CORESTA RECOMMENDED METHOD N° 38

DETERMINATION OF REDUCING CARBOHYDRATES IN TOBACCO BY CONTINUOUS FLOW ANALYSIS

(August 2010)

0. INTRODUCTION

A CORESTA Task Force studied the various widely-used procedures for the determination of reducing sugars in tobacco in order to adopt one of them as the CORESTA Recommended Method. Two procedures were adopted as CORESTA Recommended Methods N° 37 and N° 38. Studies carried out by the CORESTA Task Force between 1989 and 1993 have shown that the two methods may not produce identical results. For some tobaccos the results obtained with Method N° 37 are higher than those of Method N° 38 because Method N° 37 is sensitive to interferences from reducing substances, other than sugars, present in tobacco. This is reflected in the titles of the two methods. CORESTA has decided to publish both methods since Method N° 37 is easier to implement, while Method N° 38 is more specific. When reporting the results indicate the method used.

1. FIELD OF APPLICATION

This method is applicable to leaf samples and tobacco blends.

2. PRINCIPLE

A tobacco extract in 5% acetic acid solution (see note 1) is prepared and the reducing carbohydrates content of the extract is determined by reaction with *p*-hydroxybenzoic acid hydrazide. In alkaline medium at 85°C, a yellow osazone is formed having an absorption maximum at 410 nm.

Note 1 : Collaborative studies have shown that when extracting with distilled water, hydrolysis of sucrose occurs for some tobaccos.

3. REAGENTS

All reagents shall be used according to good laboratory practice and existing national regulations.

3.1 *Brij 35 Solution (Polyoxyethylene Lauryl Ether)*
Add 1 dm^3 distilled water to 250 g Brij 35, warm and stir until dissolved.

3.2 *Sodium Hydroxide Solution (NaOH, 0.5 M)*
Prepare 1 dm^3 of 0.5 M sodium hydroxide from ampoules or dissolve 20.0 g sodium hydroxide in 800 cm^3 distilled water. Mix and allow to cool. After total dissolution, add 0.5 cm^3 Brij 35 solution (3.1) and dilute to 1 dm^3 with distilled water.

3.3 *Calcium Chloride Solution ($CaCl_2.6H_2O$, 0.008 M)*

Dissolve 1.75 g calcium chloride hexahydrate in distilled water (see note 2) add 0.5 cm^3 Brij 35 solution (3.1) and dilute to 1 dm^3 with distilled water.

Note 2 : If a precipitate occurs, filter the solution through a Whatman N° 1 (or equivalent) filter paper.

3.4 *Acetic Acid Solution (CH_3COOH, 5 % V/V)*

Prepare a 5 % (V/V) solution of acetic acid from "glacial" acetic acid (used in preparation of standards and samples and for wash solution on continuous flow analyzer).

3.5 *Hydrochloric Acid Solution (HCl, 0.5 M)*

Place 500 cm^3 distilled water in a 1 dm^3 volumetric flask. Slowly add 42 cm^3 fuming hydrochloric acid (37 % m/m). Dilute to volume with distilled water.

3.6 *p-Hydroxybenzoic Acid Hydrazide Solution (PAHBAH),($HOC_6H_4CONHNH_2$)*

Place 250 cm^3 0.5 M hydrochloric acid (3.5) into a 500 cm^3 volumetric flask. Add 25 g *p*-hydroxybenzoic acid hydrazide and allow to dissolve. Add 10.5 g citric acid monohydrate ($HOC(CH_2COOH)_2COOH{\cdot}H_2O$). Dilute to volume with 0.5 M hydrochloric acid solution. Store at 5°C, and take out of the refrigerator only enough volume to cover the daily needs.

Note 3 : The purity of PAHBAH (> 97 % m/m) is very important since a precipitate may be formed in the analytical stream if impurities are present. The PAHBAH can be recrystallised from distilled water (see Beilstein 10, 174). The PAHBAH is not pure when the following is observed:

a) dark particles present with white PAHBAH crystals;
b) yellow colour in 5 % PAHBAH prepared in 0.5 M HCl;
c) difficulty in dissolving PAHBAH crystals in 0.5 M NaOH;
d) foreign particles floating in the reagent;
e) a wavy reagent baseline.

The PAHBAH solution can also be prepared as follows: place the 250 cm^3 0.5 M HCl solution in a beaker, warm it to 45°C and under constant stirring add the PAHBAH and the citric acid monohydrate to the HCl solution. Let the solution cool down, transfer it to a volumetric flask and dilute to volume. It has been observed that preparation of the PAHBAH solution following this procedure prevents the formation of a precipitate in the analytical stream.

