# Routine Analytical Chemistry Sub-Group

## Technical Report

## 2006 Collaborative Studies for Nicotine, Sugars and Nitrate in Tobacco.

## May 2008

**Co-ordinators:**
Linda Crumpler (Chairman), R.J. Reynolds Tobacco Co., Winston-Salem, North Carolina
Linda Drake (Secretary and Study Co-ordinator), British American Tobacco, Southampton, UK

**Statistics:**
Linda Drake

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## 1. BACKGROUND

The CORESTA Recommended Methods (CRMs) for the analysis of Nicotine, Nitrate, Reducing Substances and Reducing Carbohydrates in tobacco were last revised in 1994.

During 2005 the CORESTA Scientific Commission sanctioned the Routine Analytical Chemistry (RAC) Sub Group to carry out a collaborative study to investigate whether the R & r values quoted in the methods were still relevant.

The CRMs in question are listed below.

CRM 35
Determination of Total Alkaloids (as Nicotine) in Tobacco by Continuous Flow Analysis.

CRM36
Determination of Nitrate in Tobacco by Continuous Flow Analysis.

CRM37
Determination of Reducing Substances in Tobacco by Continuous Flow Analysis.

CRM38
Determination of Reducing Carbohydrates in Tobacco by Continuous Flow Analysis.

At the October 2005 meeting of the RAC Sub Group, British American Tobacco (BATUK) presented data on five samples that had previously been used for an internal BAT Company cross check. These samples contained a range of nicotine and sugars, had unknown nitrate levels, but were finely ground and already pouched. The Group agreed that these would be suitable to distribute for this study providing that more than one level of nitrate was represented. An initial BAT pre-study analysis on these samples showed that they contained the following nominal levels of the analytes in question.

| | Nicotine (%wwb) | Total Sugars (%wwb) | Nitrate (%wwb) |
|---|---|---|---|
| Sample A | 2.9 | 6 | 0.2 |
| Sample B | 2.6 | 16 | 0.1 |
| Sample C | 0.6 | 9 | 1.5 |
| Sample D | 3.0 | 2 | 1.4 |
| Sample E | 1.4 | <2 | 0.7 |
| Sample F * | 0.6 | 11 | 1.5 |

* Sample supplied by RJR to supplement higher nitrate levels.

23 laboratories agreed to participate in the study. The list of these participating laboratories is in APPENDIX A.

## 2. SUMMARY

In February 2006 six samples of pouched ground tobacco were despatched by BATUK to 23 laboratories. The protocol, designed by RJR, was also sent to the participating laboratories.

Three replicates were requested for each analyte and the results to be reported on an 'as received' basis with a deadline set as April 12th 2006.

22 sets of results were received by the coordinating company (BATUK) within the deadline. One laboratory was unable to take part due to heavy workload.

The raw data was presented at the April 2006 meeting of the CORESTA RAC Sub Group, alongside information on the methods used by the laboratories, as all laboratories were not able to strictly follow the protocol. This summary is to be found in APPENDIX B.

The numbers of sets of data received from laboratories that were able to follow the protocol* for each analyte were as follows:-

| | |
|---|---|
| Nicotine | 17 |
| Nitrate | 11 |
| Reducing Substances | 9 |
| Reducing Carbohydrates | 11 |

* Allowing for small deviations such as sample extraction concentration.

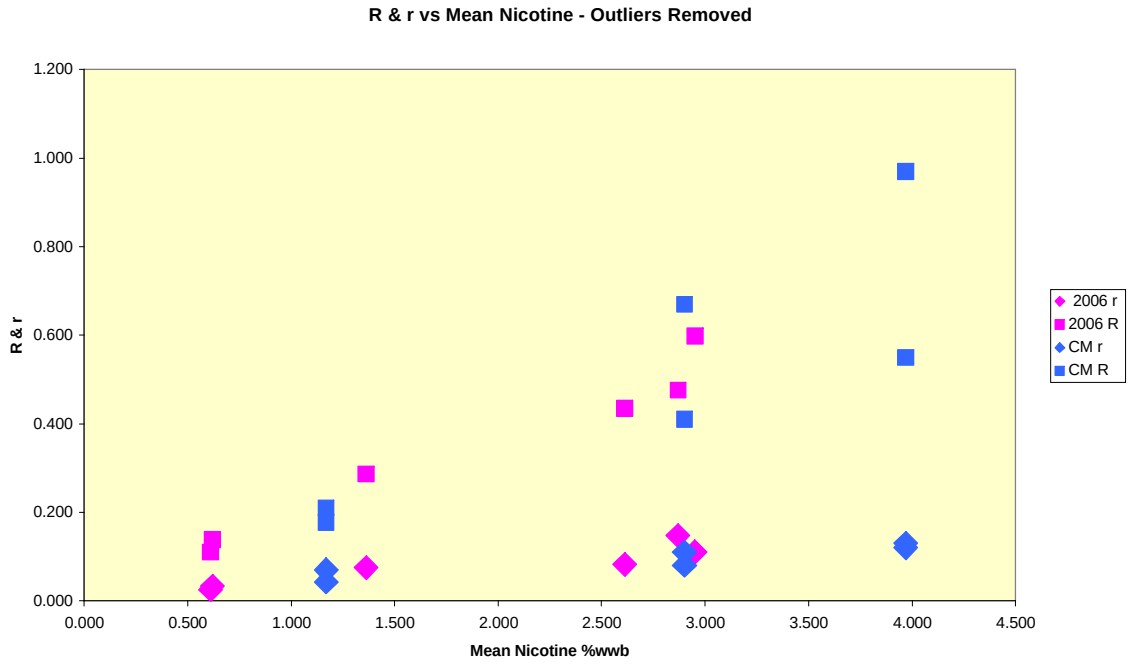
The integrity of some data needed to be confirmed with the originating laboratories as there were several apparent outliers.

In preparation for the October 2006 meeting of the Sub Group, after confirmation of the data integrity, Mandels *h* and *k* statistics were calculated on the data. This confirmed the presence of outliers which was shared at this meeting.

The final discussion on this study took place at the April 2007 Sub Group meeting, involved eliminating samples if the replicates were shown to be outliers according to either the Cochran's outlier test (for within laboratory variances) or the Grubb's test (for between laboratory variances).

