Routine Analytical Chemistry Sub-Group

Technical Report

2009 Collaborative Study on Ammonia in Tobacco using Ion Chromatography Analysis

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1 INTRODUCTION

During the past eight years, the Routine Analytical Chemistry (RAC) sub-group has carried out two collaborative studies on ammonia in tobacco using CFA technology. Each instance has failed to deliver a CORESTA Recommended Method (CRM) because insufficient participating laboratories followed the protocol.

In 2008 the RAC asked the Scientific Commission to allow a further collaborative study relating to the analysis of ammonia in tobacco using Ion Chromatography. Ion Chromatography was chosen because of its superior analytical capability in relation to CFA and potential to accommodate additional analytes. The aim of this study was to produce a CRM for ammonia in tobacco using Ion Chromatography.

At the April 2009 RAC meeting, Heintz Van Landewyck (HVL) agreed to write the protocol, supply and prepare the samples. Group Research & Development (GR&D) – British American Tobacco volunteered to arrange the distribution to participating laboratories.

Five samples were prepared, ranging in ammonium content from 0.1%-1.0%. An initial pre-study conducted by HVL on these samples revealed the following nominal levels of ammonia.

Table 1: Nominal levels of Ammonia in test samples

Sample ID	% Ammonia (wwb)
SAMPLE 1	0.4
SAMPLE 2	0.8
SAMPLE 3	0.4
SAMPLE 4	0.1
SAMPLE 5	0.2

Ten laboratories were able to provide data for the study. Data from all laboratories were coded. The list of participating laboratories is in Appendix A.

2 SUMMARY

In June 2009 five samples of ground tobacco were despatched by GR&D – British American Tobacco to 13 laboratories. The protocol, designed by HVL, was also sent to the participants.

The cross-check was designed as a balanced uniform level experiment, in which samples from 5 batches of materials, representing 5 different levels of the test were sent to participating laboratories.

Three replicates were requested and the results were reported on an “as received” basis. The moisture content of each sample was also reported.

10 sets of results were submitted within the timescale. Three laboratories were unable to take part due to instrument problems and heavy workload.

Ten laboratories submitted data; of these ten, eight followed the protocol exactly as described.

2.1 OVERVIEW OF DATA

The data were checked for the presence of outliers using the Mandel's *k* and *h* graphical consistency techniques. However, these were not used for data exclusion purposes, just as graphical illustrations and useful information for the participating laboratories. Cochran's and Grubbs' numerical outlier exclusion tests were used to discard outliers from the data but stragglers were retained. The tables below are from the data after outlier testing had been performed.

In this report, repeatability (r) refers to the variability within a laboratory and reproducibility (R) to the variability of results between laboratories.

The final repeatability and reproducibility standard deviations for all data, together with the actual r and R figures, are listed in Table 2 below.

Table 2: Actual r&R figures for Ammonia in Tobacco

Sample ID	Mean	r	S_r	R	S_R
Sample 1	0.353	0.042	0.015	0.061	0.022
Sample 2	0.736	0.036	0.013	0.046	0.017
Sample 3	0.407	0.018	0.007	0.046	0.016
Sample 4	0.111	0.005	0.002	0.022	0.008
Sample 5	0.163	0.013	0.005	0.038	0.014

Raw data from all participating laboratories is to be found in Appendix B.

3 COLLABORATIVE STUDY

3.1 PROTOCOL

The protocol for the study is to be found in Appendix C.

Laboratories were requested to carry out the analysis in triplicate by following the agreed protocol as closely as possible. Any deviations from the protocol were to be reported within the results spreadsheet.

4 DATA TREATMENT

The statistical analysis of the data followed the methods provided by ISO 5725-2 (1994) *"basic method for the determination of repeatability and reproducibility of a standard measurement method"*. Prior to the calculation of r&R figures, graphical and numerical data consistency techniques were applied to the data.

4.1 PRELIMINARY DATA

In preparation for the October 2009 meeting of the Sub Group an initial statistical evaluation was performed to obtain repeatability and reproducibility values, this initial evaluation was performed prior to any exclusion of data. The results from this are to be found in Table 3:

Table 3: Initial statistical evaluation of data

Sample	"True" Value	Repeatability r	Reproducibility R
SAMPLE 1	0.361	0.040	0.095
SAMPLE 2	0.758	0.039	0.104
SAMPLE 3	0.418	0.028	0.102
SAMPLE 4	0.114	0.022	0.072
SAMPLE 5	0.160	0.013	0.082

On further analysis it was noted that the data presented were based on the results for the ammonium ion rather than the equivalent ammonia content. The data were reanalysed and the following report is based on ammonia content.

4.2 RAW DATA

The following five plots show the raw data as received for each sample from all laboratories.