3.7 *D-Glucose ($C_6H_{12}O_6$, p.a.) for the Preparation of Standards.*

Store in a desiccator.

3.8 *Standard Glucose Solutions*

3.8.1 Stock Solution: Weigh, to the nearest 0.0001 g, approximately 10.0 g of glucose (3.7), dissolve in 800 cm^3 of 5 % acetic acid (3.4) and dilute to 1 dm^3 in a volumetric flask with 5 % acetic acid (3.4). This solution contains approximately 10 mg of glucose per cm^3. Store in a refrigerator. Prepare a fresh solution every month.

3.8.2 Working Standards: From the stock solution produce a series of at least five calibration solutions (in 5 % acetic acid) whose concentrations cover the range expected to be found in the samples *e.g.* 0.2-1.8 mg glucose per cm^3. Calculate the exact concentration for each standard. Store in a refrigerator. Prepare fresh solutions every two weeks.

4. APPARATUS

4.1 The necessary general laboratory equipment, for the preparation of samples, standards and reagents.

4.2 Continuous flow analyzer (see diagram 1) consisting of:

Sampler
Proportioning pump
Dialyser
Heating bath
Delay coils
Colorimeter (or equivalent) with 410 nm filter(s)
Recorder

5. ANALYSIS OF TOBACCO SAMPLES

5.1 Prepare the tobacco samples for analysis by grinding (the sample should totally pass through a 1 mm sieve) and determine the moisture content. If the tobacco is too wet for grinding it can be dried at a temperature not exceeding 40°C.

5.2 Weigh, to the nearest 0.0001 g, approximately 250 mg of the tobacco into a 50 cm^3 dry conical flask. Add 25 cm^3 of 5 % acetic acid (3.4), stopper the flask and shake for 30 minutes.

5.3 Filter the extract through a Whatman N° 40 (or equivalent) filter paper, reject the first few cm^3 of the filtrate, then collect the filtrate in an analyzer cup.

5.4 Run the samples and standards through the system in the normal manner (*e.g.* priming with 6 tobacco extracts, calibration standards and samples with 1 intermediate calibration solution after every 6 samples). If sample concentrations lie outside the range of the standards, the samples shall be diluted and run again.

6. CALCULATION

6.1 Plot a graph of peak height against equivalent glucose concentrations for all the calibration solutions.

6.2 Calculate the percentage reducing carbohydrates (expressed as glucose)(dry weight basis) in the tobacco using the formula:

$$\%\textbf{Reducing Carbohydrates (dwb)} = \frac{\mathbf{c \times V \times 100}}{\mathbf{m}} \times \frac{\mathbf{100}}{\mathbf{100 - M}}$$

c is the reducing carbohydrates concentration, expressed in milligrams per millilitre, obtained from the calibration curve (6.1);

V is the volume, in millilitres, of extract prepared (5.2) (normally 25 millilitres);

m is the masss, in milligrams, of the sample (5.2);

M is the moisture content, expressed as percentage by mass, of the tobacco (5.1).

The test result shall be expressed to one decimal place.

Note 4 : If this method is performed simultaneously with CORESTA Recommended Method N° 35 or CORESTA Recommended Method N° 36 combined standards may be prepared.

7. REPEATABILITY AND REPRODUCIBILITY

7.1 An international collaborative study involving 13 laboratories and 3 samples conducted in 1993 showed that when single grades of tobacco were analyzed by this method, the following values for repeatability (r) and reproducibility (R) were obtained.

The difference between two single results found on different extractions by one operator using the same apparatus within a short time interval (the time it takes to analyze 40 sample cups) and without recalibration of the equipment during the time of analysis will exceed the repeatability value (r) on average not more than once in 20 cases in the normal and correct operation of the method.

Single results reported by two laboratories will differ by more than the reproducibility value (R) on average not more than once in 20 cases in the normal and correct operation of the method.

Data analysis gave the estimates as summarized in Table 1.

Table 1 (1993 Study)

Tobacco Type	Mean content of Reducing Carbohydrates % (dwb)	Repeatability Conditions r	Reproducibility Conditions R
Burley	0.6	0.4	0.6
Oriental	14.5	1.6	3.3
Flue-Cured	20.0	1.0	4.7

For the purpose of calculating r and R, one test result was defined as the yield obtained from analyzing a single extract once.