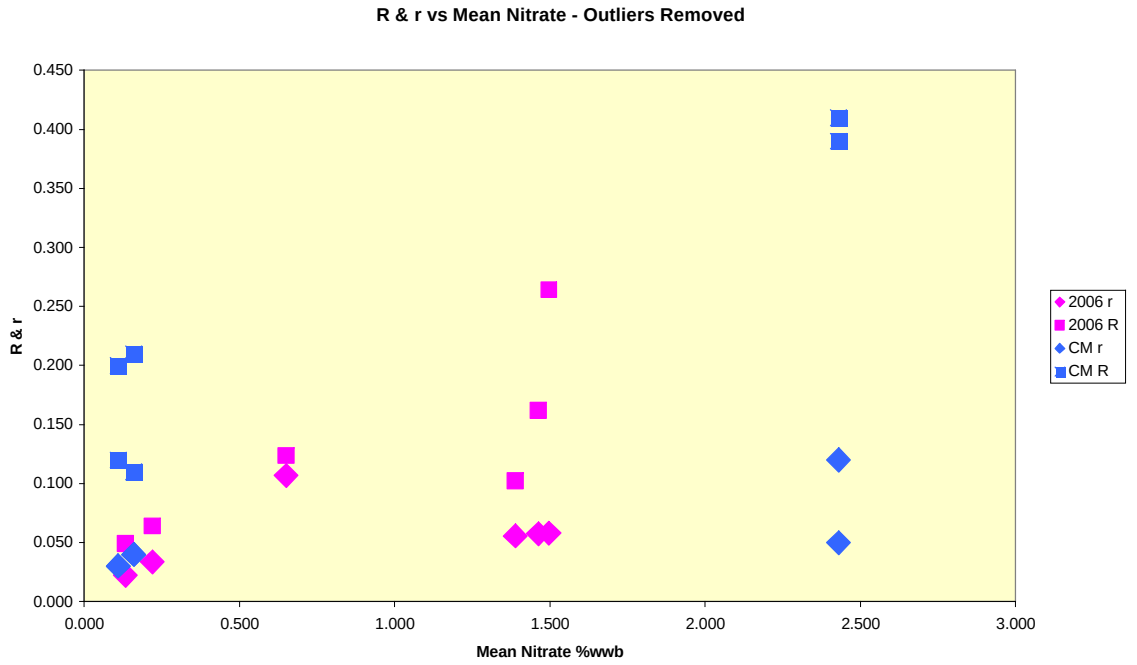
R & r values were calculated on the remaining data from laboratories following the CRMs after exclusion of outliers. The following graphs illustrate the comparison of R & r values found in the CRMs to those calculated from this 2006 study data. The tables of actual R & r values can be found in APPENDIX C.

## 2.1 NICOTINE



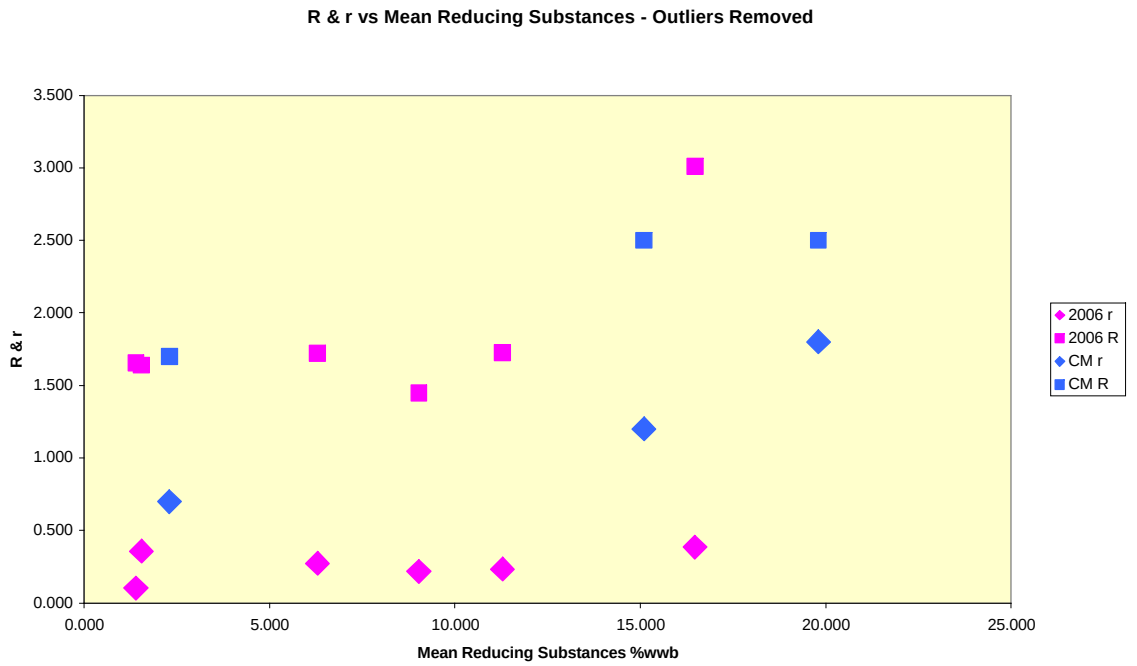

Recommendation is that the R & r values in CRM 35 be updated to include the lower values for the samples that were around 0.6% nicotine.

## 2.2 NITRATE



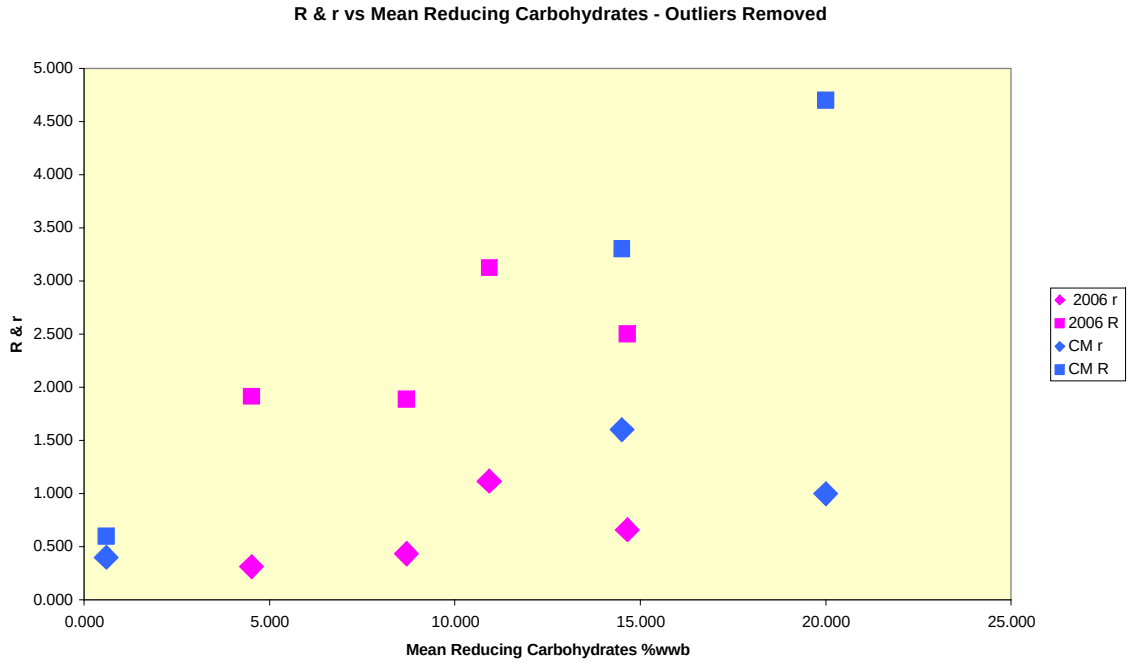

A recommendation is that the R & r values in CRM 36 be revised to replace the lower level values with those found in this study and also incorporate the other R & r values found.

## 2.3 REDUCING SUBSTANCES




Recommendation is to revise R & r table in CRM 37 to include the study values.

## *2.4 REDUCING CARBOHYDRATES*




The recommendation is to revise the R & r tables in CRM 38 to include the values found in this study.

## 3. COLLABORATIVE STUDY

### 3.1 PROTOCOL

The protocol is to be found in APPENDIX D.

Instructions were given to store the 6 sealed sample bags in an air conditioned room on receipt in order to ensure moderate ambient temperature (20-25 °C).