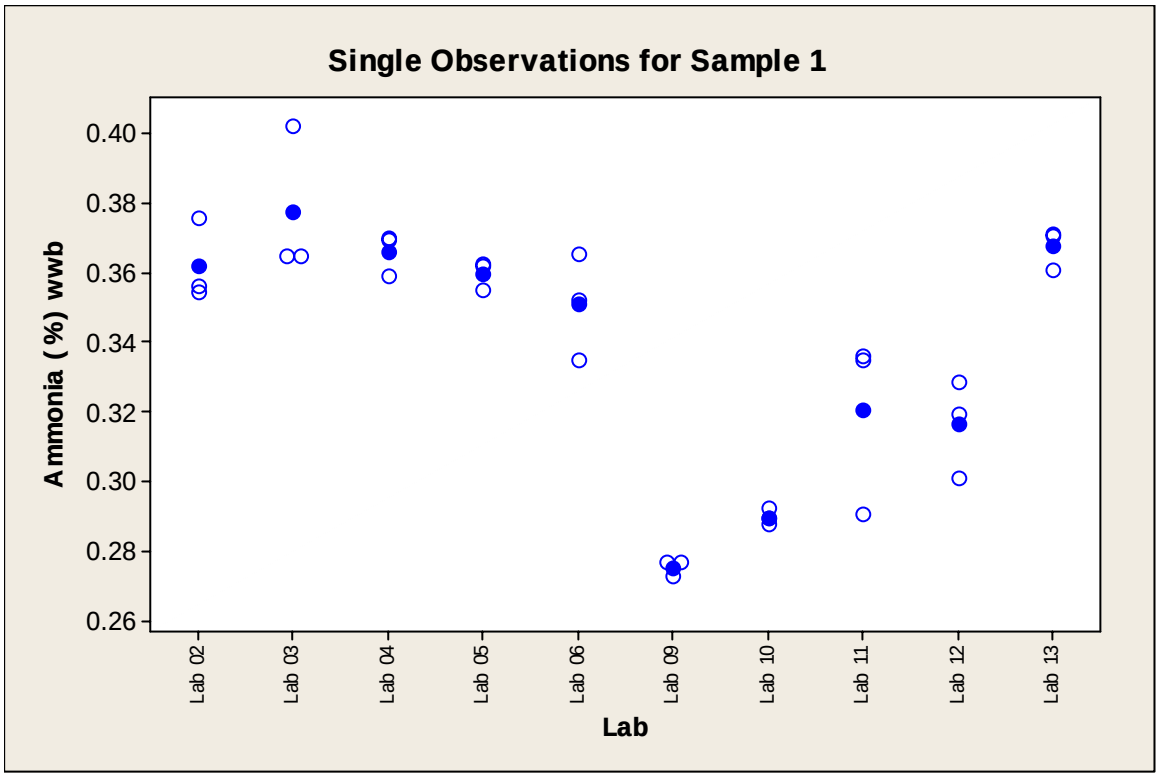


Figure 1: Single Observations for Sample 1

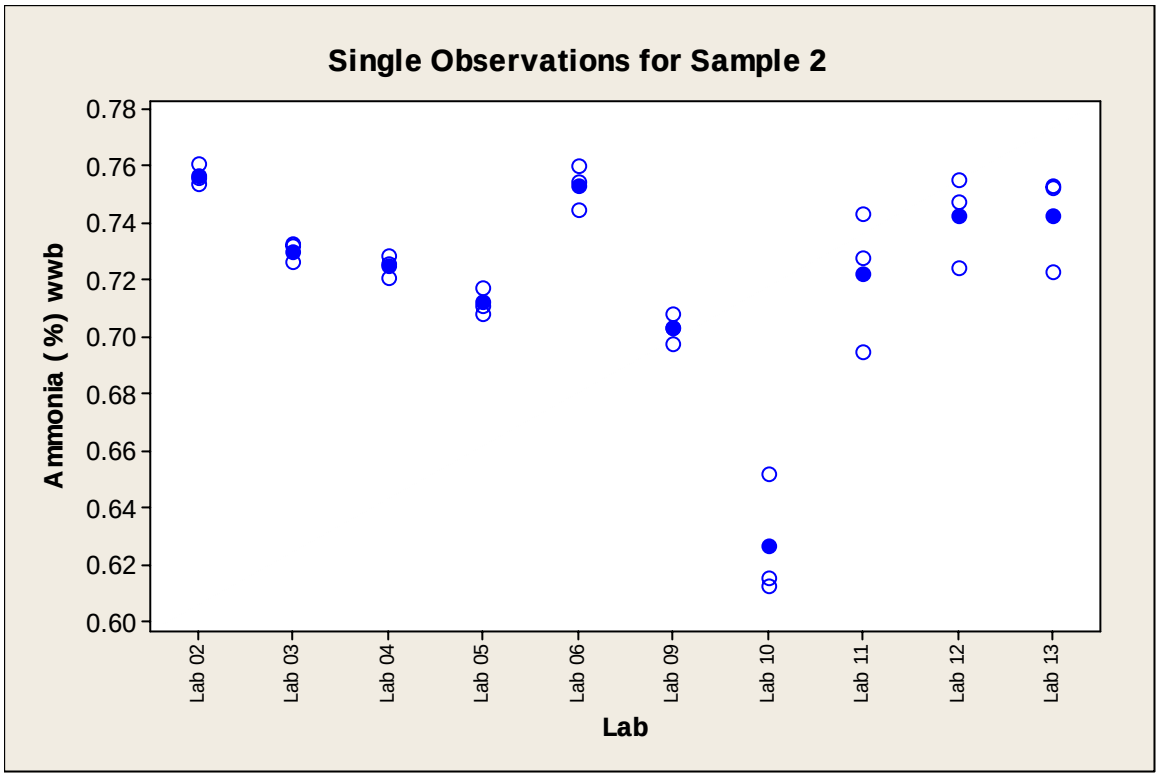


Figure 2: Single Observations for Sample 2

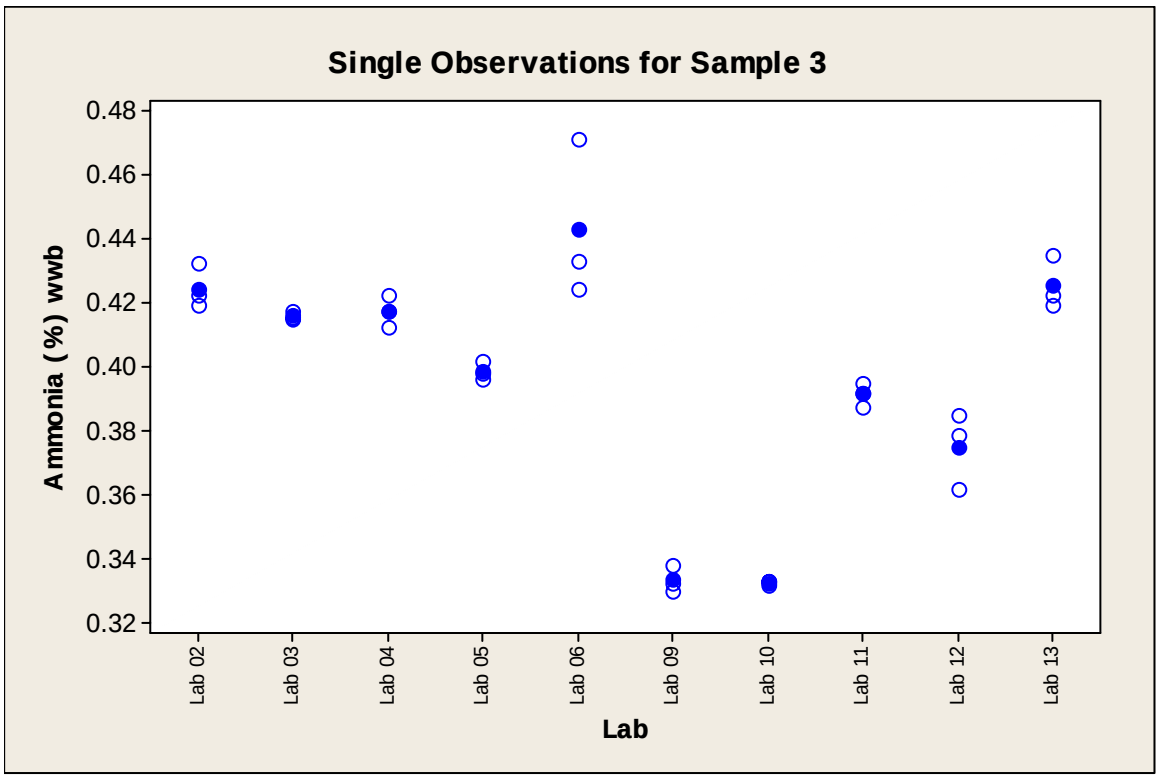


Figure 3: Single Observations for Sample 3

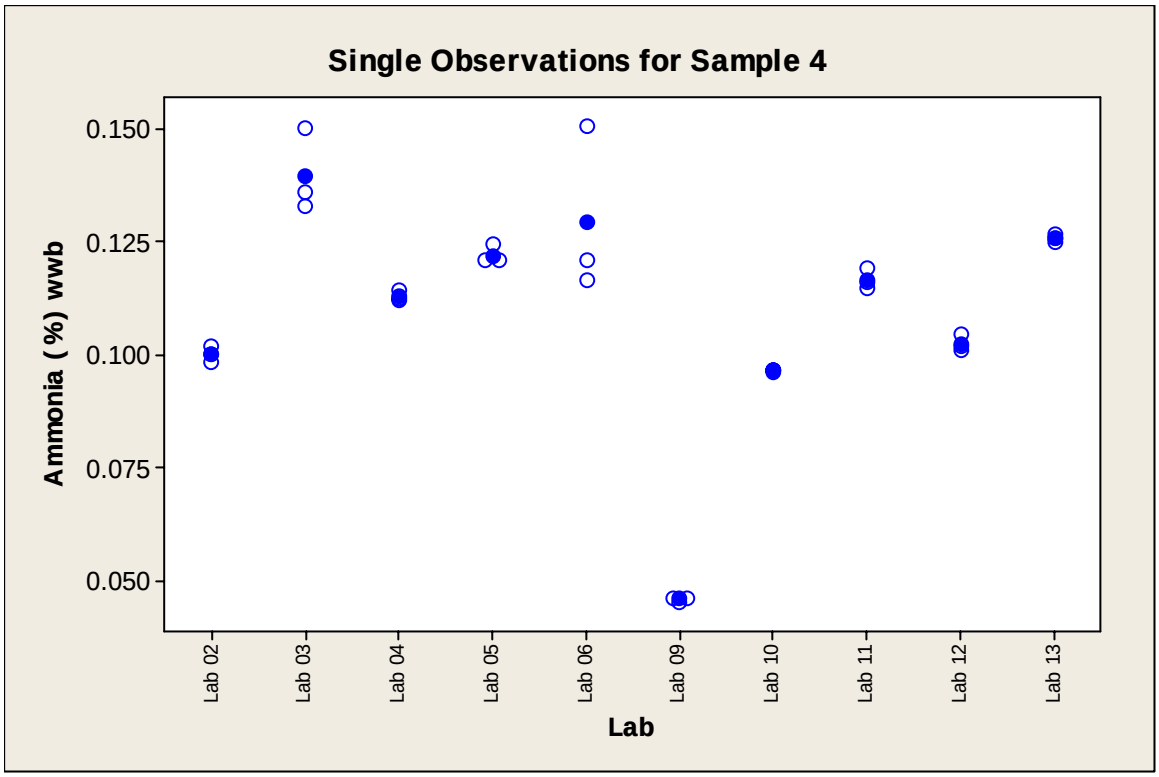


Figure 4: Single Observations for Sample 4

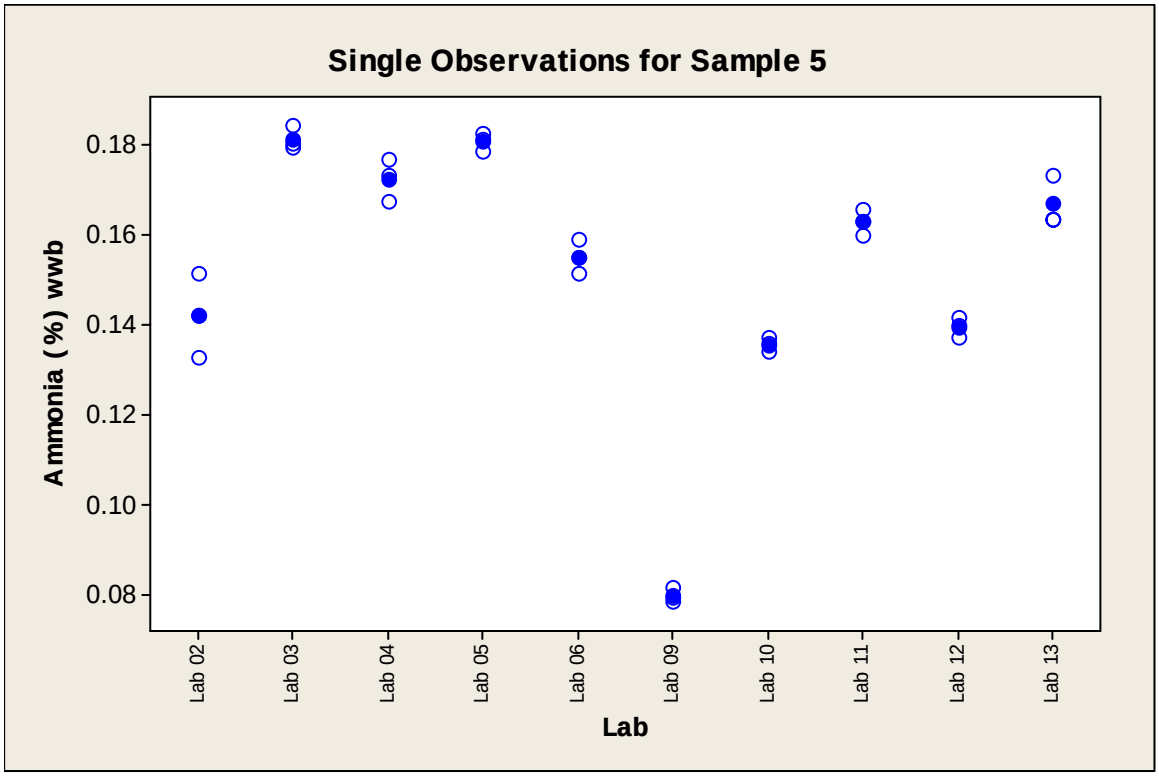


Figure 5: Single Observations for Sample 5

4.3 REVIEW OF DEVIATIONS

Following the review of deviations to the agreed protocol, laboratories 9 and 10 were excluded from further analysis.

