Smokeless Tobacco Sub-Group

Technical Report

Determination of Ammonia in Tobacco and Smokeless Tobacco Products

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1. Summary

In 2013, the CORESTA Smokeless Tobacco Sub-Group (STS) conducted a collaborative study for the determination of ammonia (as ammonium ion) in tobacco and smokeless tobacco products. Eleven laboratories participated in the study. 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 ammonia in tobacco and smokeless tobacco products. The goal of this work was to expand the scope of CORESTA Recommended Method (CRM) No. 73, *Determination of Ammonia in Tobacco by Ion Chromatographic Analysis*, to include smokeless tobacco products. The modified version of CRM No. 73 used in this study was shown to be appropriate for the determination of ammonia in both tobacco and smokeless tobacco products. Furthermore, the modified version of CRM No. 73 produced similar values for r & R as is published in CRM No. 73 for ground tobacco.

2. Introduction

In 2010, the CORESTA Smokeless Tobacco Sub-Group conducted a collaborative study that included the four CORESTA Reference Products (CRPs) and five commercial smokeless tobacco products. This study was referred to as *2010 Collaborative and Proficiency Studies*. This study included the determination of ammonia and several other analytes (nitrates, humectants, benzo[a]pyrene, select trace metals, and moisture content). The 2010 study specified the use of CRM No. 73 for the analysis of ammonia and the results showed high variability. The variability was explained by poor chromatographic resolution due to the high concentration of sodium in certain smokeless tobacco products. After review of the 2010 study results, the STS proposed the development of a CRM for the determination of ammonia in smokeless tobacco products. The focus of this report is the interlaboratory study that was conducted in support of this goal. The laboratory phase of this study took place in August 2013 through October 2013.

The ammonia content of tobacco and smokeless tobacco products was determined by extraction of the samples with a sulfuric acid solution in order to stabilize ammonium ions in solution. Ion chromatography was used to separate ammonium ions from other cations. Ammonium ions were detected conductometrically and quantified, as ammonia, against an external standard calibration. The method used for this study followed CRM No. 73 except that the gradient profile was modified to afford better separation between sodium and ammonium ions. This modification was necessary because the sodium and ammonium peaks could potentially coelute with certain smokeless tobacco products that contain higher levels of sodium. Gradient profiles used in this study and from CRM No. 73 are given in Tables 1a and 1b, respectively. Also, sample preparation was modified to allow the use of sample centrifugation and filtration with 0.45 µm syringe filters instead of only paper filtration, as is currently specified in CRM No. 73.

Table 1a: Chromatographic Gradient Profile Used for this Study

Time (min)	Concentration of MSA[1] (mM)	Eluent Generator Curve	Flow Rate (mL/min)	Suppressor Current (mA)
0.0	10	5	1.00	88
9.0	10	5	1.00	88
9.5	40	5	1.00	88
14.5	40	5	1.00	88
15.0	10	5	1.00	88

[1] Methanesulfonic acid

Table 1b: Chromatographic Gradient Profile from CRM No. 73

Time (min)	Concentration of MSA[1] (mM)	Eluent Generator Curve	Flow Rate (mL/min)	Suppressor Current (mA)
0.0	18	5	1.00	88
7.0	18	5	1.00	88
7.1	40	5	1.00	88
13.0	40	5	1.00	88
13.1	18	5	1.00	88
18.0	18	5	1.00	88

[1] Methanesulfonic acid

Data analysis for the determination of ammonia in the test samples was performed in general conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. Three replicates of ammonia were determined by each participating laboratory, for each sample. Ammonia was reported in units of µg per gram of tobacco (µg/g).

3. Organisation

3.1 Participants

A list of the participating laboratories is provided in Table 2. The laboratories are listed in alphabetical order and this order does not correspond to the same order as the data given throughout the report.

Table 2: List of Participating Laboratories

Laboratory
Altria Client Services
British American Tobacco, Southampton
China National Tobacco Quality Supervision & Test Center
Enthalpy Analytical
Essentra Scientific Services
Global Laboratory Services
Imperial Tobacco Group, Seita
Labstat International
RJ Reynolds Tobacco Company
Technical Center of Shanghai Tobacco Group Co.,Ltd.
Zhengzhou Tobacco Research Institute of China National Tobacco Corp.

3.2 Protocol

The protocol and analytical method used for this study are provided in Appendices A and B, respectively. Each laboratory was requested to report three replicate determinations for each of the eleven samples shown in Table 3. The laboratories were requested to determine the three replicates under repeatability conditions; i.e. within a short time interval and by the same operator using the same equipment. Laboratories were requested to obtain the CRPs from North Carolina State University and 3R4F from the University of Kentucky. The ground tobacco samples were provided and distributed by British American Tobacco (BAT), Southampton. American Snuff Company provided the Fine Cut Moist Snuff sample to BAT for distribution.

The following deviations from the protocol were reported: CRP1, CRP2, CRP3, and CRP4 shipped to Labs 7 and 9 were exposed to high ambient temperatures for an extended period during shipment. These deviations did not appear to affect the study results.

Table 3: Sample Identification

Product Type
CRP1 - Swedish style snus pouch
CRP2 - American-style loose moist snuff
CRP3 - American-style loose dry snuff powder
CRP4 - American-style loose-leaf chewing tobacco
Kentucky Reference Cigarette 3R4F filler (3R4F)
Fine cut moist snuff
Ground Virginia tobacco
Ground Burley tobacco
Ground Oriental tobacco
Ground fire-cured tobacco
Ground dark air-cured tobacco

4. Data - Raw

The complete data set is provided in Table 4. Raw data plots, with outliers included, are given in Appendix C. All laboratories did not provide data for all samples.

Table 4: Data Set, Concentration of Ammonia in Study Samples

		Laboratory – Ammonia (µg/g)										
Product	**Rep**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CRP1	1	662	884	738	862	840	940	1331	1016	908	1258	1037
	2	724	868	860	862	782	938	1354	988	882	1187	1063
	3	700	894	909	807	801	935	1341	895	887	1161	1056
CRP2	1	2276	2631	2259	2048	2317	2546	3332	2536	2598	2939	2851
	2	2268	2620	2234	2055	2354	2578	3256	2982	2537	2931	2900
	3	2316	2617	2183	2108	2346	2605	3252	2600	2543	3060	2883
CRP3	1	4689	4735	4603	4739	4721	4194	5423	4672	4830	5115	4892
	2	4660	4625	4611	4835	4734	4251	5408	4442	4754	4927	4919
	3	4591	4648	4607	4815	4725	4260	5393	4278	4747	5093	5032
CRP4	1	2924	3399	2394	2715	2620	2580	2887	2444	2886	4526	2963
	2	3000	2591	2407	2668	2623	2640	2842	3002	2968	3819	3063
	3	2969	2794	2314	2700	2551	2541	2864	2826	2974	3142	2930
3R4F	1	960	991	928	1182	NA	1028	NA	1063	1095	1291	1162
	2	949	1016	934	1166	NA	1028	NA	1035	1034	1411	1129
	3	949	989	941	1293	NA	1046	NA	1089	1038	1310	1138
Fine Cut Moist Snuff	1	4081	3994	3801	4057	NA	3501	NA	4438	NA	4694	4619
	2	4002	3946	3678	4059	NA	3384	NA	4394	NA	4622	4587
	3	3971	3968	3774	4068	NA	3386	NA	3965	NA	4542	4665
Virginia tobacco	1	423	411	551	816	NA	465	NA	408	NA	478	443
	2	426	399	533	787	NA	468	NA	399	NA	482	408
	3	421	405	519	795	NA	455	NA	395	NA	464	417
Burley tobacco	1	2816	2770	2687	2800	NA	2629	NA	2665	NA	2910	2756
	2	2793	2889	2798	2863	NA	2633	NA	3063	NA	2923	2864
	3	2724	2713	2807	2877	NA	2569	NA	2995	NA	3139	2899
Oriental tobacco	1	344	348	492	390	NA	356	NA	390	NA	369	355
	2	347	360	502	365	NA	361	NA	348	NA	368	378
	3	340	336	498	378	NA	364	NA	347	NA	405	347
Fire-cured tobacco	1	3021	2933	2950	3013	NA	2745	NA	2843	NA	3191	2969
	2	3034	2918	2957	3011	NA	2759	NA	3004	NA	3199	2993
	3	2998	2934	2966	2998	NA	2812	NA	2893	NA	3170	2966
Dark air-cured tobacco	1	538	513	612	559	NA	530	NA	470	NA	594	506
	2	542	534	600	549	NA	526	NA	496	NA	573	490
	3	538	518	603	560	NA	532	NA	467	NA	619	488

NA: Lab did not provide data for this sample

5. Data - Statistical Analysis

A statistical analysis was conducted in general 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. Even though ISO 5725-2:1994 does not suggest calculation of z-scores, z-scores are presented in section 5.3 so that the participating laboratories would have an additional measure of their performance compared to their peers.

