Cooperation Centre for Scientific Research Relative to Tobacco

Routine Analytical Chemistry Sub-Group

CORESTA Recommended Method No. 89

TOBACCO – DETERMINATION OF THE CONTENT OF TOTAL SUGARS – CONTINUOUS-FLOW ANALYSIS METHOD USING HYDROCHLORIC ACID / P-HYDROXY BENZOIC ACID HYDRAZIDE (PAHBAH)

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0. INTRODUCTION

In 2015 the CORESTA Routine Analytical Chemistry Sub-Group (RAC) undertook a collaborative study for the determination of 'Total Sugars' in tobacco by segmented continuous-flow analysis (CFA). 'Total sugars' is defined as the combined amount of reducing sugars and non-reducing sugars present in a sample. The predominant sugars found in tobacco are the monosaccharides fructose and glucose, which are both reducing sugars. The most common non-reducing sugar found in tobacco is the disaccharide sucrose. The CORESTA Recommended Method for reducing sugars (CRM N° 38) was used as a basis for the development of this CRM to maintain compatibility and efficiency as reducing and non-reducing sugars are often analysed in parallel. Non-reducing sugars can be converted to reducing sugars by hydrolysis with either an acid or an enzyme. Once reduced they can react with one of several colour-forming compounds (e.g. PAHBAH)[1],[2]. Additionally, the extraction solution (water or acetic acid) was examined, because of a note given in CRM N° 38 regarding that hydrolysis of sucrose may occur for some tobaccos if extracted with distilled water.

1. FIELD OF APPLICATION

This CRM specifies a method for the determination of the content of 'Total Sugars' as glucose in tobacco by CFA using hydrochloric acid (HCl) for hydrolysis and p-hydroxybenzoic acid hydrazide (PAHBAH) for colour formation.

This method is applicable to unprocessed tobacco lamina and processed tobacco such as cigarette blend tobacco and roll-your-own (RYO) tobacco.

2. NORMATIVE REFERENCES

ISO 3696
Water for analytical laboratory use - Specification and test methods

3. TERMS AND DEFINITIONS

No terms and definitions are listed in this document.

4. PRINCIPLE

An aqueous extract of the tobacco is prepared and the total sugar content (as glucose) of the extract is analysed by CFA. The extract is heated in the presence of HCl at 90 °C, which hydrolyses any sucrose present to glucose and fructose. The reduced sample extract is passed through a dialyser to eliminate interference from coloured compounds in the sample and then reacts with PAHBAH in an alkaline medium at 85 °C to produce a yellow osazone complex.

Quantitation is by external standard using a series of glucose calibration standards (0,05 mg/mL – 2,5 mg/mL) prepared with the same extraction solution. All measurements are performed at 420 nm.

A collaborative study[3] has shown that the method gives comparable results for water and 5 % acetic acid extracts. It is recommended that 5 % acetic acid extracts should be used if analysis of reducing carbohydrates (CRM N° 38) is to be carried out in parallel.

5. APPARATUS

Usual laboratory apparatus and, in particular, the following items:

5.1. Continuous-flow analyser, consisting of

- Autosampler
- Peristaltic pump
- Chemistry manifold with dialyser, heating bath and delay coils
- Photometric detector equipped with a 420 nm filter
- Data acquisition system or recorder

See Annex A for examples of suitable flow diagrams.

6. REAGENTS

Use only reagents of recognized analytical grade. All reagents shall be used according to good laboratory practice and existing national regulations. Water must be high quality distilled or deionized (DI) water (according to ISO 3696).

6.1. Polyoxyethylene lauryl ether (Brij-35®, 30 % w/w solution), CAS # 9002-92-0

6.2. Acetic acid, glacial, CAS # 64-19-7

6.3. Hydrochloric acid (HCl), 37 %, CAS # 7647-01-0

6.4. Sodium hydroxide (NaOH), CAS # 1310-73-2

6.5. Calcium chloride hexahydrate, $CaCl_2*6H_2O$, CAS # 7774-34-7

6.6. p-Hydroxy benzoic acid hydrazide (PAHBAH), CAS # 5351-23-5

6.7. Citric acid monohydrate, CAS # 5949-29-1

6.8. Benzoic acid, CAS # 65-85-0

6.9. D-glucose, CAS # 50-99-7

7. PREPARATION OF SOLUTIONS

All reagents shall be of analytical grade quality. For best results, all solutions that include the dissolution of a solid should be filtered prior to use.