Table 4: Summary of deviations to the agreed protocol

Lab ID	Followed Protocol?	Sample Extraction	Extracting Solution	Technique	Manufacturer	Model	Reagent Deviations
09	YES	As protocol	0.025N Sulphuric Acid	IC	Waters	Alliance conductivity detector 432	different eluent used: EDTA
10	YES	1g, 40ml, 45 minutes	0.025N Sulphuric Acid	IC	Dionex	ICS 3000	2.8mM MSA used as eluent

MANDEL'S *k* and *h*

Initially the raw data is checked for the presence of outliers (0.99 level) and stragglers (0.95 level) using two graphical data consistency techniques (Mandel's *k* and *h*). For convenience in data interpretation the derived *k* and *h* values are displayed in figures 6 and 7 as their corresponding standard deviations (*k* plots) or mean values (*h* plots) for each laboratory. The actual *k* and *h* values are listed in Appendix B.

Mandel's *k* checks the within laboratory data consistency by comparing the laboratories' variances for each level with straggling (0.95) and outlying (0.99) limits. Large *k* values indicate poorer repeatability in comparison with the other laboratories.

Mandel's *k* tests only the highest value in a set of standard deviations and is therefore a one-sided outlier test. Heterogeneity of variances may also occur with variances comparatively too small or zero. Consistently small *k* values could be due to excessive rounding or an insensitive measurement scale. However it seems unreasonable to reject data from a laboratory because it has accomplished a higher precision than others. Therefore, Mandel's is considered adequate.

Mandel's *k* plots display the observed standard deviations within a laboratory as points and the corresponding 0.95 and 0.99 straggling and outlying limits.

Mandel's *h* plots display the consistency of data between laboratories by comparing the overall mean results. Lines indicating 0.95 and 0.99 outlying limits are presented on the charts.

As the graphical data consistency techniques are more likely to indicate outliers than numerical techniques, no further action was taken on excluding data at this stage.

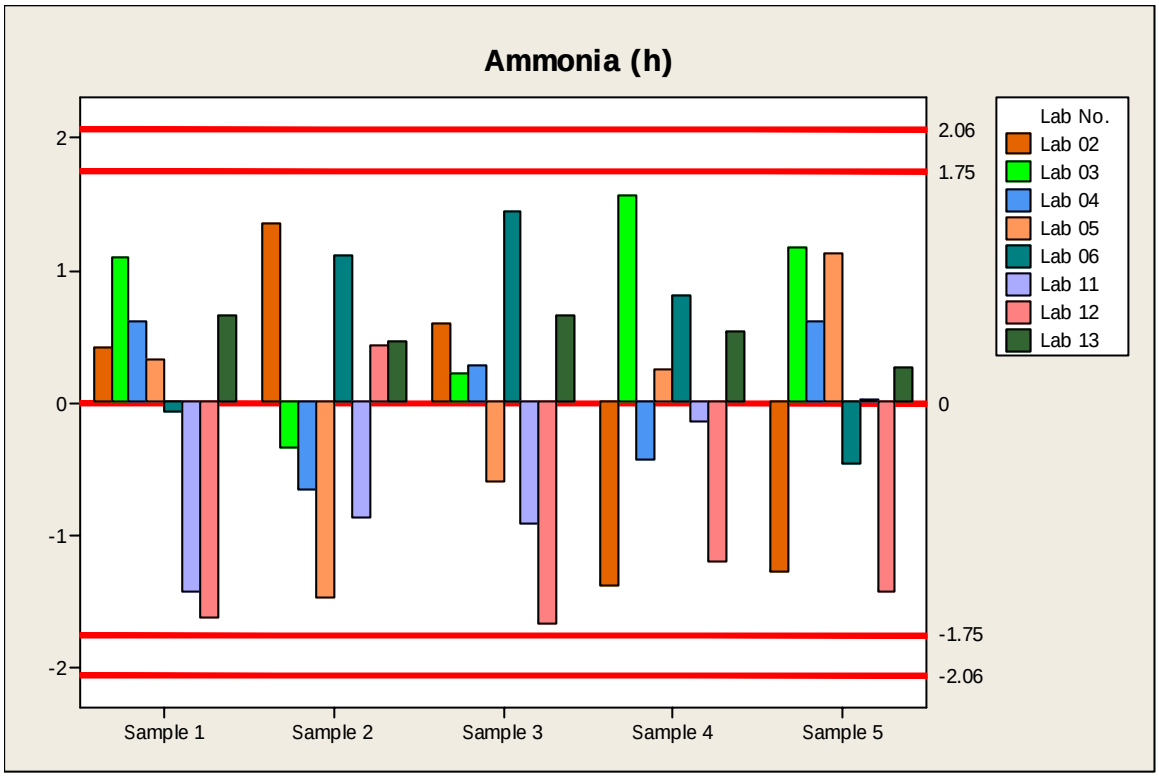


Figure 6: chart of h values showing 1% and 5% significance level

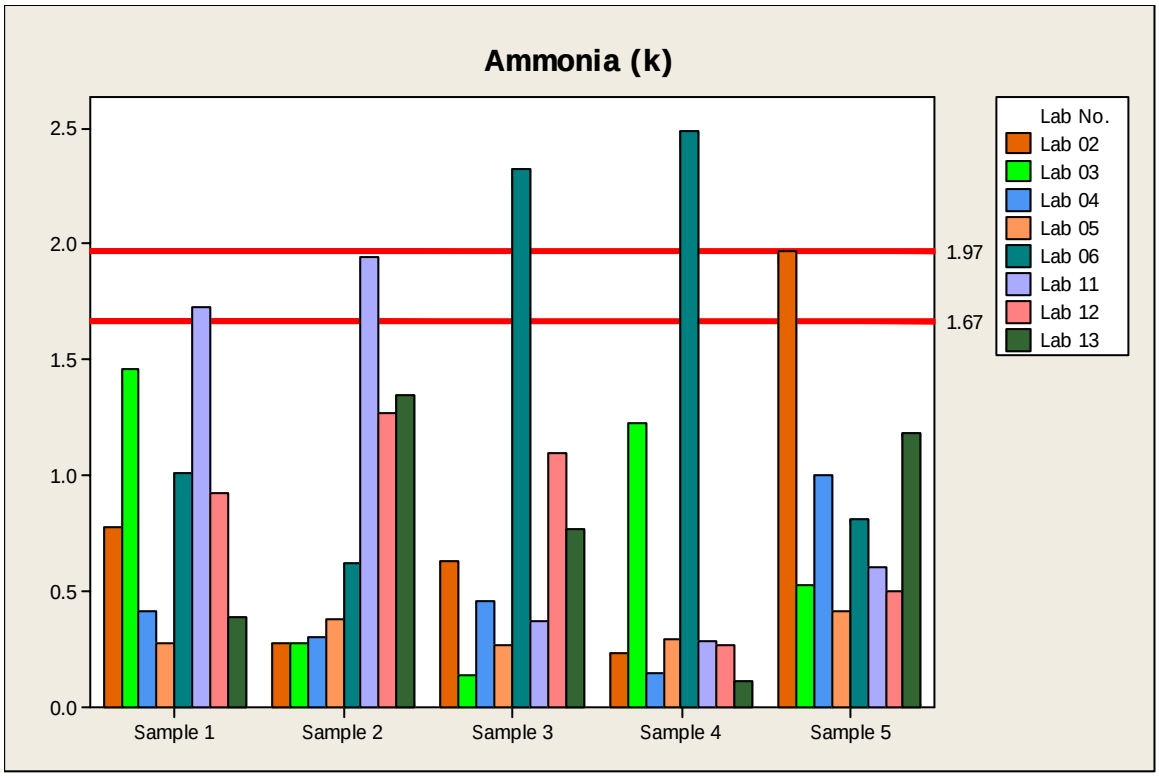


Figure 7: Chart of k values showing 1% and 5% significance level

4.4 COCHRAN'S AND GRUBBS'

Ten laboratories submitted data; of these ten, eight followed the protocol exactly as described. Laboratory 9 and Laboratory 10 were excluded from further analysis due to significant deviations from the prescribed protocol. For the purposes of this study, data from the remaining eight laboratories were included in the subsequent statistical analysis.