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. ISO 5725-2:1994 specifies the use of Cochran's test for eliminating laboratories with overly large repeatability standard deviations and Grubbs' test for eliminating laboratories with outlying mean values.

The intent of ISO 5725-2:1994 is to eliminate outliers that exceed a 1% critical value. However, Cochran's test is very sensitive to deviations from normality and, as demonstrated by Conover et al. (1981)[1], the test is prone to falsely identify laboratories as outliers much more frequently than the nominal rate of 1%. From a practical perspective, this means that data are eliminated too easily which may lead to the calculation of an unrealistically low level of method variation. The repeated application of Cochran's test is likely to exacerbate the effect. ISO 5725-2:1994 also recognizes this potential difficulty (see 7.3.3.6). For this reason, a single application of Cochran's test was employed for this analysis.

Grubbs' test and a single iteration of Cochran's test were applied at the standard nominal 1% significance level to determine outliers and the results are shown in Table 5. As noted above, it is likely that the Cochran outliers are identified with a higher probability than the nominally stated rate.

Table 5: Results of GRUBBS' and COCHRAN's Tests Outliers

Product	Cochran's Outliers Laboratory #	Grubbs' Outliers - Laboratory #
CRP1	−	−
CRP2	8	−
CRP3	8	−
CRP4	10	−
3R4F	−	−
Fine cut moist snuff	8	−
Virginia tobacco	−	4
Burley tobacco	−	−
Oriental tobacco	−	3
Fire-cured tobacco	8	−
Dark air-cured tobacco	−	−

The (−) denotes no outliers were detected.

[1] Conover, W.J., Johnson, M.E., & Johnson, M.M. (1981). A comparative study of tests for homogeneity of variances, with applications to the outer continental shelf bidding data. Technometrics, 23, 351-361

5.2 Calculation of Repeatability and Reproducibility

After removal of outlying data, as described above, the final repeatability (r) and reproducibility (R) results were calculated. The r & R results are shown in Table 6.

Table 6: Repeatability (r) and Reproducibility (R) Results

Product	Mean Ammonia (µg/g)	Number of Labs[1]	r	R	% r	% R
CRP1	951	11	113	529	11.9%	55.6%
CRP2	2581	10	103	1055	4.0%	40.8%
CRP3	4786	10	149	870	3.1%	18.2%
CRP4	2769	10	464	686	16.7%	24.8%
3R4F	1081	9	100	373	9.3%	34.5%
Fine cut moist snuff	4067	7	149	1227	3.7%	30.2%
Virginia tobacco	446	7	30	136	6.7%	30.5%
Burley tobacco	2816	8	289	392	10.3%	13.9%
Oriental tobacco	362	7	43	52	11.8%	14.4%
Fire-cured tobacco	2978	7	50	348	1.7%	11.7%
Dark air-cured tobacco	540	8	33	128	6.1%	23.6%

[1] The number of laboratory data sets remaining after removal of outliers.

5.3 Calculation of Z-Scores

Although calculation of z-scores is not suggested in ISO 5725-2:1994, z-scores were calculated so that the participating laboratories could compare their results to those of their peers. It is expected that most of the data should fall within the range of ±2. A final summary table of z-scores is presented in Table 7. Outliers detected with the GRUBBS' test and COCHRAN'S test, were removed prior to calculation of the z-scores.

Table 7: Z-Scores

	Laboratory										
Product	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CRP1	-1.37	-0.37	-0.62	-0.58	-0.77	-0.07	2.10	0.08	-0.31	1.35	0.55
CRP2	-0.79	0.11	-0.95	-1.36	-0.65	-0.01	1.86	NA[1]	-0.06	1.05	0.79
CRP3	-0.45	-0.38	-0.58	0.03	-0.19	-1.79	2.02	NA[1]	-0.03	0.84	0.52
CRP4	0.95	0.78	-1.95	-0.37	-0.84	-0.89	0.47	-0.06	0.85	NA[1]	1.06
3R4F	-0.99	-0.64	-1.13	1.02	NA[2]	-0.36	NA[2]	-0.15	-0.20	1.97	0.47
Fine cut moist snuff	-0.11	-0.22	-0.72	-0.01	NA[2]	-1.47	NA[2]	NA[1]	NA[2]	1.27	1.28
Virginia tobacco	-0.48	-0.86	1.84	NA[1]	NA[2]	0.35	NA[2]	-0.96	NA[2]	0.60	-0.49
Burley tobacco	-0.34	-0.22	-0.46	0.27	NA[2]	-1.84	NA[2]	0.82	NA[2]	1.56	0.21
Oriental tobacco	-1.31	-1.00	NA[1]	1.16	NA[2]	-0.10	NA[2]	0.00	NA[2]	1.38	-0.13
Fire-cured tobacco	0.32	-0.40	-0.17	0.24	NA[2]	-1.67	NA[2]	NA[1]	NA[2]	1.69	-0.01
Dark air-cured tobacco	-0.01	-0.41	1.46	0.36	NA[2]	-0.24	NA[2]	-1.40	NA[2]	1.24	-1.01

[1] Identified as outlier data based on numerical data consistency methods (GRUBBS' test, COCHRAN's test).
[2] Lab did not analyze this sample.

6. Data Interpretation

The values of repeatability (r) and reproducibility (R) are shown in Table 6. This table also includes values for %R for each sample; which range from approximately 11% to 55%. Within-lab variability was in the same range for all sample types. However, the CRPs and 3R4F products procured by the participants showed higher between-lab variability as compared to the ground tobacco samples distributed by BAT. This is not unexpected since the CRP and 3R4F products were not homogenized before distribution while the ground tobacco samples were.

In an effort to understand the variability seen in this study, the current study data were compared to the 2009 Routine Analytical Chemistry (RAC) collaborative study on ammonia in tobacco[1] (summarized in Table 8). The 2009 RAC study only included ground tobacco and not smokeless tobacco products. Two conclusions were evident: (1) the between-lab variability for the ground tobacco samples in the current study is similar to the 2009 RAC study and (2) the between-lab variability for the CRPs and 3R4F is slightly higher than was shown for the ground tobacco samples analyzed in the 2009 RAC study. As mentioned above, greater sample heterogeneity for the CRP and 3R4F products may be a factor in increased variability as compared to the ground tobacco samples. However, these effects are difficult to quantitate since a formal sample homogeneity study was not included in this study.

The z-score results (Table 7) do not indicate within laboratory bias where specific labs generated consistently high or low results for all sample types. This further supports the hypothesis that the observed variability in the CRP and 3R4F products may be related to sample heterogeneity and not analytical method variability.

A thorough review of the raw data and chromatography from each participating laboratory was undertaken after the study was completed. After this review, it was determined that inconsistent integration parameters could adversely affect method reproducibility; however, no other issues were identified. The integration parameters were standardized between all participating laboratories before tabulating the final data set for this report.

Table 8: 2009 Collaborative Study on Ammonia in Tobacco Using Ion Chromatography Analysis, Routine Analytical Chemistry Sub-Group, Technical Report - May 2011[1]

Sample ID	Ammonia Mean (µg/gram)	r	R	% R
Sample 1	3530	420	610	17.3
Sample 2	7360	360	460	6.3
Sample 3	4070	180	460	11.3
Sample 4	1110	50	220	19.8
Sample 5	1630	130	380	23.3

[1] 2009 Collaborative Study on Ammonia in Tobacco Using Ion Chromatography Analysis, Routine Analytical Chemistry Sub-Group, Technical Report - May 2011.

7. Recommendations

In 2009, the RAC conducted an interlaboratory study for the determination of ammonia in ground tobacco. The overall level of variability for smokeless tobacco products seen in the current study is slightly higher as compared to the 2009 RAC study, but this may be related to sample heterogeneity and not the analytical method used for the current study. The results for the current study showed similar %r values for both tobacco and smokeless tobacco products which also indicate 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 poor resolution between sodium and ammonium ions.

This report was discussed during the CORESTA STS Meeting held on April 1, 2014, in Nuremberg, Germany. The STS agreed that with minor modifications to CRM No. 73, the method would also be fit for use for the determination of ammonia in smokeless tobacco products. The following modifications are recommended for inclusion into CRM No. 73 so that the method is also suitable for the determination of ammonia in smokeless tobacco products:

1. Inclusion of the chromatographic gradient profile listed in Table 1a.
2. Peak integration should be conducted with peak area, and not peak height.
3. Peak integration should be performed with a “dropped baseline“ instead of “valley to valley” integration.
4. Modify the sample preparation section to include the use sample centrifugation and filtration with 0.45 μm syringe filters instead of paper filtration.

The STS also recommended that a technical note be added that states other IC columns, such as the ThermoFisher IonPac CS16A, may provide superior resolution between sodium and ammonia as compared to the ThermoFisher IonPac CS12A column used for this study.

