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**Tobacco — Determination of nitrate  
content — Continuous-flow analysis  
method**

*Tabac — Détermination de la teneur en nitrates — Méthode par analyse  
en flux continu*

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## Foreword

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# Tobacco — Determination of nitrate content — Continuous-flow analysis method

## 1 Scope

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

This method is applicable to manufactured and unmanufactured tobacco.

## 2 Principle

An aqueous extract (see below) of the tobacco is prepared and the nitrate content of the extract is determined by reduction of the nitrate ions to nitrite ions with hydrazinium sulfate in the presence of a copper catalyst, followed by reaction with sulfanilamide to form the diazo compound. This is coupled with *N*-1-naphthylethylenediamine dihydrochloride to form a coloured complex, which is measured at 520 nm. If there is any nitrite content, it will be detected and included in the nitrate content result.

Collaborative studies have shown that this method gives equivalent results for water and 5 % acetic acid extracts. It is recommended that 5 % acetic acid extracts should be used if nitrate and reducing substances (see ISO 15153) or reducing carbohydrate analyses (see ISO 15154) are to be carried out simultaneously.

## 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 of water to 250 g of Brij 35. Warm and stir until dissolved.

### 3.2 Sodium hydroxide solution (NaOH), p.a.

Dissolve 8,0 g of sodium hydroxide in distilled water. Add 1 ml of Brij 35 solution (3.1) and dilute to 1 litre.

### 3.3 Copper sulfate stock solution (CuSO<sub>4</sub>), p.a.

Dissolve 1,20 g of copper(II) sulfate pentahydrate in distilled water and dilute to 100 ml.

### 3.4 Hydrazinium sulfate/copper sulfate reagent

Dissolve the optimum amount of hydrazinium sulfate (N<sub>2</sub>H<sub>6</sub>SO<sub>4</sub>, p.a.) in water. Add 1,5 ml of copper sulfate stock solution (3.3) and dilute to 1 litre with water. Store in an amber glass bottle. Prepare a fresh solution every month.

### 3.5 Sulfanilamide reagent (NH<sub>2</sub>C<sub>6</sub>H<sub>4</sub>SO<sub>2</sub>NH<sub>2</sub>)

Add 25 ml of concentrated orthophosphoric acid (H<sub>3</sub>PO<sub>4</sub>, volume fraction 85 %, of grade low in nitrate) to approximately 175 ml of water. Dissolve 2,5 g of sulfanilamide in the solution followed by 0,125 g of *N*-1-naphthylethylenediamine dihydrochloride (C<sub>10</sub>H<sub>7</sub>NHCH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>·2HCl).

Dilute to 250 ml with water and filter through a Whatman No. 40<sup>1)</sup> (or equivalent) filter paper. Store in an amber glass bottle. Prepare a fresh solution every 2 days.

### 3.6 Potassium nitrate (KNO<sub>3</sub>), p.a.

### 3.7 Standard nitrate solutions

#### 3.7.1 Stock solution

Weigh, to the nearest 0,000 1 g, approximately 3,3 g of potassium nitrate (3.6). Dissolve in water and dilute to 1 litre in a volumetric flask. This solution contains approximately 2 mg/ml of nitrate. Store in a refrigerator. Prepare a fresh solution every month.

#### 3.7.2 Working standards

From the stock solution (3.7.1), produce a series of at least five calibration solutions whose concentrations cover the range expected to be found in the samples (e.g. 10 µg/ml to 200 µg/ml of nitrate). Calculate the exact concentration for each standard. Store in a refrigerator. Prepare fresh solutions every 2 weeks.

If 5 % acetic acid (five times) is to be used to extract the nicotine from the test sample in 5.2, the working standards (3.7.2) shall be made from the stock solution every 2 weeks using 5 % acetic acid instead of water.

## 4 Apparatus

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

### 4.1 Continuous flow analyser consisting of

- sampler,
- dialyser,
- heating bath,
- delay coils,
- colorimeter (or equivalent) with 520-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. Any contamination from nitrate and nitrite ions shall be minimized.

### 5.2 Test portion

Weigh, to the nearest 0,1 mg, approximately 250 mg of the tobacco in a 50 ml dry conical flask. Add 25 ml of distilled water from a dispenser. Stopper the flask and shake for 30 min.