Laboratories were requested to carry out the analyses in triplicate by following the CRMs as closely as possible and to report any deviations from the CRMs in the methods employed by the laboratories as well as any additional useful observations. The data was reported on an 'as received' basis.

### 3.2 OVERVIEW OF DATA

Raw data from all participating laboratories, including outliers, is to be found in APPENDIX E.

#### 3.2.1 NICOTINE

22 laboratories submitted data with 17 following the CRM. Some of these 17 laboratories had listed minor deviations, e.g. different sample extraction concentration, but, for the purpose of this study, data from all 17 were included in the subsequent statistical analysis. Four laboratories used continuous flow technology and significantly different reagents to the study so their data was excluded. Finally, one laboratory used GC the data from which was also excluded from the R & r calculations.

After confirming the integrity of obvious outlying data with the respective laboratories, outlier testing was carried out according to ISO 5725 using firstly Cochran's test to eliminate within laboratory outliers and then Grubb's test to eliminate between laboratory outliers.

The following table summarises those laboratories having nicotine data that were excluded using these tests.

| Sample ID | Cochran's Outliers | Grubb's Outliers |
|---|---|---|
| A | NONE | NONE |
| B | 3 | NONE |
| C | 3<br>5 | NONE |
| D | NONE | NONE |
| E | NONE | NONE |
| F | 8<br>13 | 3 |

### 3.2.2 NITRATE

20 sets of data were received submitted data with 11 following the CRM. Some of these 11 laboratories had listed minor deviations, e.g. different sample extraction concentration, but, for the purpose of this study, data from all 11 were included in the subsequent statistical analysis. 3 laboratories used CFA with different reducing reagents to the CRM, 3 laboratories used CFA with different buffers and finally 3 laboratories used IC. The data from these 9 laboratories were excluded from the statistical analysis.

After confirming the integrity of obvious outlying data with the respective laboratories, outlier testing was carried out according to ISO 5725 using Cochran's test to eliminate within laboratory variance followed by Grubb's test to eliminate between laboratory outliers.

The following table summarises those laboratories that were excluded using these tests.

| Sample ID | Cochran's Outliers | Grubb's Outliers |
|---|---|---|
| A | 5 | NONE |
| B | NONE | 11 |
| C | NONE | NONE |
| D | 6 | NONE |
| E | NONE | NONE |
| F | NONE | NONE |

### 3.2.3 REDUCING SUBSTANCES

10 laboratories submitted data with 9 following the CRM. Some of these 7 laboratories had listed minor deviations, e.g. different sample extraction concentration, but, for the purpose of this study, data from all 9 were included in the subsequent statistical analysis. One laboratory used continuous flow technology with significantly different reagents to the study so their data was excluded.

After confirming the integrity of obvious outlying data with the respective laboratories, outlier testing was carried out according to ISO 5725 using Cochran's test to eliminate within laboratory variance followed by Grubb's test to eliminate between laboratory outliers.

The following table summarises those laboratories that were excluded using these tests.

| Sample ID | Cochran's Outliers | Grubb's Outliers |
|---|---|---|
| A | 18 | NONE |
| B | NONE | 11 |
| C | NONE | NONE |
| D | NONE | NONE |
| E | 18 | NONE |
| F | 18 | NONE |

### 3.2.4 REDUCING CARBOHYDRATES

14 sets of data were submitted with 11 following the CRM. Some of these 11had listed minor deviations, e.g. different sample extraction concentration, but, for the purpose of this study, data from all 11 were included in the subsequent statistical analysis. 3 laboratories used continuous flow technology but significantly different reagents to the study so their data was excluded and finally one laboratory used HPLC the data from which was also excluded.

After confirming the integrity of obvious outlying data with the respective laboratories, outlier testing was carried out according to ISO 5725 using Cochran's test to eliminate within laboratory variance followed by Grubb's test to eliminate between laboratory outliers.

The following table summarises those laboratories that were excluded using these tests.

| Sample ID | Cochran's Outliers | Grubb's Outliers |
|---|---|---|
| A | 1 | NONE |
| B | 3 | NONE |
| C | 3 | NONE |
| F | NONE | NONE |

**APPENDICES:**

## APPENDIX A

## List of Participating Laboratories

| |
|---|
| **BAT RPC, Bayreuth, GERMANY** |
| **China Tobacco Test Center, Henan, CHINA** |
| **BAT GR&D Southampton, U.K.** |
| **IMPERIAL Tobacco, Hamburg, GERMANY** |
| **BAT, Univ. Napoli, ITALY** |
| **BAT Mfg., Zevenaar, NETHERLANDS** |
| **TABAQEIRA S.A., PORTUGAL** |
| **LTR Industries, Usine Le Mans, FRANCE** |
| **ARISTA Laboratories U.S., Richmond, U.S.** |
| **HOUSE OF PRINCE, DENMARK** |
| **ALTADIS, Fleury-Les-Aubrais, FRANCE** |
| **ITC Ltd., Bangalore, INDIA** |
| **GALLAHER LTD, Ballymena, N. IRELAND** |
| **ROTHMANS B&H, Ontario, CANADA** |
| **ALTADIS, Madrid, SPAIN** |
| **CITA, Tabacos de Canarias, S.L.** |
| **Heintz van Landewyck,R&D, LUXEMBOURG** |
| **R.J. Reynolds Tobacco Company, Winston-Salem, U.S.** |
| **Philip Morris International, Neuchatel, SWITZERLAND** |
| **KT&G, Taejon, KOREA** |
| **LORILLARD Tobacco Company, Greensboro, U.S.** |
| **SWEDISH MATCH, Stockholm, SWEDEN** |

# APPENDIX B

## Tables Summarising the Analytical Methods used by the Participating Laboratories. (*Indicates insignificant deviations from protocol)