Appendix A: Study Protocol

CORESTA Smokeless Tobacco Sub-Group

Study Protocol for a Collaborative Study on Ammonia in Tobacco and Smokeless Tobacco Products

1. Objective

The main objective of this study is to update CRM No. 73 for the determination of ammonia by IC in tobacco and smokeless tobacco products.

2. Study coordinator

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3. Analytes and Methods

3.1 Ammonia

The ammonia content of a tobacco or tobacco products including smokeless tobacco is determined by extraction into a sulphuric acid solution in order to stabilize the ammonium ion. Ion chromatographic analysis is used to separate the ammonium ions from other cations and the analyte is detected conductometrically and quantified against an external standard calibration.

Laboratories are asked to apply the draft CRM for the determination of ammonia. Laboratories are asked to record sample preparation steps any deviations from the method. Laboratories must provide analytical data in the templates distributed with the study protocol.

4. Samples

- Sample 1: CRP-1 available from NCSU
- Sample 2: CRP-2 available from NCSU
- Sample 3: CRP-3 available from NCSU
- Sample 4: CRP-4 available from NCSU
- Sample 5: 3R4F Filler available from UK
- Sample 6: Fine cut moist snuff provided by BAT Southampton
- Sample 7: Ground Virginia tobacco provided by BAT Southampton
- Sample 8: Ground Burley tobacco provided by BAT Southampton
- Sample 9: Ground Oriental tobacco provided by BAT Southampton
- Sample 10: Ground fire-cured tobacco provided by BAT Southampton
- Sample 11: Ground dark air-cured tobacco provided by BAT Southampton

All samples, with the exception of the CRPs and 3R4F filler, will be distributed by Carol Goss the first week of August.

If the participating laboratory does not already have an adequate supply of the four CRPs, they may be obtained through Karen Andres at the North Carolina State University (NCSU) Analytical Services Laboratory (http://www.tobacco.ncsu.edu/strp.html).

If the participating laboratory does not already have an adequate supply of Kentucky reference cigarettes 3R4F are available from the University of Kentucky (UK). (http://www2.ca.uky.edu/refcig/)

PLEASE NOTE: Each participating laboratory requiring CRP shipment should send the shipping address, person to whom delivery should be made, shipping account (FedEx, DHS, UPS) arrangements and any special delivery information to the following email address: Karen_Andres@ncsu.edu

Karen Andres will inform the laboratories of the actual shipping date and tracking information so that the receiving laboratories can prepare for receipt of the samples.

Participating laboratories should order CRP from NCSU if they are not confident that the CRPs have been stored at -20 ºC.

Sample Handling

The CRPs samples and the Market Type MST sample shall be removed from -20 ºC cold storage and thawed at room temperature for at least 2 hours before use. After this initial thawing, the samples shall be stored in a refrigerator in between use.

1. The Snus pouches (CRP-1) should be cut into 2 halves directly into the extraction vessel. Both snus and pouch material are to be analysed.
2. The loose leaf reference product (CRP-4) should be reduced in size for analysis by cutting with a razor blade.
3. The moist snuff reference product (CRP-2) and the dry snuff reference product (CRP-3) do not require further sample grinding.
4. The Market type MST sample and tobacco samples do not require further sample grinding.
5. 3R4F filler should be ground to a particle size of 1 mm.

All remaining samples should be stored in the freezer at -20 ºC for future CSTS studies. These studies may include B[a]P, metals, and/or additional studies.

5. Experimental plan

Three replicates for each sample are required. If possible, it is requested that all samples be analyzed on a single day. If multiple analysis days are needed it is requested that all three replicates of each sample are analyzed on a single day.

6. Data submission

The attached templates for ammonia should be used for data submission. Please supply data in the requested format without creating new cells or rows in the spreadsheet. Results should be reported back to Gene Gillman.

7. Data Analysis

The data will be analyzed statistically according to ISO 5725-2:1994 (organized by Karl Wagner, Altria).

8. Timescale

- First week of August 2013

Samples will be shipped to the laboratories; each laboratory must immediately contact Carol Goss to order samples. The CRPs should be ordered from NCSU if participants do not have sufficient quantities that have been stored at the recommended temperature of -20 ºC. 3R4F cigarettes should be ordered from University of Kentucky.

All documents will be distributed to participating laboratories:

- Protocol of the study
- Report form
- Analytical method for the study

- August to end of September 2013

Laboratories will perform the study.

- October 1, 2013

The results must be sent to the data coordinator (Gene Gillman) **on or before October 1** in order to give to the statistician sufficient time to evaluate the data. Data received after October 7th will not be included in the draft report discussed at the fall meeting.

- late October/November 2013

The statistician will perform the data analysis and the Sub-Group coordinators will format the data for presentation.

- 20th of November 2013

The CSTS will assess the results of the collaborative study during the meeting in Richmond.

- If the samples supplied by Carol Goss (ground tobacco samples and Market Type MST sample) do not arrive in sufficient time to report data by October 1, 2013, all participants should initially conduct the study with the four CRPs and 3R4F filler. Data for the additional samples may be submitted at a later date if necessary for incorporation into a revised statistical report.

Appendix B: Analytical Method

DETERMINATION OF AMMONIA IN TOBACCO AND SMOKELESS TOBACCO PRODUCTS BY ION CHROMATOGRAPHIC ANALYSIS

0. INTRODUCTION

This is a modified version of CORESTA Recommended Method No. 73. "Determination of ammonia in tobacco by ion chromatography". The procedure has been modified to include the analysis of smokeless tobacco products.

1. REFERENCES

CORESTA Recommended Method No. 73.

2. FIELD OF APPLICATION

This method is applicable to tobacco taken from single grades and finished products and smokeless tobacco products. The method is applicable for samples with ammonia content ranging from at least 0.1% to 1% on a dry weight basis.

3. PRINCIPLE

The ammonia content of a tobacco or tobacco products including smokeless tobacco is determined by extraction into a sulphuric acid solution in order to stabilize the ammonium ion. Ion chromatographic analysis is used to separate the ammonium ions from other cations and the analyte is detected conductometrically and quantified against an external standard calibration.

Note: To avoid confusion in reporting results all concentrations are reported as ammonia.

4. APPARATUS

Normal laboratory apparatus are required, in particular, the following items:

4.1. Analytical balance

4.2. Disposable 5mL syringe with filter (0.45 μm)

4.3. Volumetric flasks of capacities 100, 250 and 1000 mL

4.4. 5, 10 and 20 mL pipettes

4.5. Laboratory shaker

4.6. Extraction vessels, for example 100mL Erlenmeyer flasks

4.7. Cation exchange analytical column[1] (250 mm x 4 mm) such as Dionex IonPac CS12A or equivalent

Other column(s) may be suitable for use with this method; laboratories must verify that sodium is sufficiently resolved from ammonia before use.

[1] Dionex IonPac CS12A cation exchange analytical column is the trade name of a suitable product available commercially. This information is given for the convenience of the users of this CORESTA Recommended Method and does not constitute endorsement of this product

4.8. Cation exchange guard column such as Dionex IonPac CG12A or equivalent

4.9. Ion Chromatograph (IC) consisting of a conductivity detector, conductivity suppressor, and data collection system

4.10. An eluent degassing unit is recommended

5. REAGENTS

Use only reagents of recognized analytical grade.

5.1. Ammonium Sulphate ($(NH_4)_2SO_4$) > 99 % purity (25.78% ammonia)

5.2. (Alternatively, a certified solution of 1000 ppm ammonia)

5.3. Sulphuric Acid (H_2SO_4) > 96 % purity

5.4. Methanesulphonic Acid (MSA) > 99 % purity

5.5. Deionized water ≥18.2 MΩ-cm

5.6. Helium gas connected to the Ion Chromatograph for eluent de-gassing

6. PREPARATION OF SOLUTIONS

6.1. Sulphuric Acid, 0.025N (Standards and Extraction Solution)

Carefully add 1.277 g of H_2SO_4 to approximately 600 mL of deionized water. Mix and dilute to 1L with deionized water.

6.2. Mobile Phase A: 10 mM MSA: 650 µL methanesulfonic acid is added to 1L of deionized H_2O.

6.3. Mobile Phase B: 40 mM MSA: 2.62 mL methanesulfonic acid is added to 1L of deionized H_2O.

The methanesulphonic acid can be prepared *in-situ* by an Ion Chromatograph fitted with an eluent generator. If this equipment is not available, the above text provides detail of how to prepare this reagent.

7. STANDARDS

Prepare a series of at least five ammonia standard solutions whose concentrations cover the range expected in the test samples. The standard solutions are stable for approximately 30 days when stored at 4 °C.

7.1. Ammonia Stock Solution

Accurately weigh 0.097 g of ammonium sulphate (ammonia mass fraction = 0.2578) into a 250 mL volumetric flask. Note the exact weight in order to accurately calculate the standard concentrations. Dissolve in 0.025N H_2SO_4. Make up to volume with 0.025N H_2SO_4. This gives a final concentration of 100 µg/mL ammonia.