Appropriate safety and health practices shall be established according to national regulations.

7.1. System wash solution

Add 1 mL of Brij-35®, 30 % solution to about 800 mL DI water and mix carefully. Then dilute to 1000 mL with DI water. Replace every week in a clean bottle. Depending on the system the amount of 0,5 ml 30 % solution of Brij-35® per liter reagent might also be suitable.

7.2. Sampler wash solution

Use the extraction solution, DI water or acetic acid (5 %) (7.3), as sampler wash solution.

7.3. Acetic acid solution 5 % (v/v)

Add 50 mL of acetic acid (glacial) to about 500 mL of DI water. Dilute to 1000 mL with DI water and mix thoroughly.

7.4. Hydrochloric acid solution "A", 0,5 M (hydrolysis reagent)

Slowly add 42 mL of hydrochloric acid (37 %) to about 500 mL of DI water. Dilute to 1000 mL with DI water, add 0.5 mL of Brij-35®, 30 % solution and mix thoroughly. Stable for as long as the solution remains clear.

7.5. Hydrochloric acid solution "B", 0,5 M

Slowly add 42 mL of hydrochloric acid (37 %) to about 500 mL of DI water. Dilute to 1000 mL with DI water and mix thoroughly. Stable for as long as the solution remains clear.

7.6. Sodium hydroxide solution, 0,5 M

Dissolve 20 g of sodium hydroxide in about 700 mL of DI water. Dilute to 1000 mL, add 0,5 mL Brij-35® solution and mix thoroughly. Stable for as long as the solution remains clear.

7.7. Calcium chloride solution, 0,008 M

Dissolve 1.75 g of calcium chloride hexahydrate in about 700 mL of DI water. Dilute to 1000 mL, add 0,5 mL Brij-35® solution and mix thoroughly. If a precipitate occurs when dissolving the calcium chloride hexahydrate, then filter the solution. Stable for as long as the solution remains clear.

7.8. *p*-Hydroxy benzoic acid hydrazide (PAHBAH) solution

Place 400 mL of HCl solution (7.5) in a beaker, warm it to 45 °C and under constant stirring add 25 g PAHBAH and 10,5 g citric acid monohydrate to the HCl solution. Let the solution cool down, transfer it to a volumetric flask and dilute to volume with the HCl solution (7.5).

7.9. Benzoic acid solution 0,1 % (w/v) (stabilizing agent for the standard solutions)

Dissolve 2,0 g of benzoic acid in 2 liters of DI water.

8. STANDARDS

8.1. D-glucose stock solution

Weigh, to the nearest 0,0001 g, 10,0 g of glucose, dissolve in about 800 mL of 0,1 % benzoic acid (7.9, stabilizing agent if water extraction is used) respectively 5 % acetic acid (7.3, if acetic acid extraction is used) and dilute to volume. This solution contains 10 mg of glucose per liter. Store in a refrigerator.

8.2. D-glucose working standards

From the stock glucose solution, prepare a series of at least six calibration solutions according to the 'Total Sugars' concentration which is expected to be found in the test samples (e.g. 0,05 mg/mL – 2,5 mg/mL). Store in a refrigerator.

Table 1. D-glucose calibration standards – Nominal Concentrations (Actual concentrations will vary depending upon the amount weighed and the purity of glucose)

Standard ID	**Nominal D-glucose Concentration** (mg/mL)
1	0.05
2	0.50
3	1.00
4	1.50
5	2.00
6	2.50

9. SAMPLE PROCEDURE

9.1. Preparation of samples for analysis

Prepare the tobacco for analysis by grinding (the sample should totally pass a 1 mm sieve) and analyse. If the tobacco is too wet for grinding it can be dried at a temperature not exceeding 40 °C. For result calculation on a dry weight basis determine the moisture content.

9.2. Test portion

Weigh to the nearest 0,1 mg, approximately 250 mg, of the ground tobacco into a 50 mL conical flask. Add 25 mL of the extraction solution (water or 5 % acetic acid solution). Stopper and shake for 30 minutes at a suitable mixing speed.