### B 1 NICOTINE

| Laboratory ID | Followed CRM | Sample Extraction | Extracting soln | Technique | Manufacturer | Model | Reagent deviations | Standard preparation comments | Wavelength | Other comments |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | YES | CRM35 | CRM35 | CFA | Skalar | San plus | none | | | |
| 2 | YES* | 0.5g in 100 mls | CRM35 | CFA | B&L | Traacs 800 | none | | | |
| 3 | YES | CRM35 | CRM35 | CFA/RAA | Konelab | | none | | | |
| 4 | YES | CRM35 | CRM35 | CFA | B&L | AA3 | none | | | |
| 5 | YES* | CRM35 | CRM35 | CFA | B&L | AA3 | none | Pure nicotine used | | |
| 6 | YES* | CRM35 | CRM35 | CFA | Burkard | SFA | none | Solvent 2% benzoic acid aq | | |
| 7 | YES | CRM35 | CRM35 | CFA | B&L | AA3 | none | | | |
| 8 | YES* | 0.5g in 25 mls | CRM35 | CFA | B&L | Traacs 2000 | none | | | |
| 9 | YES* | CRM35 | 0.01N Hydrochloric acid | CFA | Skalar | San plus | none | | | |
| 10 | YES* | CRM35 | CRM35 | CFA | B&L | AA3 | none | | 10mm path cell | 45:90 sple:wash ratio |
| 11 | YES | CRM35 | CRM35 | CFA | B&L | AA3 | none | | | |
| 12 | YES | CRM35 | CRM35 | CFA/RAA | Skalar | 4000 | none | | | |
| 13 | YES | CRM35 | CRM35 | CFA | B&L | AA3 | none | | | |
| 14 | YES | CRM35 | CRM35 | CFA | Skalar | | none | | | |
| 15 | YES* | CRM35 | 1%acetic acid aq | CFA/RAA | Astoria-Pacific | 300 | none | | | |
| 16 | YES | CRM35 | CRM35 | CFA | Skalar | | none | | | |
| 17 | NO | CRM35 | CRM35 | | Systea Analytical Technologies | Flowsys 3rd generation | Aniline instead of sulphanilic acid.Potassium thiocyante and sodium hypochlorite for cyanogen chloride generation.Buffers different | | | |
| 18 | YES | CRM35 | CRM35 | CFA | B&L | AAII | Antidote B 20g sodium hydroxide/L | | | |
| 19 | NO | CRM35 | CRM35 | CFA | Alliance | Version 3.3(2004) | Buffer A 8.8g sodium hydroxide+26g sodium dihydrogen phosphate+10.4g Citric acid | Nicotine salicylate used | 480nm | |
| 20 | NO | CRM35 but 15 min shake | CRM35 | CFA/RAA | Astoria-Pacific | 300 | Buffer A 8.8g sodium hydroxide+26g sodium dihydrogen phosphate+10.4g Citric acid.Buffer B 10g sulphanilic acid+50g ammonium acetate in 1litre | Chloramine T 12g/L | | |
| 21 | NO | CRM35 | CRM35 | CFA | | | Potassium thiocyanate and sodium hypochlorite for generation of cyanogen chloride. | | | |
| 22 | NO | GC | GC | GC | | | GC | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| CRM 35 | | 0.25g in 25 mls 30 min shake | 5% acetic acid aq or water | CFA | | | | Nicotine Hydrogen Tartrate. Chloramine T 17.3g/L | 460nm 15mm pathlength | 35:30 sple:wash |

## B.2 NITRATE

| Laboratory ID | Followed CRM | Sample Extraction | Extracting soln | Technique | Manufacturer | Model | Reagent deviations | Standard preparation comments | Wavelength | Other comments |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | YES* | CRM36 | 2% acetic acid | CFA | Skalar | San plus | | | | |
| 2 | NO | | | HPLC- ion chrom with conductivity detector | | IC | | | | |
| 3 | YES | CRM36 | CRM36 | CFA/RAA | Konelab | | | | | |
| 4 | YES | CRM36 | CRM36 | CFA | B&L | AA3 | | | | |
| 5 | YES | CRM36 | CRM36 | CFA | B&L | AA3 | | | | |
| 6 | YES* | CRM36 | CRM36 | CFA | Burkard | SFA | | | 550nm | |
| 7 | YES | CRM36 | CRM36 | CFA | B&L | AA3 | | | | |
| 9 | NO | | 0.01N hydrochloric acid | HPLC- ion chrom with conductivity detector | | | | | | |
| 10 | YES* | CRM36 | CRM36 | CFA | B&L | AA3 | | | 10mm | 45:90 sple:wash |
| 11 | YES | CRM36 | CRM36 | CFA | B&L | AA3 | | | | |
| 12 | NO | CRM36 | CRM36 | CFA/RAA | Skalar | 4000 | Cadmium reduction column used. Ammonium chloride pH adjusted to 8.2 | | | |
| 13 | YES | CRM36 | CRM36 | CFA | B&L | AA3 | | | | |
| 14 | YES | CRM36 | CRM36 | CFA | Skalar | | | | | |
| 15 | NO | CRM36 | CRM36 | CFA/RAA | Astoria-Pacific | 300 | Cadmium reduction column used. | | | |
| 16 | YES | CRM36 | CRM36 | CFA | Skalar | | | | | |
| 17 | NO | CRM36 | CRM36 | CFA | Systea Analytical Technologies | Flowsys 3rd generation | 5g sodium hydroxide, 0.33g/L hydrated cupric sulphate,hydrated copper reagent also different | Chloroform added in stds | | |
| 19a | NO | CRM36 | CRM36 | CFA | Alliance | Version 3.3(2004) | 0.1N sodium hydroxide | | | |
| 19b | NO | | | HPLC- ion chrom with conductivity detector | Dionex | ICS 1000 | | | | |
| 20 | NO | CRM36 but 15 min shake | CRM36 | CFA/RAA | Astoria-Pacific | 300 | Reagents different | | | |
| 22 | NO | CRM36 | CRM36 | Flow Soln IV | | | Cadmium reduction column used. Reagents different | | 540nm | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| CRM 36 | | 0.25 in 25mls | Water or 5% acetic acid 30 min shake | CFA | | | 8g sodium hydroxide, 12g/L hydrated cupric sulphate | | 520nm 15mm flowcell | 35:30 sple:wash |

## B.3 REDUCING SUBSTANCES

| Laboratory ID | Followed CRM | Sample Extraction | Extracting soln | Technique | Manufacturer | Model | Reagent deviations | Standard preparation comments | Wavelength | Other comments |
|---|---|---|---|---|---|---|---|---|---|---|
| 4 | YES | CRM37 | water | CFA | B&L | AA3 | | | | |
| 5 | YES | CRM37 | water | CFA | B&L | AA3 | | | | |
| 7 | YES* | CRM37 | CRM37 | CFA | B&L | AA3 | Heating bath 85°C | | | |
| 10 | YES* | CRM37 | CRM37 | CFA | B&L | AA3 | 5.4ml coil | | 10mm | 45:90 sple:wash |
| 11 | YES | CRM37 | CRM37 | CFA | B&L | AA3 | | | | |
| 12 | NO | Different method | | CFA/RAA | Skalar | 4000 | Inverted by hydrochloric acid 97°C, dialysed against sodium carbonate solution. Neocuproin chelate | | 460nm | |
| 13 | YES | CRM37 | water | CFA | B&L | AA3 | | | | |
| 18 | YES* | CRM37 | CRM37 | CFA | B&L | AAII | Potassium ferricyanide 0.2g/L | | | |
| 19 | YES | CRM37 | water | CFA | Alliance | Version 3.3(2004) | | | | |
| 21 | YES* | CRM37 | CRM37 | CFA | | | 0.9% sodium chloride in a 1.4% sodium hydroxide soln | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| CRM 37 | | 0.25g in 25 mls. 30 min shake | 5% acetic acid aq | CFA | | | heating bath 90°C, 4.1ml coil, 0.15g/L potassium ferricyan,0.9%sodium chloride | | 15mm pathlength cell 420nm wavelength | 35:30 sple:wash |