7.2. Working Standards

Accurately pipette volumes according to the table below into 100 mL volumetric flasks and make up to volume with 0.025N H_2SO_4 (6.1), additional standards may be added.

Table 1 - Preparation of working standards

Standard #	Standard from which to pipette	Volume to pipette (mL)	Working standard concentration (µg/mL)
1	stock solution	10	10
2	stock solution	5	5
3	# 1	20	2
4	# 2	10	0.5
5	# 4	20	0.1

8. SAMPLE PROCEDURE

8.1. Sample Handling

For Tobacco: Mill the sample to a mesh size <1 mm using a mill suitable for the samples. If the tobacco is too moist for grinding, it should be dried at a temperature not exceeding 30 °C.

For Smokeless Tobacco: Smokeless tobacco can be chopped or cut with a razor blade to reduce particle size to approximately less than 1 mm. Portioned products should include the pouch material in the sample aliquot. The recommended procedure is to cut the pouch in half and add the tobacco and pouch material to the extraction vessel. For portioned products it may be necessary to adjust the volume of extraction solution to keep the ratio of tobacco to solution the same as loose tobacco products.

Determine the moisture content of the prepared tobacco if moisture correction of the ammonia level is desired.

8.2. Sample Preparation

Weigh approximately 0.5 ± 0.05 gram of tobacco, tobacco product or smokeless tobacco product (STP) into a suitable extraction vessel and add 50 ml of extraction solution (6.1). For portioned products it may be necessary to adjust the volume of extraction solution based on total sample weight.

Note: Volume of extraction solution may be reduced if the amount of tobacco is also reduced accordingly.

Place the extraction vessel on a laboratory shaker and shake at a moderate speed for 60 minutes. Take an aliquot and filter through a 0.45 µm syringe filter and proceed to analysis by ion chromatography. When necessary, samples may be centrifuged, for example 3000 RCF for 5 minutes before filtration through the 0.45 µm syringe filter.

8.2.1. Depending on the ammonia content of the tobacco, the extract may require dilution in order to obtain a chromatographic response covered by the calibration curve. If required the sample may be diluted with extraction solution (6.1). Typically, a dilution factor of 10 is sufficient for most samples. Samples should be diluted with 0.025N Sulphuric Acid.

Note: Typical values for ammonia for the CORESTA reference products are approximately 1000 µg/gram for CRP-1, 2500 µg/gram for CRP-2, 5000 µg/gram for CRP-3, and 2500 µg/gram for CRP-4. If these samples are prepared as recommended, a 10 fold dilution of the sample is required to bring ammonia within the calibration curve range.

8.2.2. The extracts should be analyzed as soon as possible. Their storage should however not exceed 72 hours 4 °C ± 2 °C.

9. SAMPLE ANALYSIS

9.1. Ion chromatography parameters

Set up the Ion Chromatograph, data station, and autosampler according to the manufacturer's instructions. Ensure that peaks for sodium and ammonium are well resolved. Suggested operating conditions are as follows:

A 25 µL injection loop is recommended and injection volume of all samples is 25 µL.

The initial mobile phase is 10 mM MSA and the gradient profile is stated in the Table 2.

Table 2 - Suggested gradient profile

Time (min)	Conc.Of MSA (mM)	Eluent Generator Curve	Flow Rate (mL/min)	Suppressor Current (mA)
0.0	10	5	1.00	88
9.0	10	5	1.00	88
9.5	40	5	1.00	88
14.5	40	5	1.00	88
15.0	10	5	1.00	88

Note: The gradient from CRM-73 is given in Table 3 as an alternate gradient.

Table 3 - Alternate gradient profile from CRM No. 73

Time (min)	Conc.Of MSA (mM)	Eluent Generator Curve	Flow Rate (mL/min)	Suppressor Current (mA)
0.0	18	5	1.00	88
7.0	18	5	1.00	88
7.1	40	5	1.00	88
13.0	40	5	1.00	88
13.1	18	5	1.00	88
18.0	18	5	1.00	88

OTHER TYPICAL PARAMETERS:

Needle height = 2 mm

Auto-sampler tray temperature = 10 °C

Column temperature = 30 °C

Syringe speed = 5

Pressure range: 200 psi (min) and 3000 psi (max)

Flush volume = 250 µL

Data Acquisition is throughout the period 0-15 min

Optimize the IC conditions for analyte separation and sensitivity. Once established, these conditions should be used for the analysis of all standards and samples, including the injection volume.

9.2. Calibration of the ion chromatograph

Inject an aliquot of each ammonia standard into the Ion Chromatograph. Record the peak height or area of ammonium ion. Plot a calibration curve of the peak area or height of ammonia versus expected concentration in µg/mL.

The calibration line is fitted by a quadratic function in keeping with weak base chemistry. If the coefficient of determination is <0.98 (r^2) then the calibration should be repeated. If an individual calibration point differs by 10% or more from the expected value, it should be omitted. The signal obtained for all test samples should fall within the working range of the calibration curve.

9.3. Determination of the ammonia content of samples

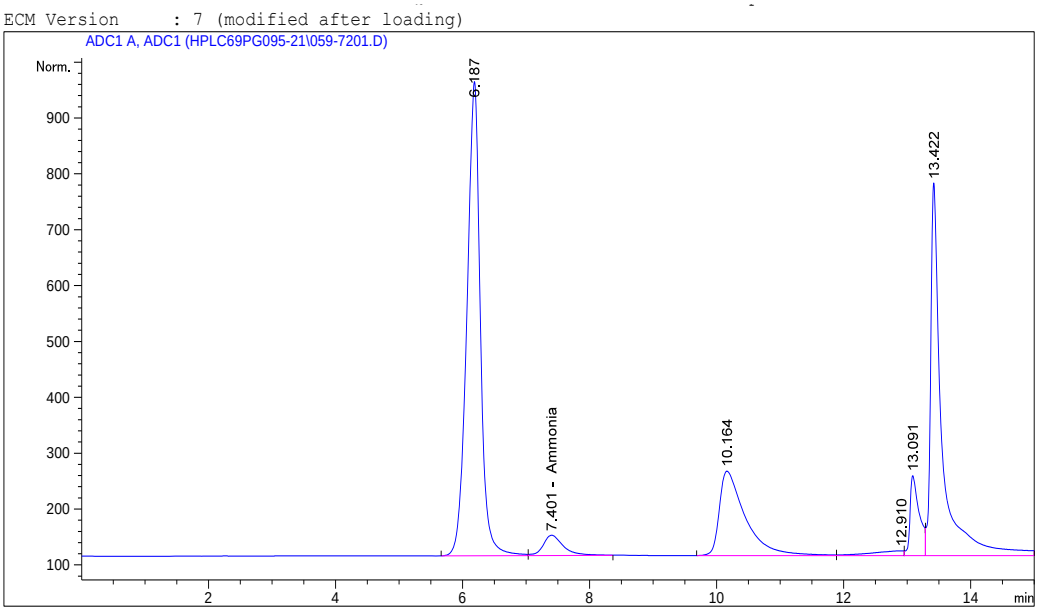
- Detection of cations is achieved using a suppressed conductivity detector in external water mode (CSRS-II). This method of detection reduces background conductivity from the mobile phase, thus increasing the sensitivity of the detector for the analyte.
- Quantitation is obtained from a five point external standard calibration using the peak height or area response of ammonium sulphate as ammonia. All calculations are based on the ammonia molar mass.

9.4. The amount of ammonia (in % of whole tobacco, not corrected for moisture content) is determined by the following calculation:

$$\% NH_3 = ((C * v * 100)/m) * \text{dilution factor}$$

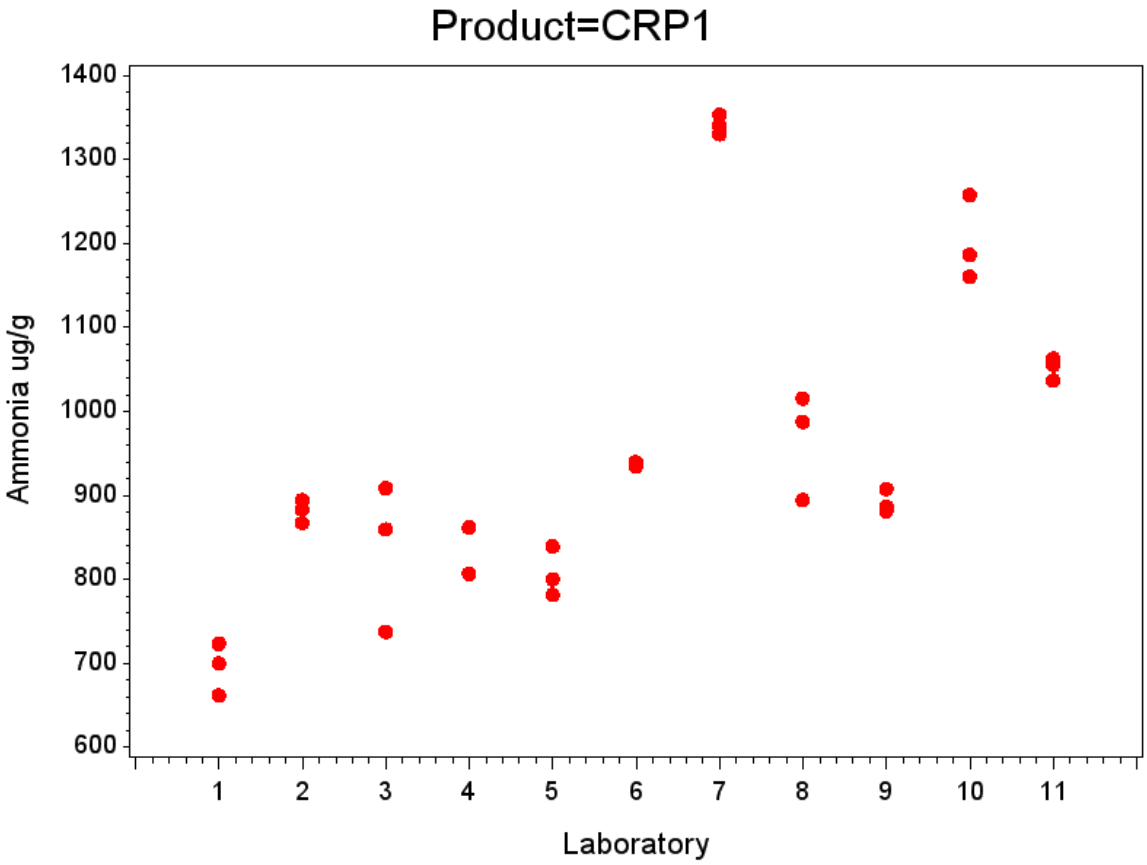
Where:

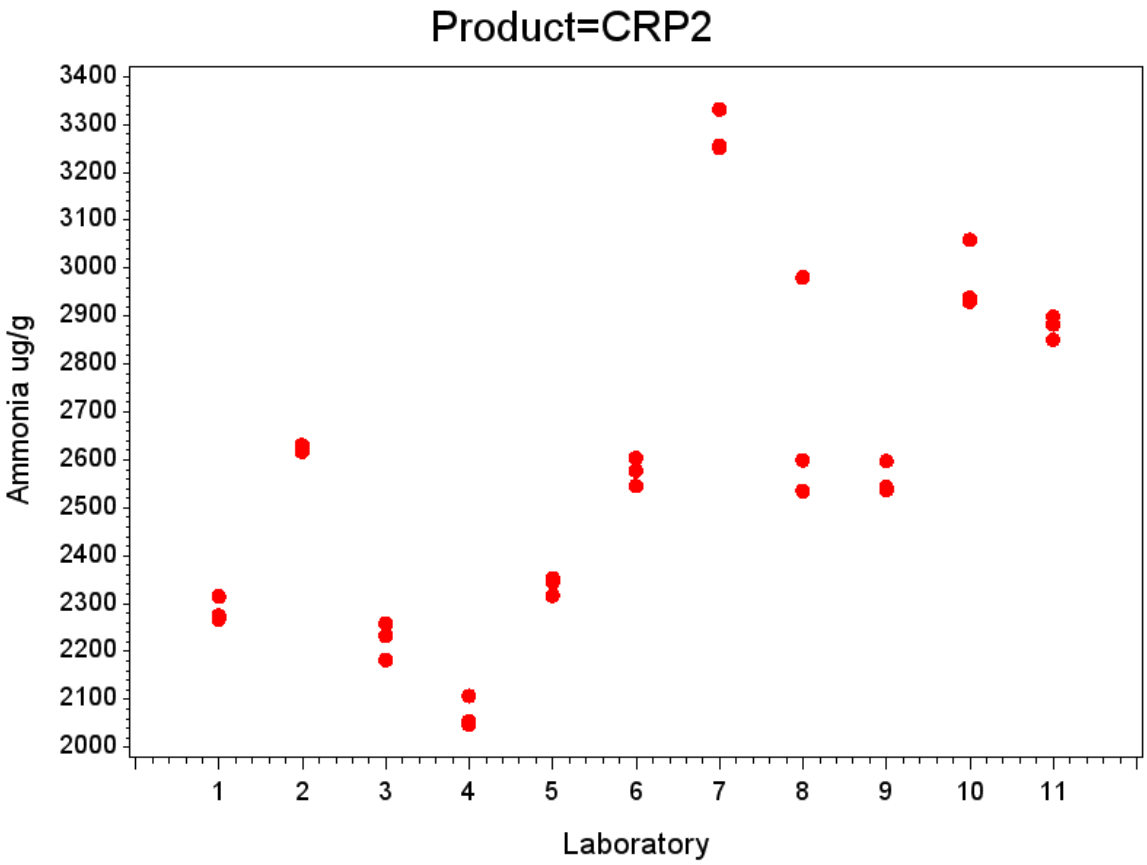
C = NH_3 concentration (in µg/mL) obtained from the calibration curve
v = extraction volume (in mL)
m = mass of the sample (in mg)
Dilution factor = factor as used in (8.2.1)

