/g)
Laboratory