9.3. Preparation of test extract

Filter the extract through a quantitative filter paper such as Whatman No 40[1] (or equivalent ashless, quantitative filter paper) filter paper, rejecting the first few mL of the filtrate, then collect the filtrate. Run the sample and standards through the system in the normal manner (e.g. priming with a high-level standard, calibration standards and samples with an intermediate calibration solution after every 25 samples). If sample concentration lies outside the range of the standards, the sample shall be diluted and run again.

When using 5 % acetic acid extracts, the wash solution shall be 5 % acetic acid.

Note: If this method is performed simultaneously with the other CFA methods, combined standards may be possible.

[1] Whatman No. 40 is an example of a suitable product available commercially. This information is given for the convenience of the users of this recommended method and does not constitute an endorsement by CORESTA of this product.

10. CALCULATION

10.1. Prepare a calibration curve by plotting Total Sugars (as d-glucose) instrument response against standard concentration. Compute sample concentration by comparing sample response with the standard curve.

10.2. Calculate the percentage of 'Total Sugars', ***w***, in the tobacco using the formula A:

$$w = \frac{c \times V \times 100}{m}$$

where

c is the 'Total sugars' concentration, expressed in milligrams per millilitre, obtained from the calibration curve (10.1);

V is the volume, in millilitres, of the sample (see 9.2), normally 25 mL;

m is the mass, in milligrams, of the sample (see 9.2);

Calculate, if applicable, the percentage of 'Total sugars' on a dry weight basis, *wd*, in the tobacco using the formula B:

$$wd = \frac{c \times V \times 100}{m} \times \frac{100}{(100 - M)}$$

where

c is the 'Total sugars' concentration, expressed in milligrams per millilitre, obtained from the calibration curve (10.1);

V is the volume, in millilitres, of the sample (see 9.2), normally 25 mL;

m is the mass, in milligrams, of the sample (see 9.2);

M the moisture content, expressed as percentage by mass, of the tobacco (see 9.1)

The test result shall be expressed to one decimal place.

11. REPEATABILITY AND REPRODUCIBILITY

In 2015 an international collaborative study involving eight laboratories and five samples (three straight grade tobaccos, one cigarette blend and one RYO tobacco) was conducted. The repeatability limit (r) and reproducibility limit (R) were calculated for this 'Total Sugars' (HCl/PAHBAH) method using both water and a 5 % acetic acid extraction (see Tables 2 and 3).

The difference between three single results, found on different extractions by one operator using the same apparatus within a short time interval (the time it takes to analyse ~ 50 sample cups) and without recalibration of the equipment during the time of analysis, will exceed the repeatability limit (*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 no more than the reproducibility limit (*R*) on average not more than once in 20 cases in the normal and correct operation of the method.

Table 2. Extraction with Water

Tobacco Type	Mean content of Total Sugars [% as received]	Repeatability r	Reproducibility R
Virginia (Low Level)	2,64	0,26	1,00
Virginia (High Level)	12,65	0,85	3,37
Burley	0,25	0,14	0,31
Cut Rag / Cig Blend	7,68	0,47	2,38
Cut Rag / RYO	5,49	0,39	2,06

Table 3. Extraction with 5 % Acetic Acid

Tobacco Type	Mean content of Total Sugars [% as received]	Repeatability r	Reproducibility R
Virginia (Low Level)	2,50	0,21	0,63
Virginia (High Level)	12,49	0,89	3,28
Burley	0,20	0,10	0,24
Cut Rag / Cig Blend	7,48	0,31	1,81
Cut Rag / RYO	5,33	0,29	1,65

12. TEST REPORT

The test report shall provide the Total Sugars results to precision of one decimal place. It shall also provide all details necessary for the identification of the sample.

ANNEX A - SUITABLE FLOW DIAGRAMS

(Informative)

Note: For both Macro and Micro Flow systems, the position of the de-bubbler should be as near as possible to the pump. For good reproducible results a well-shaped flow bubble pattern is necessary.

In Table 4 the flow rates of the tubing are given in (mL/min).