## B.4 REDUCING CARBOHYDRATES

| Laboratory ID | Followed CRM | Sample Extraction | Extracting soln | Technique | Manufacturer | Model | Reagent deviations | Standard preparation comments | Wavelength | Other comments |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | YES* | CRM38 | 2% acetic acid aq | CFA | Skalar | San plus | | | | |
| 2 | YES* | 0.5g in 100 mls | ? | CFA | B&L | Traacs 800 | | | | |
| 3 | YES* | CRM38 | CRM38 | CFA/RAA | Konelab | | | 50/50 fruct/gluc used for stds | | |
| 6 | NO | CRM38 | water | CFA | Burkard | SFA | invertase/neocuproine | | | |
| 7 | YES | CRM38 | CRM38 | CFA | B&L | AA3 | | | | |
| 8a | YES* | 0.5g in 25mls | CRM38 | CFA | B&L | Traacs 2000 | | | | |
| 8b | YES* | 0.5g in 25mls | CRM38 | CFA | B&L | Traacs 2000 | | 50/50 fruct/gluc used for stds | | |
| 10 | YES* | CRM38 | CRM38 | CFA | B&L | AA3 | coil Al jacket - no heating | | 10mm | 45:90 sple:wash |
| 11 | YES | CRM38 | CRM38 | CFA | B&L | AA3 | | | | |
| 14 | YES* | CRM38 | CRM38 | CFA | Skalar | | | 50/50 fruct/gluc used for stds | | |
| 15 | YES* | CRM38 | 1%acetic acid | CFA/RAA | Astoria-Pacific | 300 | | 50/50 fruct/gluc used for stds | | |
| 16 | YES* | CRM38 | water | CFA | Skalar | | | 50/50 fruct/gluc used for stds | | |
| 17 | NO | CRM38 | water | CFA? | Systea Analytical Technologies | Flowsys 3rd generation | neocuproine | | | |
| 20 | NO | CRM38 15 min shake | CRM38 | CFA/RAA | Astoria-Pacific | 300 | 1N sodium hydroxide, different PAHBAH, 0.002M calcium chloride | | | |
| 22 | NO | CM 61 | | HPLC | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| CRM 38 | | 0.25g in 25 mls 30 min shake | 5% acetic acid aq | CFA | | | | Glucose | 410nm 15mm flowcell | 35:30 sple:wash |

# APPENDIX C

## Comparison of R &r Values Found in this Study Against Those in the Published CORESTA Recommended Methods

**NICOTINE**

| SAMPLE | NO. LABORATORIES | AVERAGE %WWB | REPEATABILITY r | REPRODUCIBILITY R |
|---|---|---|---|---|
| A | 17 | 2.87 | 0.15 | 0.48 |
| B | 16 | 2.61 | 0.08 | 0.43 |
| C | 15 | 0.62 | 0.03 | 0.14 |
| D | 17 | 2.95 | 0.11 | 0.60 |
| E | 17 | 1.36 | 0.07 | 0.29 |
| F | 14 | 0.61 | 0.03 | 0.11 |
| CRM A* | 12 | 1.17 | 0.04 | 0.18 |
| CRM B* | 12 | 2.90 | 0.08 | 0.41 |
| CRM C* | 12 | 3.97 | 0.12 | 0.55 |
| CRM A** | 12 | 1.17 | 0.07 | 0.21 |
| CRM B** | 12 | 2.90 | 0.11 | 0.67 |
| CRM C** | 12 | 3.97 | 0.13 | 0.97 |
| | | | | |

KEY

* Water extraction

** 5% Acetic Acid Extraction

**NITRATE**

| SAMPLE | NO. LABORATORIES | AVERAGE %WWB | REPEATABILITY r | REPRODUCIBILITY R |
|---|---|---|---|---|
| A | 10 | 0.22 | 0.03 | 0.06 |
| B | 10 | 0.13 | 0.02 | 0.05 |
| C | 11 | 1.50 | 0.06 | 0.26 |
| D | 10 | 1.39 | 0.06 | 0.10 |
| E | 11 | 0.65 | 0.11 | 0.12 |
| F | 11 | 1.46 | 0.06 | 0.16 |
| CRM A* | 12 | 0.11 | 0.03 | 0.12 |
| CRM B* | 12 | 0.16 | 0.04 | 0.11 |
| CRM C* | 12 | 2.43 | 0.12 | 0.41 |
| CRM A** | 12 | 0.11 | 0.03 | 0.20 |
| CRM B** | 12 | 0.16 | 0.04 | 0.21 |
| CRM C** | 12 | 2.43 | 0.05 | 0.39 |
| | | | | |

KEY

* Water extraction

** 5% Acetic Acid Extraction

**REDUCING SUBSTANCES**

| SAMPLE | NO. LABORATORIES | AVERAGE %WWB | REPEATABILITY r | REPRODUCIBILITY R |
|---|---|---|---|---|
| A | 8 | 6.3 | 0.3 | 1.7 |
| B | 9 | 16.5 | 0.4 | 3.0 |
| C | 7 | 9.0 | 0.2 | 1.4 |
| D | 8 | 1.5 | 0.4 | 1.6 |
| E | 7 | 1.4 | 0.1 | 1.7 |
| F | 8 | 11.3 | 0.2 | 1.7 |
| CRM A | 12 | 2.3 | 0.7 | 1.7 |
| CRM B | 12 | 15.1 | 1.2 | 2.5 |
| CRM C | 12 | 19.8 | 1.8 | 2.5 |
| | | | | |

**REDUCING CARBOHYDRATES**

| SAMPLE | NO. LABORATORIES | AVERAGE %WWB | REPEATABILITY r | REPRODUCIBILITY R |
|---|---|---|---|---|
| A | 10 | 4.5 | 0.3 | 1.9 |
| B | 9 | 14.7 | 0.7 | 2.5 |
| C | 10 | 8.7 | 0.4 | 1.9 |
| F | 11 | 10.9 | 1.1 | 3.1 |
| CRM A | 13 | 0.6 | 0.4 | 0.6 |
| CRM B | 13 | 14.5 | 1.6 | 3.3 |
| CRM C | 13 | 20.0 | 1 | 4.7 |
| | | | | |

# APPENDIX D

## CORESTA SUB-GROUP ROUTINE ANALYTICAL CHEMISTRY

## JOINT EXPERIMENT 2006

## TEST PROTOCOL

### 1. Analytical Methods:

a. CORESTA Recommended Method 35, Determination of Total Alkaloids (As Nicotine) In Tobacco By Continuous Flow Analysis
b. CORESTA Recommended Method 36, Determination of Nitrate In Tobacco By Continuous Flow Analysis
c. CORESTA Recommended Method 37, Determination of Reducing Substances In Tobacco By Continuous Flow Analysis, or
d. CORESTA Recommended Method 38, Determination of Reducing Carbohydrates In Tobacco By Continuous Flow Analysis

Report any deviations from the methods (a.-d.) listed above, as well as any observations you deem useful to share on attached Data Sheet 1.

If a methodology other than those specified for Total Alkaloids, Nitrate, Reducing Substances, or Reducing Carbohydrates is used, please document deviations carefully on the attached Data Sheet 1.

### 2. SAMPLES

Upon receipt of the six samples store the sealed bags in an air conditioned room in order to ensure moderate ambient temperature (20-25 °C). Samples consist of various weights of finely ground and homogenized tobacco.

### 3. TESTS

Samples should be analyzed **"as received"** and reported on Data Sheet 2 with **no Oven Volatiles Correction**. Perform each extraction in **triplicate**.

Although results will not be moisture corrected, each sample **should be tested for Oven Volatiles twice (if sufficient sample)** and results included on the attached Data Sheet 2. This information may be useful in resolving questions of outliers.

Note: Please retain samples (well sealed and stored at moderate ambient temperature) until results for the Joint Experiment are finalized.

### 4. REPORTING

Report results to Linda Drake by **April 12$^{th}$ 2006** at the latest for inclusion in the data analysis to be presented at the April 25th meeting of the Routine Analytical Chemistry Sub-Group. Data received later than **April 12$^{th}$ 2006** will be included in a later report.