After confirming the integrity of the data reported by the respective laboratories, outlier testing was carried out according to ISO 5725-2 using firstly Cochran's test to eliminate within laboratory outliers and then Grubbs' test to eliminate between laboratory outliers.

Cochran's test is applied first and is an iterative procedure. Similar to Mandel's *k*, it analyses the within laboratory performance by comparing the observed data variances. The first iteration of Cochran's test identified the following outliers and stragglers.

Table 5: Results of the first iteration of Cochran's outlier detection technique

Sample	Lab Code	Class
SAMPLE 3	06	OUTLIER
SAMPLE 4	06	OUTLIER

Both outliers were discarded from analysis.

The second iteration of the Cochran's test identified the following outlier.

Table 6: Results of the second iteration of Cochran's outlier detection technique

Sample	Lab Code	Class
SAMPLE 4	03	OUTLIER

The outlier was excluded from further analysis. A third iteration of Cochran's test was performed but failed to identify any further outliers or stragglers. The iteration stopped. The remaining data was submitted for Grubbs' test.

The Grubbs' test was performed on individuals to assess between laboratory outliers. None of the remaining laboratories were identified with an unsatisfactory z score. Z scores are considered satisfactory if <2.

4.5 Z SCORES

Following the assessment of outliers using the Grubbs test, it is possible to represent the Z score graphically. The Z score graphs follow.