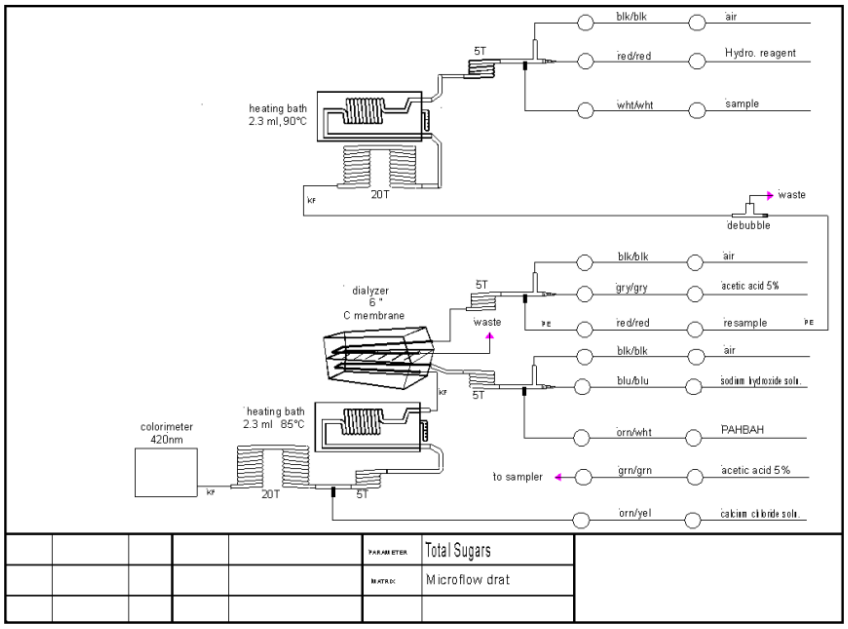

Figure 1: Suggested Flowchart for Macro Flow (mL/min) Systems

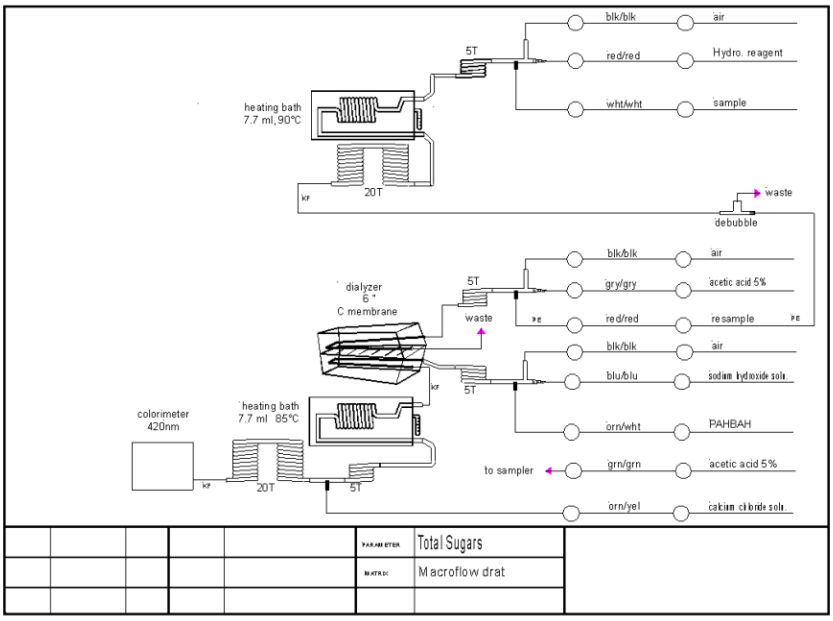

Figure 2: Suggested Flowchart for Micro Flow (µL/min) Systems

Table 4: Flow rates of the tubing

PUMP TUBE	FLOW RATE (mL/min)	
	1 mm manifold (micro flow)	2 mm manifold (macro flow)
orn/yel	0.08	0.16
orn/wht	0.11	0.23
blk/blk	0.15	0.32
wht/wht	0.26	0.60
red/red	0.32	0.80
gry/gry	0.38	1.00
blu/blu	0.54	1.60
grn/grn	0.64	2.00

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[1] Clarke, M.B.; Bezabeh, D.Z.; Howard, C.T. Determination of Carbohydrates in tobacco Products by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry: A Comparison with Ion Chromatography and Application to Product Discrimination. *J. Agric. Food Chem.* 2006, 54, 1975-1981

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