Results should be sent electronically on the attached data sheets to e-mail address Linda_Drake@bat.com

# APPENDIX E

## Raw Data

### E 1 NICOTINE

| Lab No. | Method used | SAMPLE A | | | SAMPLE B | | | SAMPLE C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 1 | CRM | 2.85 | 2.85 | 2.85 | 2.56 | 2.53 | 2.54 | 0.62 | 0.61 | 0.60 |
| 2 | CRM* | 2.98 | 3.00 | 2.96 | 2.72 | 2.72 | 2.76 | 0.67 | 0.63 | 0.65 |
| 3 | CRM | 3.15 | 3.05 | 3.30 | 2.92* | 2.17* | 2.43* | 1.05* | 0.88* | 0.89* |
| 4 | CRM | 2.73 | 2.74 | 2.75 | 2.48 | 2.50 | 2.47 | 0.60 | 0.60 | 0.60 |
| 5 | CRM* | 3.06 | 3.05 | 3.10 | 2.91 | 2.90 | 2.79 | 0.64* | 0.6* | 0.55* |
| 6 | CRM* | 2.66 | 2.88 | 2.65 | 2.41 | 2.40 | 2.44 | 0.58 | 0.58 | 0.58 |
| 7 | CRM | 2.49 | 2.52 | 2.52 | 2.28 | 2.28 | 2.30 | 0.53 | 0.57 | 0.58 |
| 8 | CRM* | 3.06 | 3.07 | 3.05 | 2.75 | 2.83 | 2.81 | 0.69 | 0.66 | 0.66 |
| 9 | CRM* | 2.89 | 2.90 | 2.90 | 2.65 | 2.66 | 2.67 | 0.61 | 0.61 | 0.60 |
| 10 | CRM* | 2.62 | 2.64 | 2.65 | 2.43 | 2.42 | 2.43 | 0.52 | 0.51 | 0.53 |
| 11 | CRM | 3.01 | 3.04 | 2.98 | 2.76 | 2.76 | 2.76 | 0.71 | 0.71 | 0.71 |
| 12 | CRM | 3.16 | 3.12 | 3.13 | 2.86 | 2.83 | 2.79 | 0.62 | 0.62 | 0.61 |
| 13 | CRM | 2.86 | 3.02 | 2.94 | 2.84 | 2.78 | IS | 0.65 | 0.67 | 0.64 |
| 14 | CRM | 2.74 | 2.77 | 2.77 | 2.56 | 2.56 | 2.61 | 0.62 | 0.62 | 0.63 |
| 15 | CRM* | 2.99 | 2.98 | 2.92 | 2.71 | 2.71 | 2.74 | 0.76 | 0.75 | 0.74 |
| 16 | CRM | 2.82 | 2.82 | 2.81 | 2.56 | 2.59 | 2.56 | 0.60 | 0.60 | 0.60 |
| 18 | CRM | 2.56 | 2.51 | 2.45 | 2.35 | 2.31 | 2.27 | 0.57 | 0.56 | 0.55 |
| 17 | CFA | 3.08 | 2.91 | 3.01 | 2.84 | 2.78 | 2.84 | 0.71 | 0.68 | 0.71 |
| 19 | CFA | 2.89 | 2.96 | 2.83 | 2.63 | 2.64 | 2.62 | 0.61 | 0.63 | 0.67 |
| 20 | CFA | 2.75 | 2.73 | 2.79 | 2.52 | 2.54 | 2.58 | 0.62 | 0.60 | 0.61 |
| 21 | CFA | 2.77 | 2.77 | 2.77 | 2.45 | 2.48 | 2.48 | 0.59 | 0.60 | 0.60 |
| 22 | GC | 3.12 | 3.14 | 3.15 | 2.80 | 2.81 | 2.85 | 0.67 | 0.67 | 0.66 |

| Lab No. | Method used | SAMPLE D | | | SAMPLE E | | | SAMPLE F | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 1 | CRM | 3.00 | 2.96 | 2.97 | 1.38 | 1.39 | 1.37 | 0.60 | 0.60 | 0.59 |
| 2 | CRM* | 2.89 | 2.91 | 2.94 | 1.39 | 1.42 | 1.41 | 0.61 | 0.63 | 0.62 |
| 3 | CRM | 3.57 | 3.42 | 3.52 | 1.69 | 1.69 | 1.64 | 0.88** | 0.87** | 0.86** |
| 4 | CRM | 2.94 | 3.01 | 2.97 | 1.29 | 1.36 | 1.32 | 0.58 | 0.57 | 0.57 |
| 5 | CRM* | 3.04 | 3.01 | 3.07 | 1.43 | 1.35 | 1.33 | 0.63 | 0.61 | 0.62 |
| 6 | CRM* | 2.81 | 2.77 | 2.77 | 1.28 | 1.28 | 1.25 | 0.59 | 0.57 | 0.58 |
| 7 | CRM | 2.53 | 2.55 | 2.57 | 1.21 | 1.22 | 1.17 | 0.56 | 0.56 | 0.56 |
| 8 | CRM* | 3.28 | 3.32 | 3.32 | 1.48 | 1.46 | 1.43 | 0.63* | 0.67* | 0.61* |
| 9 | CRM* | 3.13 | 3.12 | 3.08 | 1.41 | 1.37 | 1.40 | 0.63 | 0.62 | 0.63 |
| 10 | CRM* | 2.65 | 2.62 | 2.65 | 1.22 | 1.22 | 1.18 | 0.56 | 0.57 | 0.58 |
| 11 | CRM | 3.08 | 3.12 | 3.09 | 1.46 | 1.45 | 1.47 | 0.68 | 0.68 | 0.68 |
| 12 | CRM | 3.00 | 3.02 | 2.99 | 1.42 | 1.33 | 1.40 | 0.62 | 0.66 | 0.64 |
| 13 | CRM | 3.04 | 2.93 | 3.07 | 1.34 | 1.38 | 1.39 | 0.69* | 0.63* | IS |
| 14 | CRM | 2.90 | 2.86 | 2.92 | 1.33 | 1.31 | 1.34 | 0.63 | 0.63 | 0.64 |
| 15 | CRM* | 3.04 | 3.01 | 2.98 | 1.51 | 1.48 | 1.47 | 0.72 | 0.71 | 0.73 |
| 16 | CRM | 2.90 | 2.94 | 2.99 | 1.30 | 1.31 | 1.28 | 0.59 | 0.59 | 0.59 |
| 18 | CRM | 2.50 | 2.37 | 2.39 | 1.17 | 1.18 | 1.14 | 0.56 | 0.56 | 0.54 |
| 17 | CFA | 3.14 | 3.09 | 3.01 | 1.37 | 1.39 | 1.35 | 0.70 | 0.73 | 0.72 |
| 19 | CFA | 2.96 | 3.07 | 3.16 | 1.33 | 1.45 | 1.44 | 0.71 | 0.69 | 0.69 |
| 20 | CFA | 2.84 | 2.84 | 2.86 | 1.30 | 1.30 | 1.32 | 0.65 | 0.63 | 0.64 |
| 21 | CFA | 2.89 | 2.89 | 2.89 | 1.27 | 1.27 | 1.29 | 0.57 | 0.58 | 0.59 |
| 22 | GC | 3.52 | 3.55 | 3.56 | 1.57 | 1.53 | 1.55 | 0.64 | 0.65 | 0.63 |

| KEY | |
|---|---|
| * | Cochran's Failure |
| ** | Grubb's Failure |
| | |
| CRM | No.35 |
| CRM* | No.35 with minor deviations |
| CFA | Same technique as CRM with major deviations |
| GC | Gas Chromatography |