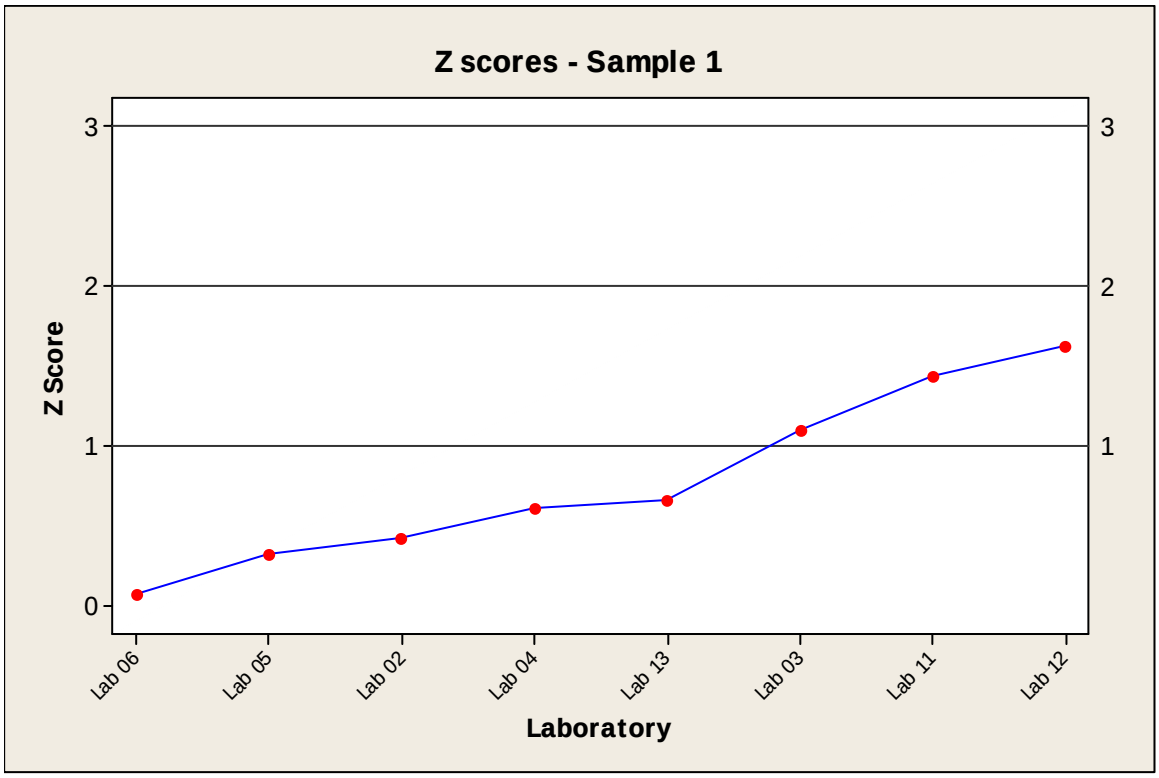


Figure 8: Z scores Sample 1

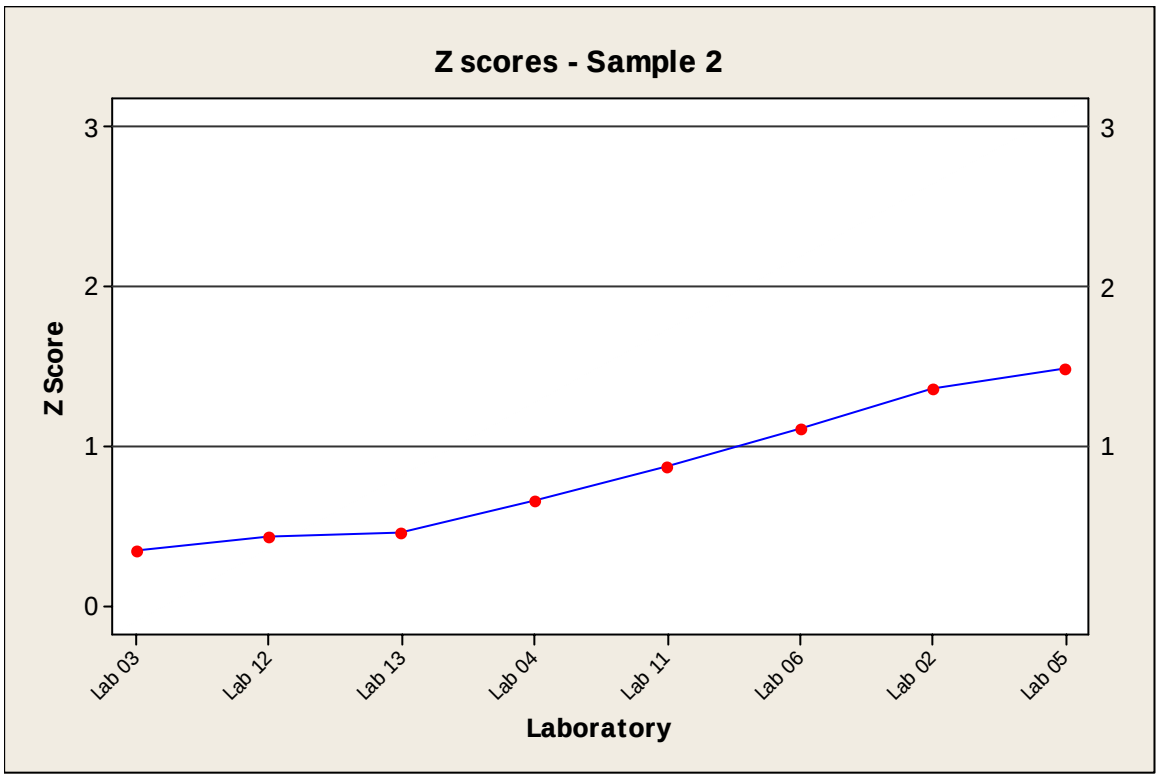


Figure 9: Z scores Sample 2

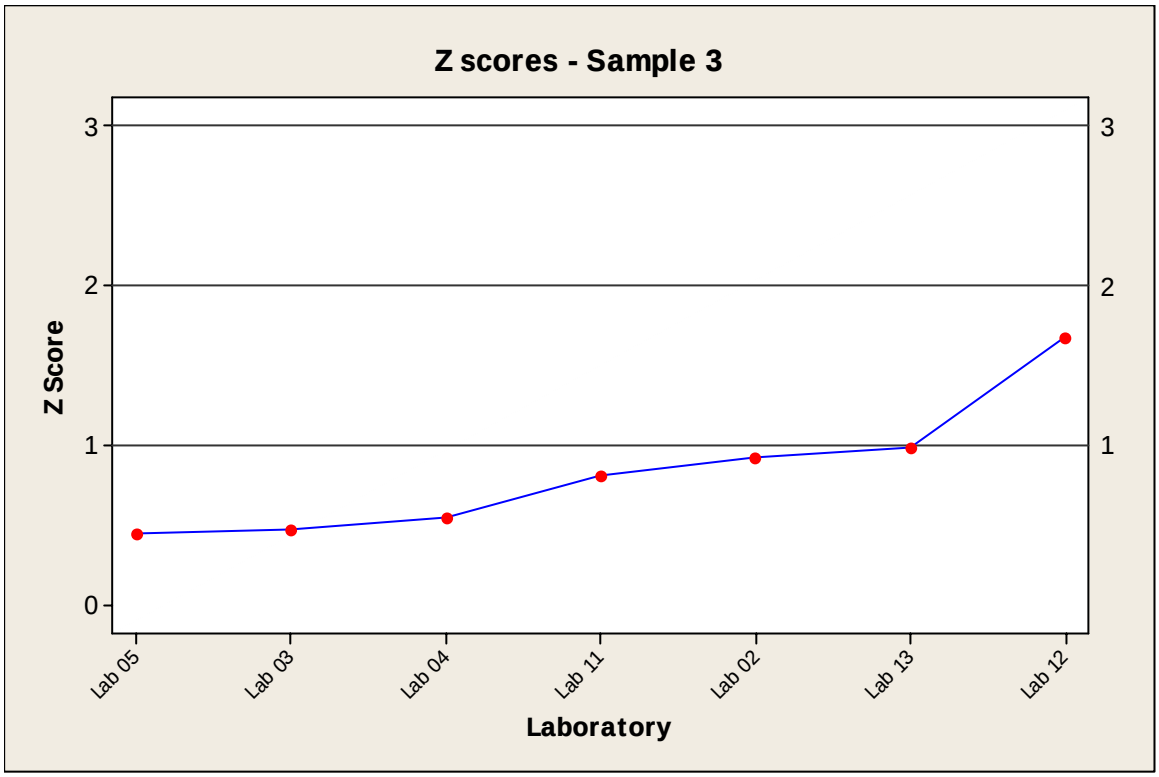


Figure 10: Z scores Sample 3

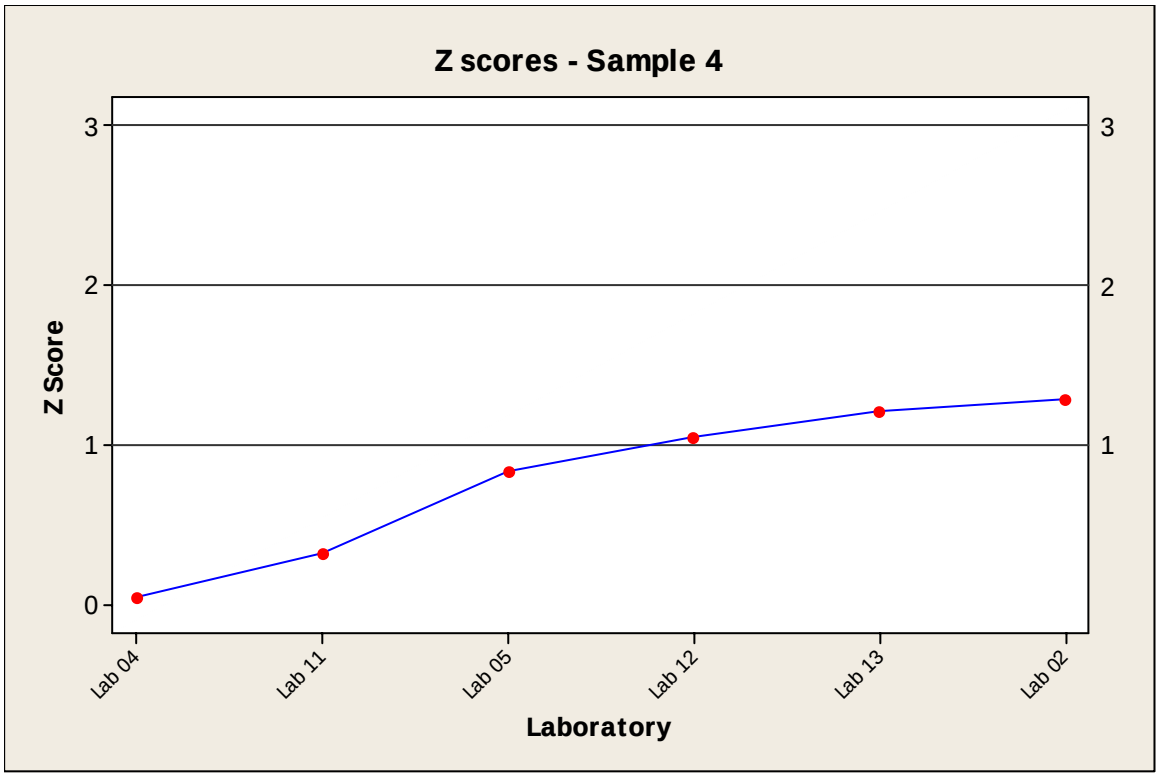


Figure 11: Z scores Sample 4

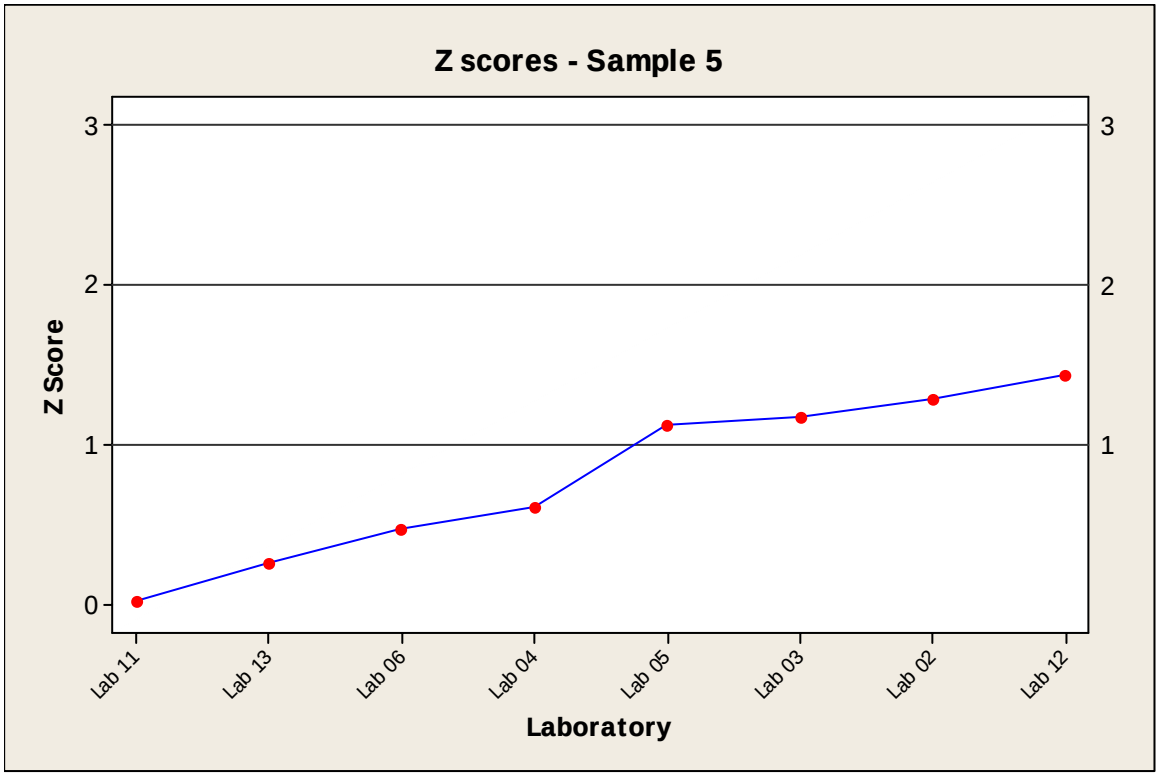


Figure 12: Z scores Sample 5

4.6 REPEATABILITY AND REPRODUCIBILITY

In order to calculate r and R, participating laboratories with unacceptable Cochran's test scores or z-scores greater than 2 were excluded from the calculation.

The final repeatability and reproducibility standard deviations, together with the actual r and R figures are listed below.

Table 7: Actual r and R figures for ammonia in tobacco

Sample ID	Mean	r SD	R SD	r	R
Sample 1	0.353	0.015	0.022	0.042	0.061
Sample 2	0.736	0.013	0.017	0.036	0.046
Sample 3	0.407	0.007	0.016	0.018	0.046
Sample 4	0.111	0.002	0.008	0.005	0.022
Sample 5	0.163	0.005	0.014	0.013	0.038

Note: Appendix B:4 contains all the raw data submitted for this study

5 INVESTIGATION INTO SAMPLE PREPARATION

Following the presentation of preliminary data the Routine Analytical Chemistry sub-group agreed a further study was required in order to investigate any potential changes in ammonia content when samples were subjected to drying or milling prior to analysis.

The laboratory of Heintz Van Landewyck, Luxembourg, performed the following experiment.

5.1 SAMPLES

Three types of sample were sourced:

- Cigarette blends (11 samples)
- Fine cut blends (RYO/MYO) (11 samples)
- Leaf grades (3 samples)

5.2 SAMPLE PREPARATION AND ANALYSIS

All samples were subjected to environmental conditions of 22 °C and 60% RH overnight. The cigarette blend and fine cut samples were analysed at two conditions :

- after environmental conditioning
- after environmental conditioning and milling to a <1 mm mesh size.

The leaf grades were analysed at two conditions :

- after environmental conditioning and cut into pieces (approximately 2 cm^2)
- after environmental conditioning and milling to a <1 mm mesh size.

The samples were analysed in accordance with the test protocol for the 2009 collaborative study.