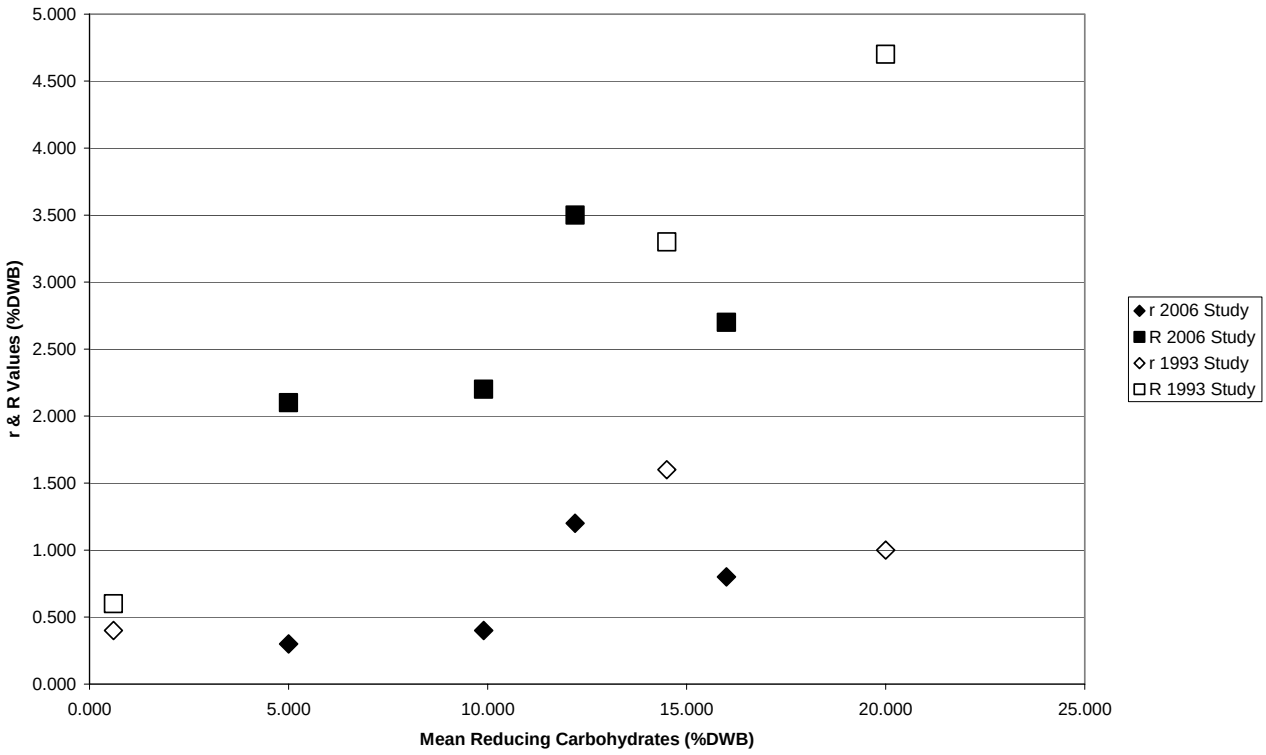
7.2 During 2005 the CORESTA Scientific Commission sanctioned the CORESTA Routine Analytical Chemistry Sub-group to carry out a collaborative study to confirm these r & R values. This international study involved 11 laboratories and 4 samples and was conducted during 2006. The resulting data are to be found in Table 2.

Table 2. Results from the 2006 RAC Collaborative Study

Tobacco Type	Mean Content of Reducing Carbohydrates % (dwb)	Repeatability conditions r	Repeatability coefficient of variation r CV	Reproducibility conditions R	Reproducibility coefficient of variation R CV
Flue-Cured Sample A	5.0	0.3	6.0	2.1	42.0
Flue-Cured Sample B	9.9	0.4	4.0	2.2	22.2
Flue-Cured Sample C	12.2	1.2	9.8	3.5	28.7
Flue-Cured Sample F	16.0	0.8	5.0	2.7	16.9

A plot comparing this data to that of the original study can be found below:

Comparison of r and R Results from the 1993 and 2006 Studies



APPENDIX 1

An injection fitting with a large internal diameter (2 mm) shall be used when introducing the PAHBAH solution into the analytical stream in order to prevent precipitation of PAHBAH. In addition the concentration of the PAHBAH solution may be reduced as long as it is ascertained that the PAHBAH is in excess in the analytical stream. This prevents precipitation as well.

It is preferable to use on-line mixing of PAHBAH/NaOH (see diagram 1). If however a precipitate forms on-line it is possible to pre-mix daily the PAHBAH/NaOH solutions and introduce the combined reagent to the analytical stream. Experiments have shown that similar results are obtained provided the combined reagent is not kept longer than 8 hours. If a combined reagent is used baseline correction may be required due to increased background signal.

DIAGRAM 1 *RM38*
Reducing Carbohydrates

(Technicon part numbers only given for information)
(Wash and sample times only intended as a guide)