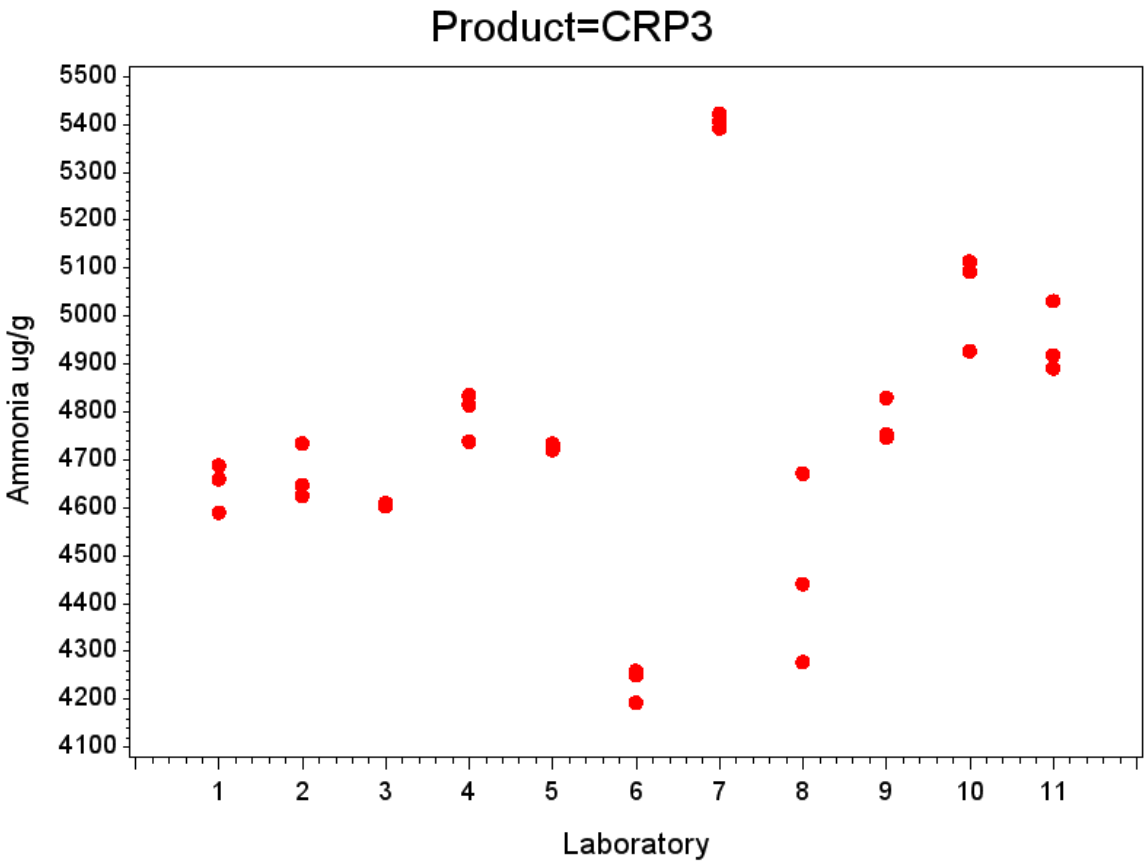


Chromatogram of ammonia in Smokeless tobacco product CRP-2

Appendix C: Raw data plots



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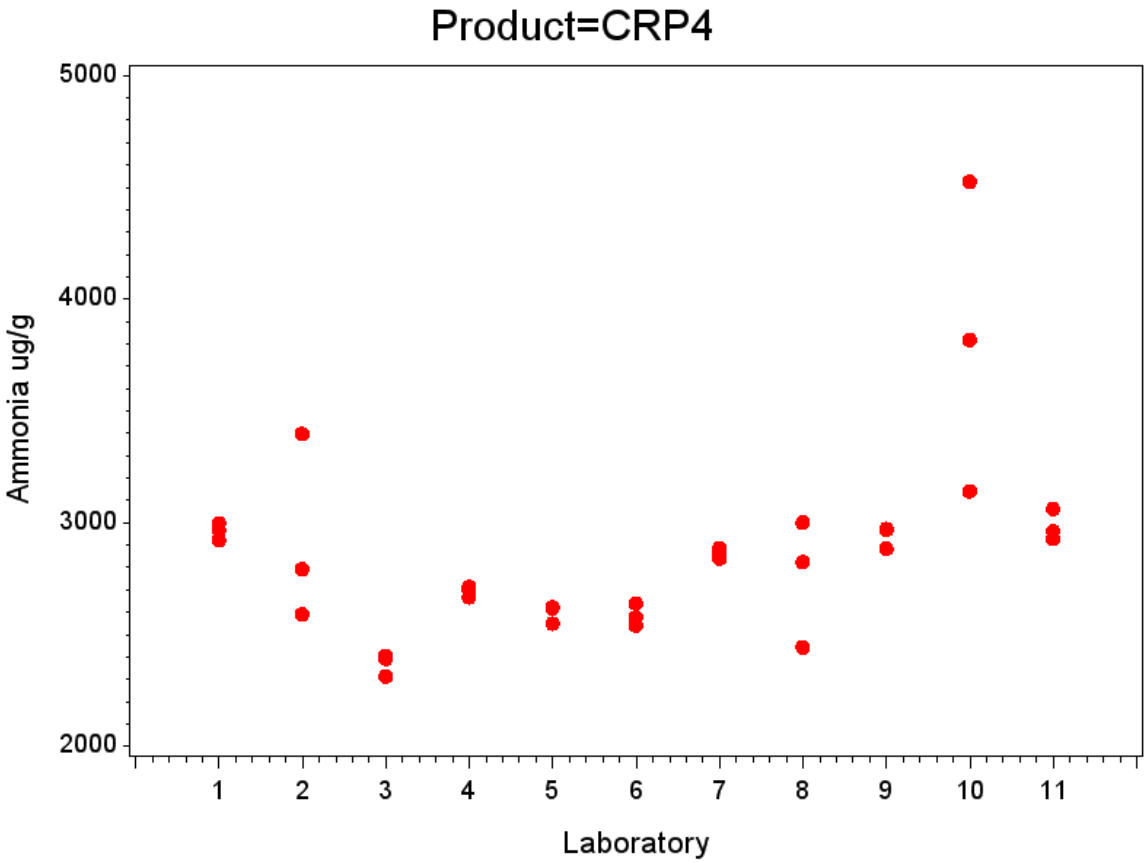
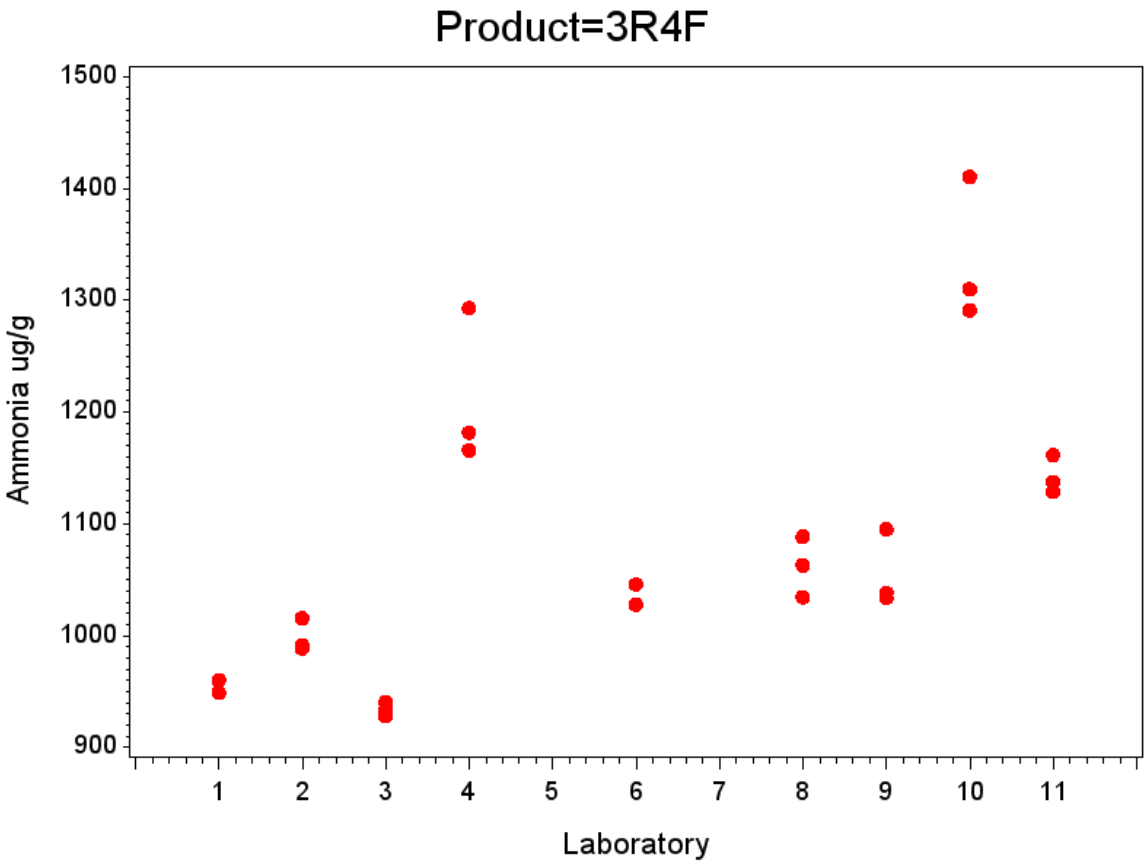