5.3 RESULTS

The cigarette blends and the fine cut tobacco samples were analysed in triplicate and the mean results plotted in the following graphs (Figure 13 and Figure 14). The Leaf grade samples were analysed and a mean of 8 replicates plotted in Figure 15.

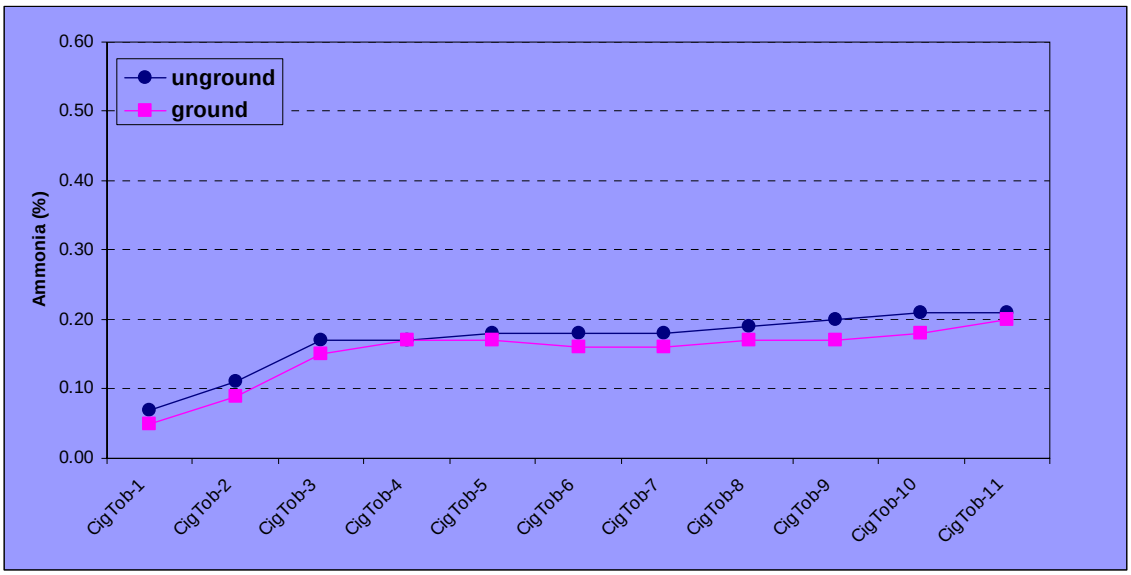


Figure 13: Cigarette Blends

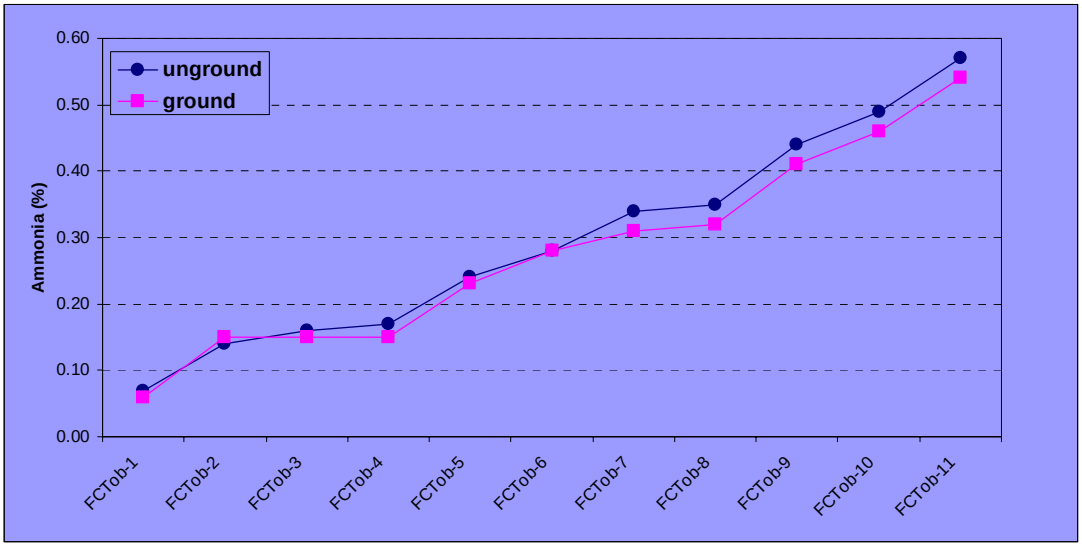


Figure 14: Fine Cut Tobacco (RYO/MYO)

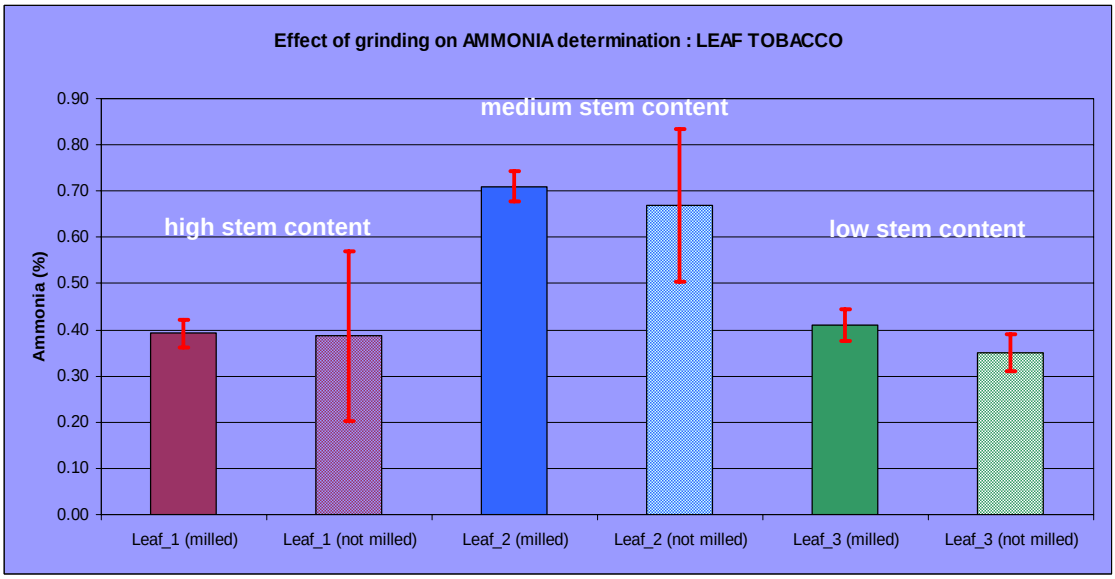


Figure 15: Leaf Grades

5.4 CONCLUSIONS

In the case of cigarette blends and fine cut tobacco there is no significant difference between ground and unground samples.

Leaf grades which have been analysed as received show also comparable values, however with a high variability in results obtained and this is likely to be due to non-homogenous samples.

In view of these results and for the sake of consistency with similar recommended methods, the Subgroup recommends that all samples should be ground to a <1 mm mesh size prior to analyses.

APPENDIX A - List of Participating Laboratories

PT HM Sampoerna Tbk, Indonesia
KT&G, Taejon, Korea
China National Tobacco
Heintz Van Landewyck, Luxembourg
LNE, France
JTI, Germany
Filtrona Technology Centre, UK
Labstat International, Canada
SEITA (Imperial), France
British American Tobacco GR&D, UK

APPENDIX B – Data Tables

Appendix B: 1 Mean observations and standard deviations for the test samples per laboratory

Laboratory Code	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lab02	0.362	0.012	0.757	0.004	0.424	0.007	0.100	0.002	0.142	0.009
Lab03	0.377	0.022	0.730	0.004	0.416	0.001	0.140	0.009	0.181	0.003
Lab04	0.366	0.006	0.725	0.004	0.417	0.005	0.113	0.001	0.172	0.005
Lab05	0.360	0.004	0.712	0.005	0.398	0.003	0.122	0.002	0.181	0.002
Lab06	0.351	0.015	0.753	0.008	0.443	0.025	0.130	0.018	0.155	0.004
Lab09	0.275	0.002	0.703	0.005	0.333	0.004	0.046	0.001	0.080	0.001
Lab10	0.290	0.002	0.627	0.022	0.332	0.001	0.097	0.000	0.135	0.002
Lab11	0.321	0.026	0.722	0.025	0.391	0.004	0.117	0.002	0.163	0.003
Lab12	0.316	0.014	0.743	0.016	0.375	0.012	0.103	0.002	0.139	0.002
Lab13	0.367	0.006	0.743	0.017	0.425	0.008	0.126	0.001	0.167	0.006

Appendix B: 2 MANDEL's k-values per laboratory

Laboratory Code	*k*				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Lab 02	0.78	0.28	0.63	0.23	1.97
Lab 03	1.45	0.28	0.13	1.23	0.52
Lab 04	0.41	0.31	0.46	0.14	1.00
Lab 05	0.27	0.38	0.27	0.29	0.41
Lab 06	1.01	0.62	2.32	2.48	0.81
Lab 11	1.73	1.95	0.37	0.28	0.60
Lab 12	0.93	1.27	1.10	0.27	0.50
Lab 13	0.39	1.35	0.77	0.11	1.18