## E.2 NITRATE

| Lab No. | Method used | SAMPLE A | | | SAMPLE B | | | SAMPLE C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 1 | CRM* | 0.21 | 0.22 | 0.20 | 0.12 | 0.12 | 0.14 | 1.47 | 1.48 | 1.50 |
| 3 | CRM | 0.20 | 0.24 | 0.24 | 0.14 | 0.15 | 0.14 | 1.47 | 1.53 | 1.53 |
| 4 | CRM | 0.20 | 0.21 | 0.19 | 0.11 | 0.10 | 0.12 | 1.44 | 1.42 | 1.43 |
| 5 | CRM | 0.28* | 0.19* | 0.21* | 0.13 | 0.12 | 0.13 | 1.49 | 1.56 | 1.51 |
| 6 | CRM* | 0.18 | 0.18 | 0.18 | 0.13 | 0.13 | 0.13 | 1.46 | 1.46 | 1.46 |
| 7 | CRM | 0.25 | 0.23 | 0.21 | 0.14 | 0.14 | 0.14 | 1.68 | 1.66 | 1.61 |
| 10 | CRM* | 0.24 | 0.24 | 0.22 | 0.12 | 0.12 | 0.11 | 1.51 | 1.51 | 1.52 |
| 11 | CRM | 0.26 | 0.26 | 0.27 | 0.24** | 0.24** | 0.24** | 1.53 | 1.52 | 1.53 |
| 13 | CRM | 0.19 | 0.19 | 0.19 | 0.12 | 0.11 | IS | 1.22 | 1.19 | 1.22 |
| 14 | CRM | 0.25 | 0.25 | 0.23 | 0.18 | 0.16 | 0.19 | 1.59 | 1.60 | 1.61 |
| 16 | CRM | 0.23 | 0.22 | 0.22 | 0.15 | 0.15 | 0.15 | 1.56 | 1.56 | 1.58 |
| 12 | CFA | 0.24 | 0.21 | 0.21 | 0.13 | 0.12 | 0.11 | 1.55 | 1.60 | 1.64 |
| 15 | CFA | 0.24 | 0.22 | 0.19 | 0.15 | 0.15 | 0.12 | 1.70 | 1.63 | 1.69 |
| 17 | CFA | 0.29 | 0.25 | 0.24 | 0.14 | 0.14 | 0.14 | 1.45 | 1.47 | 1.51 |
| 19 | CFA | 0.20 | 0.19 | 0.17 | 0.08 | 0.08 | 0.07 | 1.38 | 1.46 | 1.38 |
| 19 | IC | 0.19 | 0.20 | 0.20 | 0.12 | 0.13 | 0.13 | 1.54 | 1.55 | 1.59 |
| 2 | IC | 0.18 | 0.20 | 0.19 | 0.15 | 0.13 | 0.14 | 1.55 | 1.54 | 1.53 |
| 20 | CFA | 0.26 | 0.21 | 0.22 | 0.13 | 0.12 | 0.13 | 1.54 | 1.51 | 1.54 |
| 22 | CFA | 0.23 | 0.20 | 0.21 | 0.14 | 0.13 | 0.13 | 1.59 | 1.56 | 1.60 |
| 9 | IC | 0.23 | 0.22 | 0.25 | 0.12 | 0.12 | 0.12 | 1.38 | 1.40 | 1.48 |
| Lab No. | Method used | SAMPLE D | | | SAMPLE E | | | SAMPLE F | | |
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 1 | CRM* | 1.37 | 1.41 | 1.41 | 0.68 | 0.62 | 0.63 | 1.44 | 1.45 | 1.44 |
| 3 | CRM | 1.39 | 1.35 | 1.40 | 0.66 | 0.66 | 0.62 | 1.46 | 1.50 | 1.43 |
| 4 | CRM | 1.38 | 1.39 | 1.39 | 0.62 | 0.76 | 0.66 | 1.51 | 1.49 | 1.49 |
| 5 | CRM | 1.41 | 1.35 | 1.35 | 0.75 | 0.66 | 0.61 | 1.46 | 1.44 | 1.47 |
| 6 | CRM* | 1.33* | 1.37* | 1.46* | 0.71 | 0.62 | 0.62 | 1.51 | 1.46 | 1.46 |
| 7 | CRM | 1.43 | 1.42 | 1.45 | 0.67 | 0.67 | 0.69 | 1.52 | 1.51 | 1.48 |
| 10 | CRM* | 1.38 | 1.36 | 1.33 | 0.64 | 0.62 | 0.60 | 1.45 | 1.45 | 1.45 |
| 11 | CRM | 1.38 | 1.38 | 1.39 | 0.62 | 0.65 | 0.63 | 1.44 | 1.43 | 1.46 |
| 13 | CRM | 1.32 | 1.29 | 1.33 | 0.55 | 0.57 | 0.56 | 1.32 | 1.27 | IS |
| 14 | CRM | 1.46 | 1.46 | 1.46 | 0.68 | 0.69 | 0.68 | 1.58 | 1.58 | 1.57 |
| 16 | CRM | 1.41 | 1.39 | 1.41 | 0.70 | 0.72 | 0.67 | 1.51 | 1.49 | 1.48 |
| 12 | CFA | 1.43 | 1.44 | 1.41 | 0.68 | 0.64 | 0.64 | 1.48 | 1.48 | 1.51 |
| 15 | CFA | 1.52 | 1.47 | 1.43 | 0.65 | 0.74 | 0.67 | 1.53 | 1.56 | 1.64 |
| 17 | CFA | 1.45 | 1.32 | 1.31 | 0.70 | 0.74 | 0.75 | 1.49 | 1.45 | 1.53 |
| 19 | CFA | 1.42 | 1.32 | 1.40 | 0.59 | 0.67 | 0.65 | 1.42 | 1.44 | 1.44 |
| 19 | IC | 1.37 | 1.46 | 1.39 | 0.63 | 0.65 | 0.65 | 1.50 | 1.50 | 1.56 |
| 2 | IC | 1.47 | 1.49 | 1.49 | 0.68 | 0.66 | 0.65 | 1.49 | 1.47 | 1.46 |
| 20 | CFA | 1.39 | 1.33 | 1.33 | 0.63 | 0.59 | 0.63 | 1.48 | 1.45 | 1.46 |
| 22 | CFA | 1.39 | 1.40 | 1.41 | 0.63 | 0.67 | 0.67 | 1.55 | 1.54 | 1.58 |
| 9 | IC | 1.35 | 1.31 | 1.33 | 0.62 | 0.62 | 0.63 | 1.38 | 1.33 | 1.35 |

| KEY | |
|---|---|
| * | Cochran's Failure |
| ** | Grubb's Failure |
| | |
| CRM | No.36 |
| CRM* | No.36 with minor deviations |
| CFA | Same technique as CRM with major deviations |
| IC | Ion Chromatography |

