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Tobacco — Determination of the content of reducing carbohydrates — Continuous-flow analysis method

Tabac — Détermination de la teneur en hydrates de carbone réducteurs — Méthode par analyse en flux continu

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15154 was prepared by Technical Committee ISO/TC 126, *Tobacco and tobacco products*, Subcommittee SC 2, *Leaf tobacco*.

Introduction

A CORESTA[1)] 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 ISO 15153 and this International Standard. 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 the method given in ISO 15153 are higher than those of the method in this International Standard, because the latter is sensitive to interferences from reducing substances, other than sugars, present in tobacco. Collaborative studies have shown that when extracting with distilled water, hydrolysis of sucrose occurs with some tobaccos.

1) CORESTA: Cooperation Centre for Scientific Research Relative to Tobacco.

Tobacco — Determination of the content of reducing carbohydrates — Continuous-flow analysis method

1 Scope

This International Standard specifies a method for the determination of the content of reducing carbohydrates in tobacco by continuous-flow analysis.

This method is applicable to manufactured and unmanufactured tobacco.

2 Principle

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

3 Reagents

All reagents shall be used according to good laboratory practice and existing national regulations. Use distilled water or water of at least equivalent purity.

3.1 Polyoxyethylene lauryl ether (Brij 35 solution).

Add 1 litre water to 250 g of Brij 35. Warm and stir until dissolved.

3.2 Sodium hydroxide solution, c(NaOH) = 0,5 mol/l.

Prepare 1 litre of 0,5 mol/l sodium hydroxide from ampoules or dissolve 20,0 g of sodium hydroxide in 800 ml of water. Mix and allow to cool. After total dissolution, add 0,5 ml of Brij 35 solution (3.1) and dilute to 1 litre with water.

3.3 Calcium chloride solution, $c(CaCl_2)$ = 0,008 mol/l.

Dissolve 1,75 g calcium chloride hexahydrate in water. Add 0,5 ml of Brij 35 solution (3.1) and dilute to 1 litre with water.

If a precipitate occurs, filter the solution through a Whatman No. 1[2)] (or equivalent) filter paper.

3.4 Acetic acid solution (CH_3COOH), volume fraction of 5 %.

Prepare a 5 % (volume fraction) solution of acetic acid from glacial acetic acid. (This is used in the preparation of standards and samples and for the wash solution for the continuous flow analyser.)

3.5 Dilute hydrochloric acid, c(HCl) = 0,5 mol/l.

Place 500 ml of water in a 1 litre volumetric flask. Slowly add 42 ml of concentrated hydrochloric acid (a mass fraction of 37 %). Dilute to volume with water.

2) Whatman No. 1 is an example of a suitable product available commercially. This information is given for the convenience of the users of and does not constitute an endorsement by ISO of this product.

3.6 *p*-Hydroxybenzoic acid hydrazide solution (PAHBAH), ($HOC_6H_4CONHNH_2$).

Place 250 ml of dilute hydrochloric acid (3.5) in a 500 ml volumetric flask. Add 25 g of *p*-hydroxybenzoic acid hydrazide and allow to dissolve. Add 10,5 g of citric acid monohydrate [$HOC(CH_2COOH)_2COOH{\cdot}H_2O$]. Dilute to volume with hydrochloric acid solution (3.5). Store at 5 °C, and take out of the refrigerator only enough to cover the daily needs.

The purity of PAHBAH (mass fraction of $>$ 97 %) is very important since a precipitate can be formed in the analytical stream if impurities are present. The PAHBAH can be recrystallised from water (see [4]). 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 mol/l HCl;

c) difficulty in dissolving PAHBAH crystals in 0,5 mol/l 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 ml of dilute hydrochloric acid in a beaker. Warm it to 45 °C ± 1 °C and, under constant stirring, add the PAHBAH and the citric acid monohydrate to the dilute hydrochloric acid. 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.

Annex A gives other methods for the prevention of precipitation of PAHBAH.

3.7 D-Glucose ($C_6H_{12}O_6$)

Store in a desiccator.

3.8 Standard glucose solutions

3.8.1 Stock solution

Weigh, to the nearest 0,1 mg, approximately 10,0 g of glucose (3.7). Dissolve in 800 ml of 5 % acetic acid (3.4) and dilute to 1 litre in a volumetric flask with the acetic acid. This solution contains approximately 10 mg/ml of glucose. Store in a refrigerator. Prepare a fresh solution every month.

3.8.2 Working standards

From the stock solution (3.8.1) prepare by dilution with the 5 % acetic acid (3.4) a series of at least five calibration solutions, the glucose concentrations of which cover the range expected to be found in the test samples (e.g. 0,2 mg/ml to 1,8 mg/ml). Calculate the exact concentration for each standard. Store in a refrigerator. Prepare fresh solutions every 2 weeks.

4 Apparatus

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

4.1 Continuous flow analyser, consisting of

— sampler,

— proportioning pump,

— dialyser,

— heating bath,

— delay coils,

— colorimeter (or equivalent) with 410 nm filter(s), and

— recorder.

See Annex B for an example of a suitable layout.

5 Procedure

5.1 Preparation of samples for analysis

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 may be dried at a temperature not exceeding 40 °C.

5.2 Test portion

Weigh, to the nearest 0,1 mg, approximately 250 mg of the tobacco into a 50 ml dry conical flask. Add 25 ml of acetic acid (3.4), stopper the flask and shake for 30 min.

5.3 Preparation of test extract

Filter the extract through a Whatman No. 40[3)] (or equivalent) filter paper. Reject the first few millilitres of the filtrate, then collect the filtrate in an analyser cup.

Run the samples and standards through the system in the normal manner (e.g. priming with six tobacco extracts, calibration standards and samples with one intermediate calibration solution after every six samples). If the sample concentrations lie outside the range of the standards, the samples shall be diluted with the acetic acid (3.4) 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 method described in ISO 15152 or ISO 15517, combined standards may be prepared. Combined stock solutions may precipitate after about 2 weeks.

6 Calculation

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

6.2 Calculate the percentage of reducing carbohydrates, w (expressed as glucose), on a dry weight basis, in the tobacco using the formula

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

where

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

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

3) Whatman No. 40 is an example of a suitable product available commercially. This information is given for the convenience of the users of this International Standard and does not constitute an endorsement by ISO of this product.

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

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

The test result shall be expressed to one decimal place.

7 Repeatability and reproducibility

An international collaborative study involving 13 laboratories and 3 samples conducted in 1993 showed that when single grades of tobacco were analysed by this method, the following values for repeatability limit (r) and reproducibility limit (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 analyse 40 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 by more than the reproducibility limit (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. For the purpose of calculating r and R, one test result was defined as the yield obtained from analysing a single extract once.

Table 1 — Values of repeatability and reproducibility limits

Tobacco type	Mean content of reducing carbohydrates % (dry weight)	Repeatability limit r	Reproducibility limit R
Burley	0,6	0,4	0,6
Oriental	14,5	1,6	3,3
Flue cured	20,0	1,0	4,7

8 Test report

When reporting results, the method used shall be specified.

Annex A
(informative)

Prevention of precipitation of PAHBAH

An injection fitting with a large internal diameter (2 mm) should 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 Figure B.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 h. If a combined reagent is used, baseline correction may be required due to increased background signal.