

CORESTA RECOMMENDED METHOD N° 36

DETERMINATION OF NITRATE IN TOBACCO BY CONTINUOUS FLOW ANALYSIS

(November 1994)

1. FIELD OF APPLICATION

This method is applicable to unmanufactured and manufactured tobacco.

2. PRINCIPLE

An aqueous (see note 1) extract of the tobacco is prepared and the nitrate content of the extract is determined by reduction of the nitrate to nitrite with hydrazinium sulphate in the presence of a copper catalyst, followed by reaction with sulphanilamide 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 result.

Note 1 : 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 CORESTA Recommended Method N° 37) or reducing carbohydrates (see CORESTA Recommended Method N° 38) analysis are to be carried out simultaneously.

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 dm³ distilled water to 250 g Brij 35, warm and stir until dissolved.

3.2 *Sodium Hydroxide Solution (NaOH, p.a.)*

Dissolve 8.0 g sodium hydroxide in distilled water, add 1 cm³ Brij solution (3.1) and dilute to 1 dm³.

3.3 *Copper Sulphate Stock Solution (CuSO₄·5H₂O, p.a.)*

Dissolve 1.20 g hydrated cupric sulphate in distilled water and dilute to 100 cm³.

3.4 *Hydrazinium Sulphate - Copper Sulphate Reagent*

This reagent has to be optimised according to appendix 1.

Dissolve the optimum amount of hydrazinium sulphate (N₂H₆SO₄, p.a.) in water, add 1.5 cm³ of copper sulphate stock solution (3.3) and dilute to 1 dm³ with distilled water. Store in an amber glass bottle. Prepare a fresh solution every month.

3.5 *Sulphanilamide Reagent (NH₂C₆H₄SO₂NH₂)*

Add 25 cm³ concentrated orthophosphoric acid (H₃PO₄ 85% (V/V), low in nitrate grade) to approximately 175 cm³ distilled water. Dissolve 2.5 g sulphanilamide in the solution followed by 0.125 g N-1-naphthylethylenediamine dihydrochloride (C₁₀H₇NHCH₂CH₂NH₂·2HCl).

Dilute to 250 cm³ with distilled water and filter through a Whatman N° 40 (or equivalent) filter paper. Store in an amber glass bottle. Prepare a fresh solution every two days.

3.6 *Potassium Nitrate (KNO₃, p.a.) for the Preparation of Standards.*

3.7 *Standard Nitrate Solutions*

3.7.1 Stock Solution : Weigh, to the nearest 0.0001 g, approximately 3.3 g of potassium nitrate in distilled water and dilute to 1.00 dm³ in a volumetric flask. This solution contains approximately 2 mg nitrate per cm³. Store in a refrigerator. Prepare a fresh solution every month.

3.7.2 Working Standards : From the stock solution produce a series of at least 5 calibration solutions whose concentrations cover the range expected to be found in the samples *e.g.* 10-200 µg nitrate per cm³. 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 520 nm filter(s)
Recorder

5. ANALYSIS OF TOBACCO SAMPLES

5.1 Prepare the tobacco 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. Any contamination from nitrate and nitrite shall be minimised.

5.2 Weigh, to the nearest 0.0001 g, approximately 250 mg of the tobacco in a 50 cm³ dry conical flask. Add 25 cm³ distilled water from a dispenser, 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 cm³ 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 nitrate concentrations for all the calibration solutions.
- 6.2** Calculate the percentage nitrate (dry weight basis) in the tobacco using the formula:

$$\% \text{ Nitrate (dwb)} = \frac{c \times V \times 100}{m \times 1000} \times \frac{100}{100 - M}$$

- c** is the nitrate concentration, expressed in micrograms per millilitre, obtained from the calibration curve (5.1);
- V** is the volume, in millilitres, of extract prepared (5.2) (normally 25 millilitres);
- m** is the mass, 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 two decimal places.

Notes

- 2** When using 5 % acetic acid extracts the standard nitrate solutions (3.7) must be made up with 5 % acetic acid and the wash cycle must be with 5 % acetic acid.
- 3** If this method is performed simultaneously with CORESTA Recommended Method N° 35, CORESTA Recommended Method N° 37 or CORESTA Recommended Method N° 38 combined standards may be prepared.

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 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 and 2.

TABLE 1 : Extraction with Water

Tobacco Type	Mean Content of Nitrate % (dwb)	Repeatability Conditions r	Reproducibility Conditions R
Oriental	0.11	0.03	0.12
Flue-Cured	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 % (dwb)	Repeatability Conditions r	Reproducibility Conditions R
Oriental	0.11	0.03	0.20
Flue-Cured	0.16	0.04	0.21
Burley	2.43	0.05	0.39

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

APPENDIX 1

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

1. Standard Nitrite Solutions

- 1.1 *Stock Solution* : Dissolve 0.900 g sodium nitrite (NaNO_2 , p.a.) in distilled water and dilute to volume in a 1 dm^3 volumetric flask. This solution contains 0.6 mg nitrite per cm^3 .
- 1.2 *Working Solution* : Pipette a 25 cm^3 aliquot of the stock solution into a 100 cm^3 volumetric flask and dilute to volume with distilled water. This solution contains $150 \mu\text{g}$ nitrite per cm^3 .

2. Optimisation of the Hydrazinium Sulphate Reagent

- 2.1 Dilute 0.75 cm^3 of the copper sulphate stock solution (3.3) to 1 dm^3 with distilled water.
- 2.2 Dissolve 0.5 g hydrazinium sulphate in distilled water and dilute to 100 cm^3 in a volumetric flask.
- 2.3 From a burette, dispense $1.0, 2.0, 3.0 \dots 10.0 \text{ cm}^3$ aliquots of the hydrazinium sulphate solution (appendix 1, 2.2) into 25 cm^3 volumetric flasks and dilute to volume with distilled water. These solutions contain $0.2, 0.4, 0.6 \dots 2.0 \text{ g}$ hydrazinium sulphate per dm^3 .
- 2.4 Connect the hydrazinium/copper reagent line to the analyzer sampler. Connect the water line to the dilute copper sulphate solution reservoir. Connect the sample line to the standard nitrite working solution (appendix 1, 1.2) reservoir.
- 2.5 Start the analyzer pump, pumping all other reagents as normal.
- 2.6 Place sample cups of the hydrazinium solutions (appendix 1, 2.3) in the sampler in ascending order of concentration.
- 2.7 When the reaction colour reaches the flow cell, adjust the recorder response to 90% full scale deflection and start the sampler.
- 2.8 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.
- 2.9 Replace the standard nitrite working solution (appendix 1, 1.2) with the highest nitrate standard. Wait for the reagent base line to be re-established, then re-run the hydrazinium sulphate solutions.
- 2.10 The optimum hydrazinium sulphate concentration is that which ensures complete reduction of nitrate to nitrite but does not result in the reduction of nitrite to nitrogen.

DIAGRAM 1 RM36 Nitrate

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