Appendix B: 3 MANDEL's h-values per laboratory

Laboratory Code	*h*				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Lab 02	0.42	1.36	0.60	-1.38	-1.28
Lab 03	1.10	-0.34	0.21	1.56	1.17
Lab 04	0.61	-0.66	0.28	-0.43	0.61
Lab 05	0.33	-1.48	-0.60	0.25	1.13
Lab 06	-0.07	1.11	1.45	0.80	-0.47
Lab 11	-1.43	-0.87	-0.92	-0.14	0.02
Lab 12	-1.62	0.43	-1.67	-1.20	-1.44
Lab 13	0.66	0.46	0.65	0.54	0.26

Appendix B: 4 All raw data, Ammonia in tobacco (%wwb)

		Concentration of NH_3 (%wwb)				
Laboratory	**Replicate**	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Lab 2	1	0.356	0.756	0.419	0.102	0.151
Lab 2	2	0.375	0.761	0.432	0.099	0.142
Lab 2	3	0.354	0.754	0.422	0.100	0.132
Lab 3	1	0.365	0.726	0.417	0.133	0.180
Lab 3	2	0.365	0.732	0.415	0.136	0.179
Lab 3	3	0.402	0.733	0.416	0.150	0.184
Lab 4	1	0.359	0.726	0.412	0.114	0.173
Lab 4	2	0.370	0.729	0.422	0.113	0.177
Lab 4	3	0.369	0.721	0.417	0.112	0.167
Lab 5	1	0.362	0.711	0.401	0.121	0.181
Lab 5	2	0.355	0.708	0.396	0.125	0.179
Lab 5	3	0.363	0.718	0.398	0.121	0.182
Lab 6	1	0.352	0.761	0.433	0.151	0.159
Lab 6	2	0.365	0.745	0.471	0.121	0.151
Lab 6	3	0.335	0.755	0.424	0.117	0.155
Lab 9	1	0.277	0.708	0.338	0.046	0.081
Lab 9	2	0.273	0.698	0.330	0.046	0.078
Lab 9	3	0.277	0.704	0.332	0.045	0.079
Lab 10	1	0.292	0.616	0.333	0.097	0.137
Lab 10	2	0.290	0.612	0.333	0.097	0.134
Lab 10	3	0.288	0.652	0.331	0.096	0.136
Lab 11	1	0.336	0.743	0.392	0.119	0.160
Lab 11	2	0.335	0.695	0.387	0.116	0.166
Lab 11	3	0.291	0.728	0.395	0.115	0.163
Lab 12	1	0.329	0.724	0.384	0.102	0.142
Lab 12	2	0.319	0.756	0.362	0.101	0.140
Lab 12	3	0.301	0.748	0.379	0.105	0.137
Lab 13	1	0.371	0.723	0.422	0.125	0.163
Lab 13	2	0.361	0.753	0.435	0.127	0.173
Lab 13	3	0.370	0.754	0.419	0.126	0.164

APPENDIX C – Protocol for Inter-laboratory Ammonia in Tobacco Cross Check

1. INTRODUCTION

1.1 This procedure describes a method for conducting a study to determine the precision of the Ammonia Nitrogen Ion-chromatographic method as attached. Results are reported as % Ammonia, on a wet weight basis.

1.2 The cross-check is designed as a ***balanced uniform level experiment***, in which samples from 5 batches of materials, representing 5 different ***levels*** of the test are sent to participating laboratories. The laboratories have volunteered and are members of the CORESTA Routine Analytical Chemistry Sub Group.

The aim of the study is to assess the Repeatability and Reproducibility (*see Section 5 for definitions*) of all laboratories, and the Repeatability and Reproducibility of the Ammonia Nitrogen method with a view to recommending it for adoption by CORESTA as a Standard Method.

2. SCOPE

2.1 This document describes how the cross-check procedure shall be conducted by an individual participating laboratory. This document does not describe the actual recommended test method for Ammonia Nitrogen, which is to be found in the APPENDIX D. Laboratories will carry out the testing using method that reflects as close as possible the recommended method and note any deviations from the method when submitting their results.

2.2 Statistical analysis of the data is not discussed in this document, but details can be obtained from Jacqui_vella@bat.com

3. PROCEDURE

3.1 5 pouches of homogenised tobacco, at different ammonia nitrogen levels have been prepared by Southampton R&D, and distributed to the participating laboratories. The ammonia levels of the samples (expressed as $\%NH_4^+$) will be in the range of 0.1 - 1.0 %.

3.2 Each laboratory shall analyse **exactly 3** test portions, from each of the 5 pouches, **under repeatability conditions**:

> i.e. within a short interval of time by the same operator, without any intermediate recalibration of the apparatus, unless this is an integral part of performing the measurement.

This will result in 3 measurements for each of the 5 pouches being reported in the spreadsheet (i.e. 15 measurements in all).

3.3 If an operator becomes unavailable, another one can complete the measurements, provided the change does not occur within a group of 3 tests but only between two of the 5 levels. Any such change shall be reported with the results.

3.4 All measurements shall be completed within 2 months of receiving the sample pouches.

3.5 Where the measurement is carried out by a team of operators, each of whom performs some specific part of the procedure, the team shall be regarded as the "operator". Any change in the team shall be regarded as a different "operator".

3.6 In a precision experiment, the test results shall not be rounded, and ideally should be reported to at least one more digit than specified in the standard method used by the laboratory for the test.

4. REPORTING OF RESULTS

Using the spreadsheet provided by BAT Southampton,

The laboratory supervisor should report the following information:

4.1 The individual test results entered on the spreadsheet provided. The results shall not be rounded, and ideally, reported to at least one more digit than specified in the standard method used by the laboratory for the test.

4.2 The original observed moisture values from which the results were derived, entered on the spreadsheet provided.

4.3 Comments from the operators on any deviation from the documented analytical procedure should be reported in the comments column of the spreadsheet.

4.4 Information regarding any irregularities or disturbances during the measurement, including change of operator, together with a statement as to which measurements were performed by which operator, and the reasons for any missing results.

4.5 The date when the samples were received.

4.6 The date when the samples were measured.

4.7 Information regarding the equipment used.

4.8 Any other relevant information.

4.9 The completed spreadsheet shall be returned to Jacqui Vella at GR&D Southampton, e-mail address Jacqui_vella@bat.com

telephone: +44 23 8058 8111

fax: +44 23 8079 3962

4.10 Any questions or concerns regarding this protocol should also be addressed to Jacqui Vella.

5. DEFINITIONS

5.1 **Repeatability:**

The variability between independent test results obtained within a single laboratory in the shortest practical period of time by a single operator on the same set of test apparatus using test specimens from a single quantity of homogeneous material.

5.2 **Reproducibility:**

The variability between test results, obtained in different laboratories, using test specimens from a single quantity of homogeneous material.

5.3 **Test result:**

A test result is the value obtained by carrying out the complete test method once

APPENDIX D – Test Method for the Determination of Ammonia in Tobacco by Ion Chromatography

1. SCOPE

This method is intended for use in the quantitative determination of ammonia in aqueous extracts of tobacco matrices by Liquid Chromatography (HPLC) and conductivity detection.

2. PRINCIPLE

An aqueous extract of tobacco is prepared and ammonia is determined by HPLC with a suitable chromatographic column and conductivity detection. Quantitation is obtained from an external standard calibration.

3. APPARATUS AND EQUIPMENT

3.1. Analytical balance
3.2. Disposable 5 cc syringe with filter (0.45 µm).
3.3. 100, 250 and 1000 mL (class A) volumetric flasks.
3.4. 5, 10 and 20 mL (class A) pipettes
3.5. High Performance Liquid Chromatograph (HPLC) consisting of a conductivity detector, conductivity suppresser and data collection system. An eluent degassing unit is recommended.
3.6. Dionex IonPac CS12A cation exchange analytical column (250 mm X 4 mm) or equivalent.
3.7. Dionex IonPac CG12A cation exchange guard column or equivalent.

4. REAGENTS AND SUPPLIES

***Note**: All reagents shall be of analytical grade quality.*

4.1. Ammonium Sulphate ($(NH_4)_2SO_4$) > 99 % purity.
4.2. Sulphuric Acid (H_2SO_4) > 96 % purity.
4.3. Methanesulphonic Acid (MSA) > 99 % purity.
4.4. Reagent-grade water

***Note** : The water should have a resistivity greater than 18.0 MΩ.cm @ 25 °C.*

5. PREPARATION OF SOLUTIONS

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

- Carefully add 1.277 g of H_2SO_4 (4.2) to approx. 600 mL of water (4.4).
- Mix and dilute to 1 L with water (4.4).

5.2. MSA 20mM (Ion Chromatography Eluent)

- Carefully add 1.922 g of Methanesulphonic Acid (4.3) to approx. 600 mL of water (4.4).
- Mix and dilute to 1 L with water (4.4).

6. PREPARATION OF STANDARDS

6.1. Ammonium Stock solution :

- Accurately weigh 0.092 g of ammonium sulphate (4.1) 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 (5.1).
- Make up to volume with 0.025N H_2SO_4 (5.1).

This solution, stored below 4 °C, is stable for approx. 30 days.

***Note**: This corresponds approximately to a 100 mg/L NH_4^+ ion stock solution*

6.2. Working Standards :

- Accurately pipette volumes according to the table below into 100 ml volumetric flasks and make up to volume with 0.025 N H_2SO_4 (5.1).