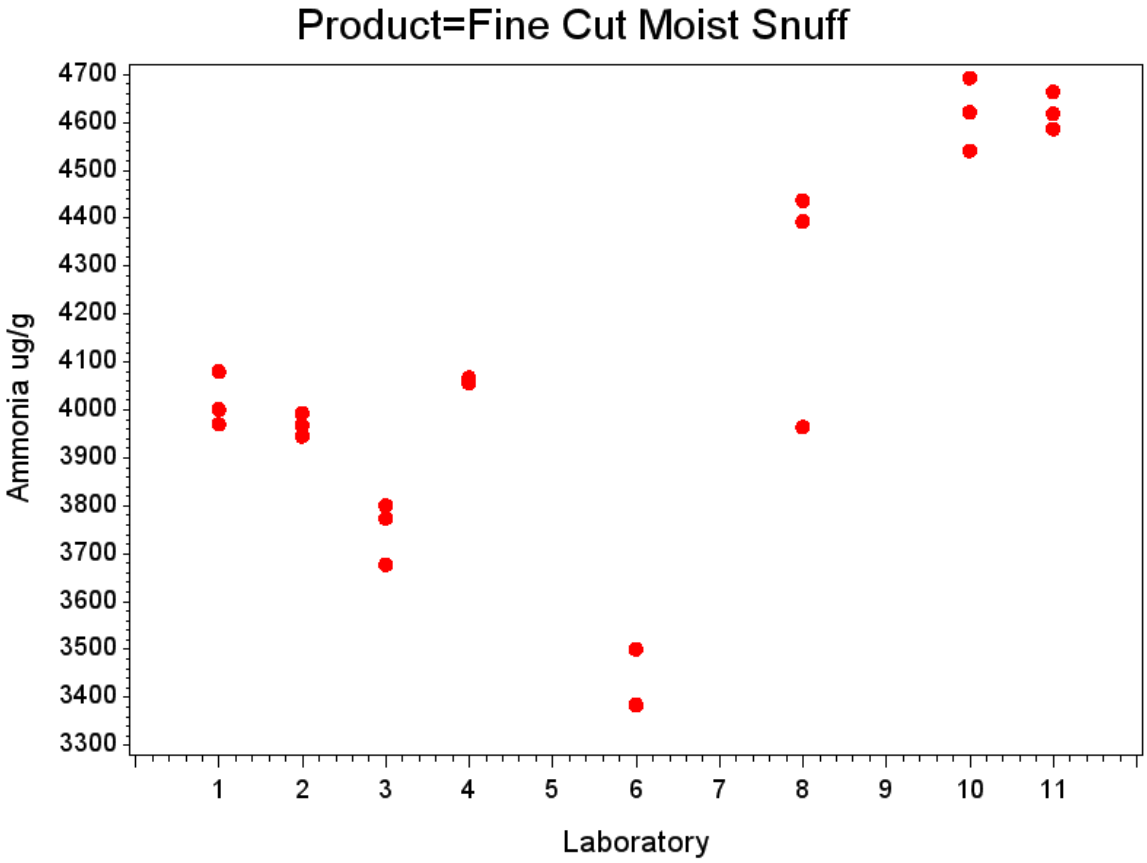
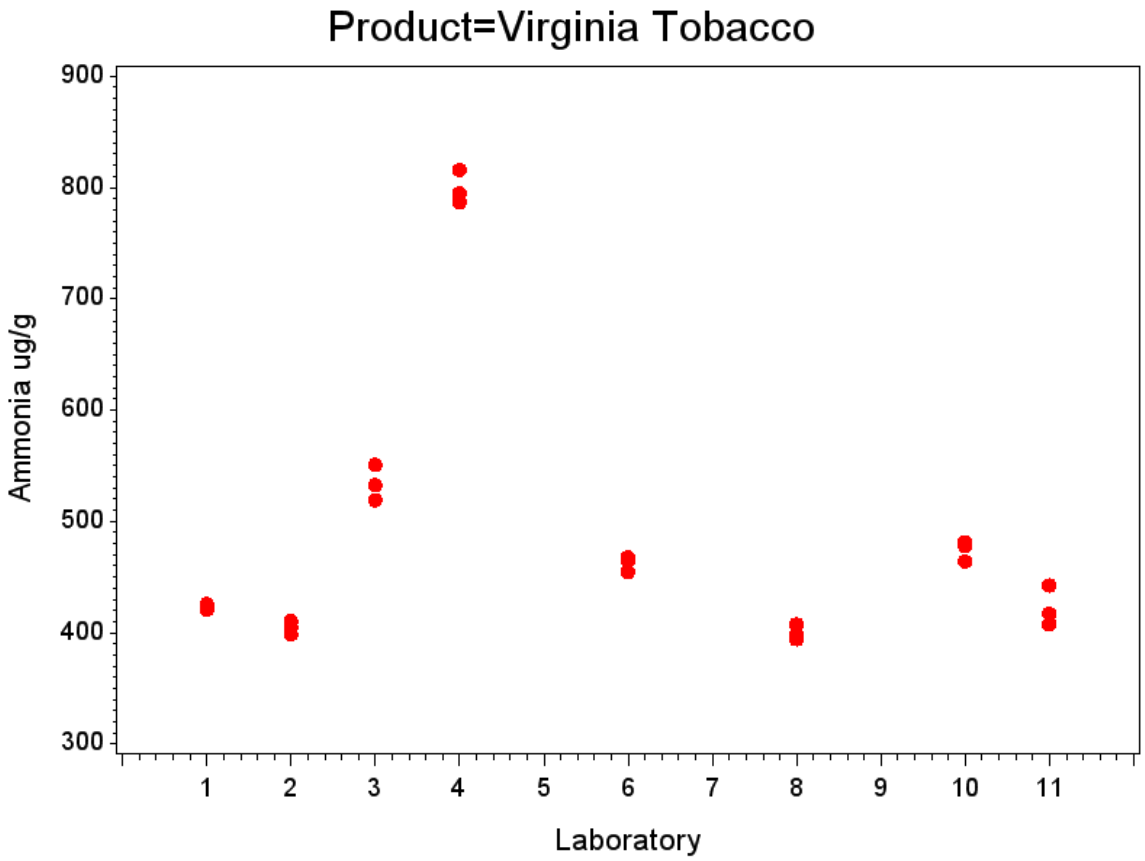
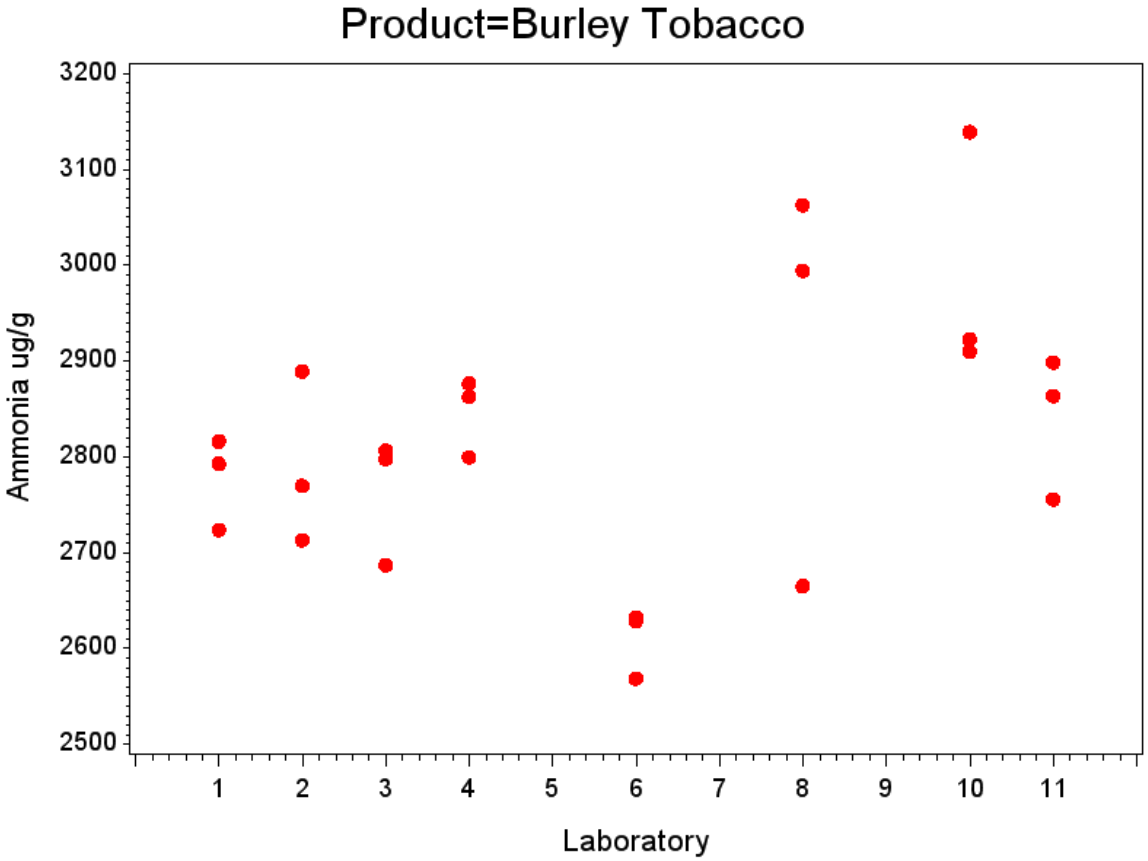
Ammonia ug/g
5500
5400
5300
5200
5100
5000
4900
4800
4700
4600
4500
4400
4300
4200
4100
1
2
3
4
5
6
7
8
9
10
11
Laboratory