**E.3 REDUCING SUBSTANCES**

| Lab No. | Method used | SAMPLE A | | | SAMPLE B | | | SAMPLE C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 4 | CRM | 6.30 | 6.30 | 6.10 | 15.50 | 15.90 | 15.70 | 9.20 | 9.10 | 8.90 |
| 5 | CRM | 6.38 | 6.45 | 6.45 | 16.23 | 15.88 | 16.17 | 9.44 | 9.37 | 9.30 |
| 7 | CRM* | 5.57 | 5.45 | 5.45 | 15.10 | 15.06 | 15.04 | 8.34 | 8.45 | 8.47 |
| 10 | CRM* | 5.80 | 5.80 | 5.50 | 15.80 | 15.80 | 15.80 | 8.80 | 8.90 | 8.90 |
| 11 | CRM | 7.00 | 7.20 | 6.90 | 19.4** | 19.4** | 19.6** | 10.40 | 10.10 | 9.10 |
| 13 | CRM | 5.73 | 5.80 | 5.80 | 16.66 | 16.76 | IS | 9.16 | 9.06 | 9.02 |
| 18 | CRM* | 6.93* | 7.15* | 6.5* | 17.01 | 17.21 | 16.71 | 9.78 | 10.18 | 9.57 |
| 19 | CRM | 7.69 | 7.73 | 7.67 | 17.00 | 16.99 | 16.99 | 10.17 | 10.11 | 10.15 |
| 21 | CRM* | 5.96 | 6.00 | 5.96 | 15.34 | 15.52 | 15.56 | 8.26 | 8.24 | 8.24 |
| 12 | CFA | 6.10 | 5.90 | 6.00 | 15.80 | 17.90 | 16.10 | 9.60 | 9.10 | 9.40 |
| | | | | | | | | | | |
| Lab No. | Method used | SAMPLE D | | | SAMPLE E | | | SAMPLE F | | |
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 4 | CRM | 1.80 | 2.20 | 2.10 | 1.60 | 1.70 | 1.70 | 11.70 | 11.60 | 11.50 |
| 5 | CRM | 1.96 | 1.83 | 2.08 | 1.91 | 1.95 | 1.98 | 11.25 | 11.04 | 11.19 |
| 7 | CRM* | 0.80 | 0.81 | 0.82 | 0.88 | 0.84 | 0.86 | 10.50 | 10.50 | 10.48 |
| 10 | CRM* | 1.10 | 1.00 | 1.00 | 0.90 | 0.90 | 0.90 | 11.10 | 10.90 | 11.00 |
| 11 | CRM | <DL | | | <DL | | | 11.8 | 12.20 | 12.30 |
| 13 | CRM | 1.00 | 0.94 | 0.95 | 0.91 | 0.88 | 0.92 | 11.54 | 11.59 | IS |
| 18 | CRM* | 2.00 | 2.07 | 1.60 | 1.86* | 1.97* | 1.53* | 11.49* | 12.02* | 11.72* |
| 19 | CRM | 2.73 | 2.73 | 2.79 | 2.63 | 2.67 | 2.74 | 12.04 | 12.26 | 12.04 |
| 21 | CRM* | 0.91 | 0.97 | 0.94 | 0.79 | 0.82 | 0.85 | 10.12 | 10.11 | 10.05 |
| 12 | CFA | 1.60 | 1.60 | 1.70 | 1.90 | 1.60 | 1.40 | 11.60 | 11.60 | 11.70 |

| KEY | |
|---|---|
| * | Cochran's Failure |
| ** | Grubb's Failure |
| | |
| CRM | No.37 |
| CRM* | No.37 with minor deviations |
| CFA | Same technique as CRM with major deviations |
| | |

## E.4 REDUCING CARBOHYDRATES

| Lab No. | Method used | SAMPLE A | | | SAMPLE B | | | SAMPLE C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 | REP1 | REP2 | REP3 |
| 1 | CRM* | 4.77* | 4.1* | 4.07* | 14.43 | 14.02 | 14.15 | 8.45 | 8.38 | 8.19 |
| 2 | CRM* | 4.00 | 4.00 | 4.10 | 15.10 | 15.20 | 15.30 | 9.00 | 8.90 | 9.00 |
| 3 | CRM* | 4.63 | 4.57 | 4.51 | 14.56* | 13.03* | 14.53* | 12.45* | 14.22* | 14.43* |
| 7 | CRM | 3.72 | 3.70 | 3.76 | 13.85 | 13.76 | 13.94 | 7.89 | 8.01 | 8.10 |
| 8a | CRM* | 4.65 | 4.31 | 4.31 | 14.49 | 15.42 | 15.13 | 8.72 | 8.78 | 8.72 |
| 8b | CRM* | 4.17 | 3.94 | 3.82 | 13.51 | 14.05 | 13.78 | 8.16 | 8.27 | 8.07 |
| 10 | CRM* | 5.00 | 5.00 | 5.00 | 16.00 | 16.10 | 16.20 | 10.20 | 10.20 | 10.20 |
| 11 | CRM | 6.30 | 6.50 | 6.30 | 15.90 | 15.90 | 15.70 | 9.20 | 9.30 | 9.20 |
| 14 | CRM* | 3.92 | 3.94 | 3.92 | 13.28 | 13.31 | 13.34 | 7.75 | 7.81 | 7.71 |
| 15 | CRM* | 5.40 | 5.20 | 5.30 | 15.90 | 15.50 | 15.90 | 9.80 | 9.40 | 9.70 |
| 16 | CRM* | 4.04 | 3.81 | 3.76 | 13.81 | 13.81 | 13.85 | 7.88 | 7.98 | 7.83 |
| 6 | CFA | 3.80 | 4.20 | 3.90 | 14.50 | 14.50 | 14.60 | 9.10 | 9.30 | 9.30 |
| 17 | CFA | 5.39 | 5.35 | 5.36 | 15.40 | 15.54 | 15.41 | 9.25 | 9.27 | 9.28 |
| 20 | CFA | 4.55 | 4.40 | 4.58 | 15.62 | 15.85 | 16.05 | 9.83 | 9.60 | 9.62 |
| 22 | HPLC | 2.68 | 2.79 | 2.77 | 11.62 | 11.42 | 11.10 | 7.55 | 7.63 | 7.65 |
| | | | | | | | | | | |

| Lab No. | Method used | SAMPLE F | | |
|---|---|---|---|---|
| | | REP1 | REP2 | REP3 |
| 1 | CRM* | 10.10 | 10.03 | 9.94 |
| 2 | CRM* | 11.10 | 11.20 | 11.20 |
| 3 | CRM* | 14.18 | 13.57 | 13.11 |
| 7 | CRM | 10.06 | 10.16 | 10.15 |
| 8a | CRM* | 9.89 | 11.02 | 10.54 |
| 8b | CRM* | 9.78 | 9.80 | 9.90 |
| 10 | CRM* | 12.40 | 12.60 | 12.60 |
| 11 | CRM | 11.20 | 11.20 | 11.20 |
| 14 | CRM* | 9.54 | 9.52 | 9.47 |
| 15 | CRM* | 11.80 | 11.90 | 12.00 |
| 16 | CRM* | 10.06 | 9.56 | 9.76 |
| 6 | CFA | 12.50 | 12.70 | 12.40 |
| 17 | CFA | 10.89 | 10.95 | 11.00 |
| 20 | CFA | 11.87 | 11.85 | 11.84 |
| 22 | HPLC | 10.69 | 10.84 | 10.71 |
| | | | | |

| KEY | |
|---|---|
| * | Cochran's Failure |
| ** | Grubb's Failure |
| | |
| CRM | No.38 |
| CRM* | No.38 with minor deviations |
| CFA | Same technique as CRM with major deviations |
| HPLC | Liquid Chromatography |