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 International Standard and does not constitute an endorsement by ISO of this product.

### 5.3 Preparation of test extract

Filter the extract through a Whatman No. 40 (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 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 nitrate concentrations for all the standard solutions.

**6.2** Calculate the percentage of nitrate,  $w$ , on a dry weight basis, in the tobacco using the formula:

$$w = \frac{C \times V \times 100}{m \times 1000} \times \frac{100}{100 - M}$$

where

$C$  is the nitrate concentration, expressed in micrograms 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);

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

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

The test result shall be expressed to two decimal places.

When using 5 % acetic acid extracts, the standard nitrate solutions (3.7) shall be made up with 5 % acetic acid and the wash cycle shall be with 5 % acetic acid.

## 7 Repeatability and reproducibility

An international collaborative study involving 12 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 Tables 1 and 2. For the purposes of calculating  $r$  and  $R$ , one test result was defined as the yield obtained from analysing a single extract once.

Table 1 — Extraction with water

Tobacco type	Mean content of nitrate % (dry weight)	Repeatability limit <i>r</i>	Reproducibility limit <i>R</i>
Flue cured	0,11	0,03	0,12
Oriental	0,16	0,04	0,11
Burley	2,43	0,12	0,41

Table 2 — Extraction with 5 % acetic acid

Tobacco type	Mean content of nitrate % (dry weight)	Repeatability limit <i>r</i>	Reproducibility limit <i>R</i>
Flue cured	0,11	0,03	0,20
Oriental	0,16	0,04	0,21
Burley	2,43	0,05	0,39

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## Annex A (normative)

### Optimization of hydrazinium sulfate/copper sulfate

#### A.1 General

The optimization of the hydrazinium sulfate-copper sulfate reagent (see 3.4) shall be carried out when initially setting up the instrument. It should also be carried out when fresh batches of hydrazinium sulfate are purchased.

#### A.2 Reagents

##### A.2.1 Standard nitrite stock solution

Dissolve 0,900 g of sodium nitrite ( $\text{NaNO}_2$ , p.a.) in distilled water and dilute to volume in a 1 litre volumetric flask. This solution contains 0,6 mg/ml of nitrite.

##### A.2.2 Standard nitrite working solution

Pipette a 25 ml aliquot of the stock solution (A.2.1) into a 100 ml volumetric flask and dilute to volume with distilled water. This solution contains 150  $\mu\text{g/ml}$  of nitrite.

#### A.3 Optimization of the hydrazinium sulfate reagent

**A.3.1** Dilute 0,75 ml of the copper sulfate stock solution (3.3) to 1 litre with distilled water.

**A.3.2** Dissolve 0,5 g of hydrazinium sulfate in distilled water and dilute to 100 ml in a volumetric flask.

**A.3.3** From a burette, dispense 1,0 ml, 2,0 ml, 3,0 ml, ..., 10,0 ml aliquots of the hydrazinium sulfate solution (refer to A.3.2) into 25 ml volumetric flasks and dilute to volume with distilled water. These solutions contain 0,2 g, 0,4 g, 0,6 g, ..., 2,0 g hydrazinium sulfate per litre.

**A.3.4** Connect the hydrazinium/copper reagent line to the analyser sampler. Connect the water line to the dilute copper sulfate solution reservoir. Connect the sample line to the standard nitrite working solution reservoir (refer to A.2.2).

**A.3.5** Start the analyser pump, pumping all other reagents as normal.

**A.3.6** Place sample cups containing the hydrazinium solutions (A.3.3) in the sampler in ascending order of concentration.

**A.3.7** When the reaction colour reaches the flow cell, adjust the recorder response to 90 % full-scale deflection and start the sampler.

**A.3.8** When all the hydrazinium solutions have been run, note the concentration which produces a loss in colour due to the reduction of the nitrite to nitrogen.

**A.3.9** Replace the standard nitrite working solution (A.2.2) with the highest nitrate standard (see 3.7.2). Wait for the reagent base line to be re-established, then re-run the hydrazinium sulfate solutions.

**A.3.10** The optimum hydrazinium sulfate concentration is that which ensures complete reduction of nitrate to nitrite but does not result in the reduction of nitrite to nitrogen.