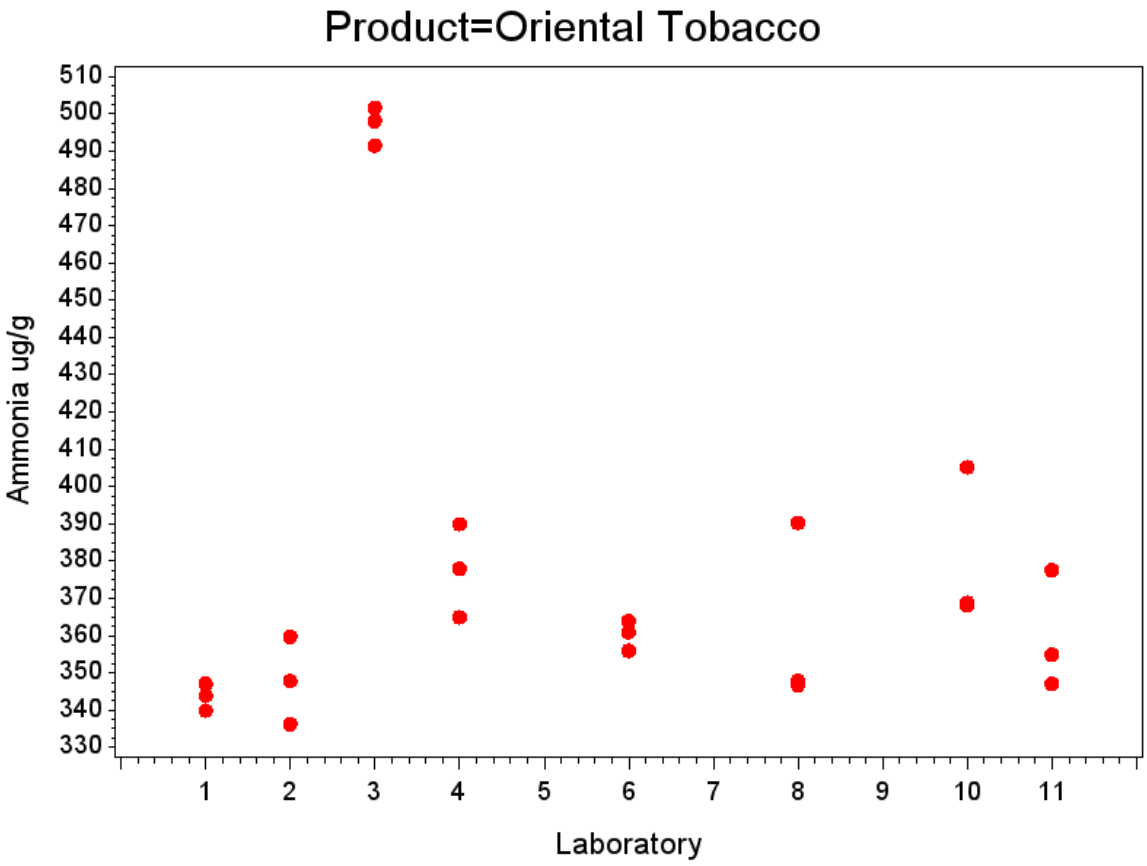

Product=CRP4
Ammonia ug/g
5000
4000
3000
2000
1
2
3
4
5
6
7
8
9
10
11
Laboratory

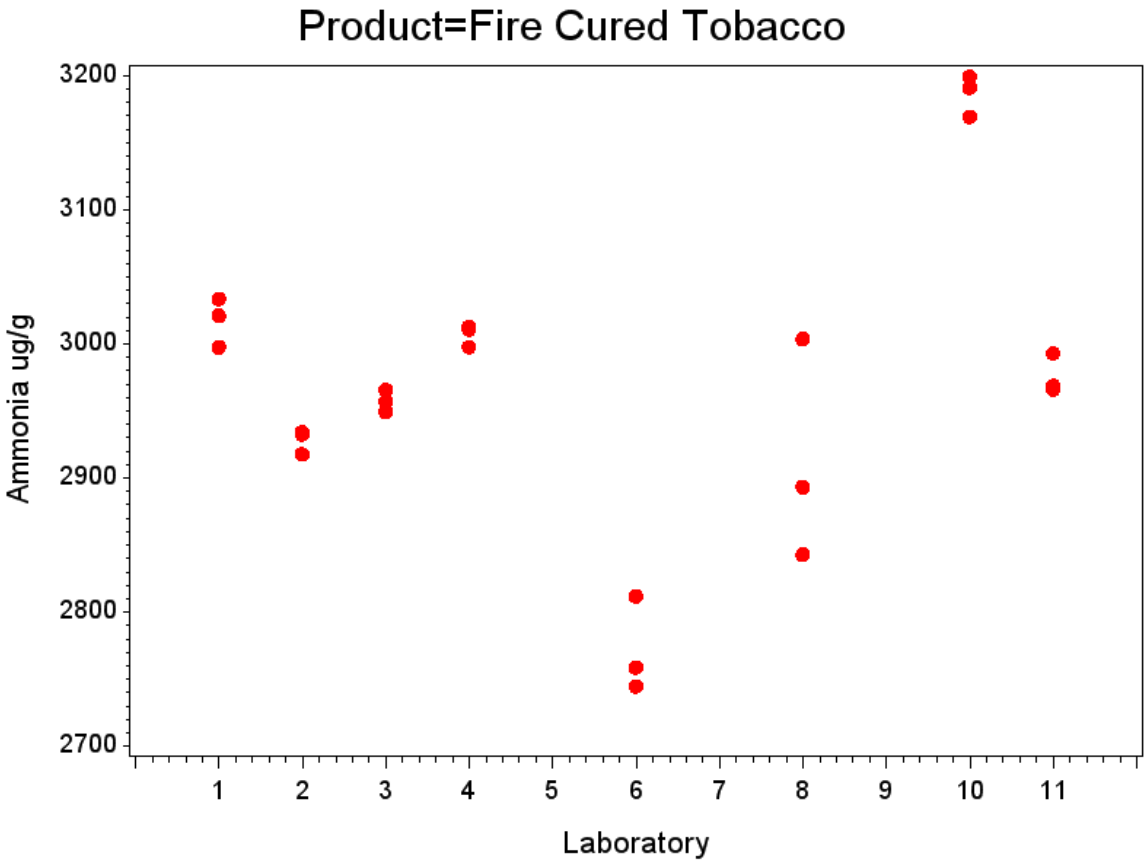

Product=3R4F
Ammonia ug/g
1500
1400
1300
1200
1100
1000
900
1
2
3
4
5
6
7
8
9
10
11
Laboratory

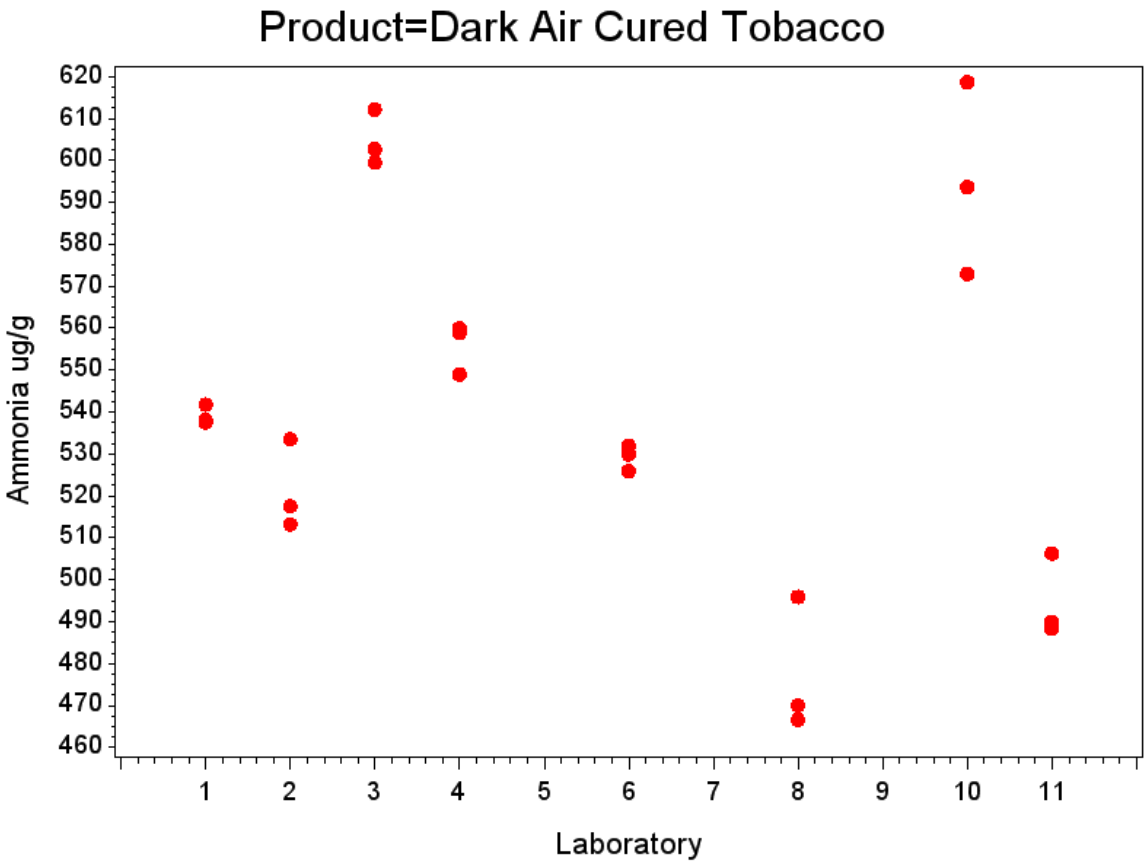

Product=Fine Cut Moist Snuff
Ammonia ug/g
4700
4600
4500
4400
4300
4200
4100
4000
3900
3800
3700
3600
3500
3400
3300
1
2
3
4
5
6
7
8
9
10
11
Laboratory


Product=Virginia Tobacco
Ammonia ug/g
900
800
700
600
500
400
300
1
2
3
4
5
6
7
8
9
10
11
Laboratory


Product=Burley Tobacco
Ammonia ug/g
3200
3100
3000
2900
2800
2700
2600
2500
1
2
3
4
5
6
7
8
9
10
11
Laboratory


Product=Oriental Tobacco
Ammonia ug/g
510
500
490
480
470
460
450
440
430
420
410
400
390
380
370
360
350
340
330
1
2
3
4
5
6
7
8
9
10
11
Laboratory


Product=Fire Cured Tobacco
Ammonia ug/g
3200
3100
3000
2900
2800
2700
1
2
3
4
5
6
7
8
9
10
11
Laboratory


Product=Dark Air Cured Tobacco
Ammonia ug/g
620
610
600
590
580
570
560
550
540
530
520
510
500
490
480
470
460
1
2
3
4
5
6
7
8
9
10
11
Laboratory