Standard #	standard from which to pipette :	volume to pipette (ml)	Working standard concentration (mg/L)
1	stock solution	10	10
2	stock solution	5	5
3	# 1	20	2
4	# 2	10	0.5
5	# 4	20	0.1

These standard solutions, stored below 4 °C, are stable for approx. 30 days.

7. SAMPLE PREPARATION

7.1. Mill the tobacco sample to a mesh size < 1 mm. If the tobacco is too moist for grinding, it should be dried down at a temperature not exceeding 40 °C.
7.2. Determine the water content of the ground tobacco
7.3. Weigh 0.250 g ± 0.001 g of the ground tobacco into a 100 ml Erlenmeyer flask and add 50 mL of the extraction solution (5.1).
7.4. Place the Erlenmeyer on a wrist action shaker for 60 minutes.
7.5. Filter the extract through a Whatman paper filter (n° 40 or equivalent) into a clean 100 mL flask.
7.6. Take an aliquot and dilute (see note 1) with extraction solution (5.1)
7.7. Filter through a 0.45 µm syringe filter and proceed to analysis by cation exchange chromatography.

***Note 1**: Depending on the ammonia content of the tobacco, the extract may be more or less diluted in order to obtain a chromatographic response covered by the calibration curve. Usually, a dilution factor of 10 is sufficient.*

***Note**: The extracts should be analysed as soon as possible. Their storage should however not exceed 72 hours at below 4 °C.*

8. ANALYSES AND CALCULATIONS

- 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.

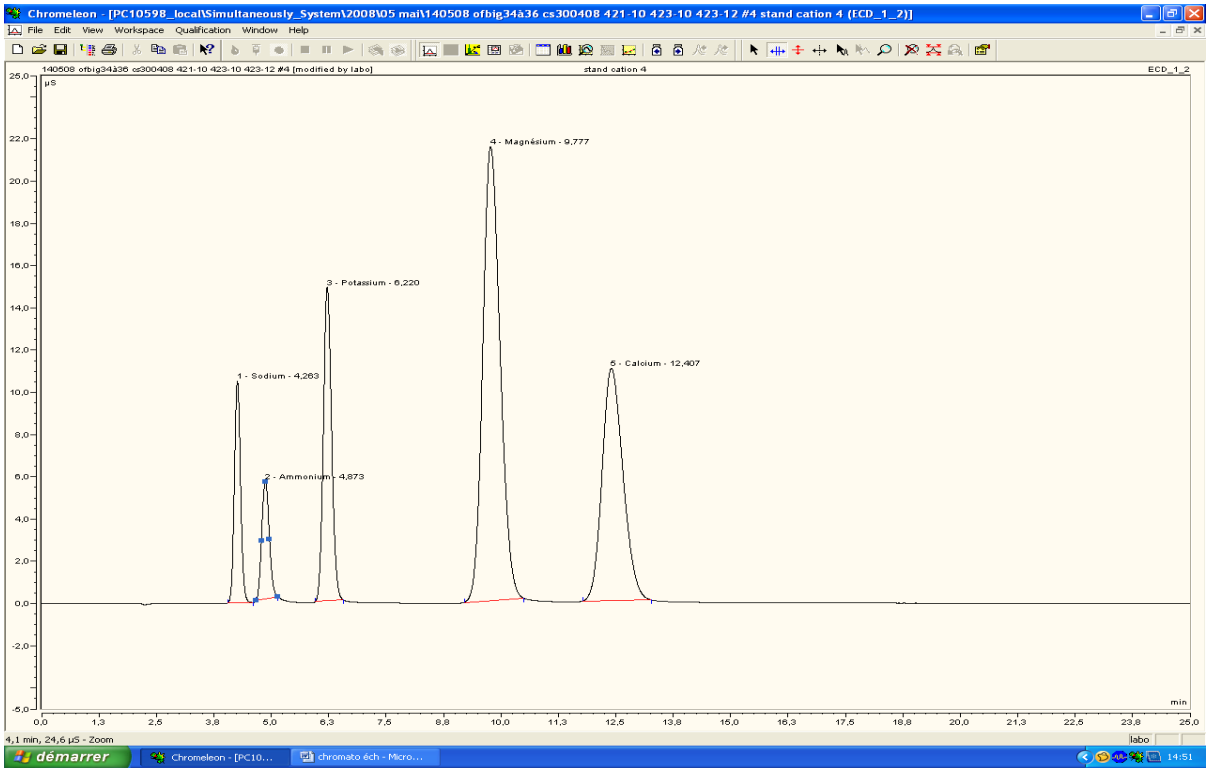
The amount of ammonia (in % of whole tobacco, not corrected for moisture content) is determined by the following calculation

$$\%_{NH_4^+} = \frac{c * v * 100}{m} * dilution\ \ factor$$

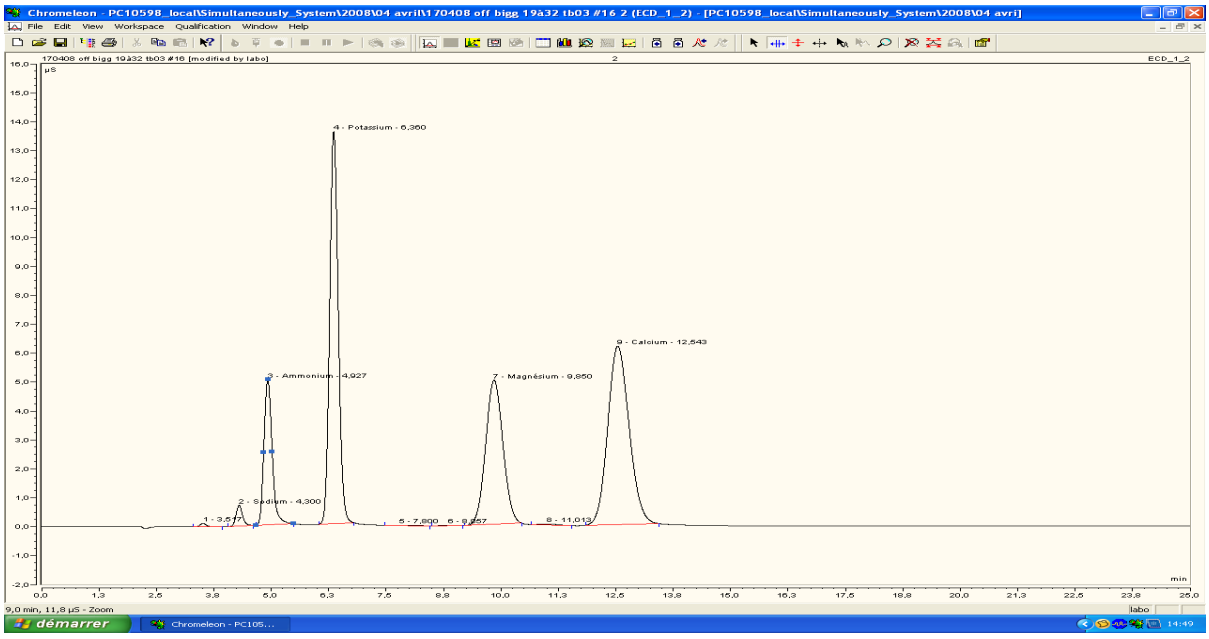
where :

c = NH_4^+ concentration (in mg/mL) obtained from the calibration curve
v = extraction volume (in mL)
m = mass of the sample (in mg)
dilution factor = factor as used in 7.6

9. Typical Chromatograms



Typical chromatogram of a standard



Typical chromatogram of a tobacco extract

APPENDIX E: Laboratory Summary

Summary table of Analytical Method used by the participating laboratories

Lab ID	Followed Protocol?	Sample Extraction	Extracting Solution	Technique	Manufacturer	Model	Reagent Deviations
02	YES	As protocol	0.025N Sulphuric Acid	IC	Dionex	ICS 1500	none
03	YES	As protocol	0.025N Sulphuric Acid	IC	Dionex	ICS 2000	none
04	YES	As protocol	0.025N Sulphuric Acid	IC	Dionex	ICS 3000	none
05	YES	As protocol	0.025N Sulphuric Acid	IC	Dionex	ICS 1000	none
06	YES	As protocol	0.025N Sulphuric Acid	IC	Dionex	ICS 3000	none
09	YES	As protocol	0.025N Sulphuric Acid	IC	Waters	Alliance conductivity detector 432	different eluent used: EDTA
10	YES	1 g, 40 ml, 45 minutes	0.025N Sulphuric Acid	IC	Dionex	ICS 3000	2.8mM MSA used as eluent
11	YES	As protocol	0.025N Sulphuric Acid	IC	Varian/ Dionex	not supplied	0.003 N MSA, Water, 0.2 N H_2SO_4
12	YES	As protocol	Sulphuric Acid	IC	Dionex	ICS 1500	none
13	YES	0.2 g, 40 ml, 60 minutes	0.025N Sulphuric Acid	IC	Dionex	ICS 2000	none
Protocol		0.25g, 50 ml, 60 minutes	0.025N Sulphuric acid	IC			