

# CORESTA RECOMMENDED METHOD N° 36

## DETERMINATION OF NITRATE IN TOBACCO AND SMOKELESS TOBACCO PRODUCTS BY REDUCTION TO NITRITE AND CONTINUOUS FLOW ANALYSIS

(January 2015)

### 1. FIELD OF APPLICATION

This method is applicable to leaf samples, tobacco blends and smokeless tobacco products.

### 2. REFERENCES

- 2.1. CSTS Smokeless Tobacco Glossary, available on the CORESTA website:  
[http://www.coresta.org/Reports/STS\\_Smokeless-Tobacco-Glossary.pdf](http://www.coresta.org/Reports/STS_Smokeless-Tobacco-Glossary.pdf)  
(accessed 13 January 2015)
- 2.2. Determination of Nitrate in Smokeless Tobacco Products by Continuous Flow Analysis - 2010 Collaborative and Proficiency Studies, available on the CORESTA website:  
[http://www.coresta.org/Reports/STS-CTR\\_Nitrate-Smokeless-Tob-Prod\\_Jan2015.pdf](http://www.coresta.org/Reports/STS-CTR_Nitrate-Smokeless-Tob-Prod_Jan2015.pdf)  
(accessed 13 January 2015)
- 2.3. CORESTA Guide No. 11 - Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products

### 3. PRINCIPLE

An aqueous (see note 1) extract of the tobacco or smokeless tobacco product 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, for which the absorbance is measured at 520 nm.

**Note 1:** If nitrite is also present in the tobacco, it will be detected by this method and may thus increase the apparent nitrate concentration.

**Note 2:** Collaborative studies have shown that this method gives equivalent results for extracts prepared in water and in 5 % acetic acid. It is recommended that 5 % acetic acid should be used if the simultaneous determination of nitrate and either reducing substances (see CORESTA Recommended Method N° 37) or reducing carbohydrates (see CORESTA Recommended Method N° 38) is to be conducted.

**Note 3:** This method is not recommended for samples containing nitrate at concentrations below 0,2 g / 100 g

### 4. REAGENTS

All reagents should be of analytical reagent grade unless otherwise stated and used within the assigned stability period.

**4.1. Brij 35 Solution (Polyoxyethylene Lauryl Ether)**

Add 1 dm<sup>3</sup> distilled water to 250 g Brij 35, warm and stir until dissolved.

**4.2. Sodium Hydroxide Solution (NaOH)**

Dissolve 8,0 g sodium hydroxide in distilled water, add 1 cm<sup>3</sup> Brij solution (4.1) and dilute to 1 dm<sup>3</sup>.

**4.3. Copper Sulphate Stock Solution (CuSO<sub>4</sub>·5H<sub>2</sub>O)**

Dissolve 1,20 g copper (II) sulphate pentahydrate in distilled water and dilute to 100 cm<sup>3</sup>.

**4.4. Hydrazinium Sulphate - Copper Sulphate Reagent**

The concentration of this reagent must be optimised according to Appendix 1.

Dissolve the optimum mass of hydrazinium sulphate (N<sub>2</sub>H<sub>6</sub>SO<sub>4</sub>) in water, add 1,5 cm<sup>3</sup> of copper sulphate stock solution (4.3) and dilute to 1 dm<sup>3</sup> with distilled water. Store in an amber glass bottle. Prepare a fresh solution every month.

**4.5. Sulphanilamide Reagent (NH<sub>2</sub>C<sub>6</sub>H<sub>4</sub>SO<sub>2</sub>NH<sub>2</sub>)**

Add 25 cm<sup>3</sup> concentrated orthophosphoric acid (H<sub>3</sub>PO<sub>4</sub> 85% (V/V), low in nitrate grade) to approximately 175 cm<sup>3</sup> distilled water. Dissolve 2,5 g sulphanilamide in the solution, followed by 0,125 g 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 cm<sup>3</sup> 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.

**4.6. Potassium Nitrate (KNO<sub>3</sub>) for the Preparation of Standards**

**4.7. Standard Nitrate Solutions**

**4.7.1. Stock Solution:** Weigh 3,30 g of potassium nitrate, recording the mass to the nearest 0,001 g, and dilute with distilled water to 1,00 dm<sup>3</sup> in a volumetric flask. This solution contains approximately 2 mg nitrate per cm<sup>3</sup>. Store in a refrigerator. Prepare a fresh solution every month.

**4.7.2. 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.* 5-200 µg nitrate per cm<sup>3</sup>. Calculate the exact concentration for each standard. Store in a refrigerator. Prepare fresh solutions every two weeks.

## 5. APPARATUS

**5.1. General laboratory equipment for the preparation of samples, standards and reagents.**

**5.2. 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

## 6. ANALYSIS OF TOBACCO SAMPLES

- 6.1. 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 should be minimised. Refer to 2.3 for guidelines on the handling of smokeless tobacco samples.
- 6.2. Weigh  $250 \pm 10$  mg of the tobacco into a  $50 \text{ cm}^3$  dry conical flask and record the mass to 0,0001 g. Add  $25 \text{ cm}^3$  distilled water from a dispenser, stopper the flask and shake for 30 minutes.
- 6.3. Filter the extract through a Whatman N° 40 (or equivalent) filter paper, reject the first  $5 \text{ cm}^3$  of the filtrate, then collect the filtrate in an analyzer cup.
- 6.4. Run the samples and standards through the analyzer 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 the measured concentration exceeds the highest standard, the extract should be diluted and re-analysed.

## 7. CALCULATION

- 7.1. Plot a graph of peak height against equivalent nitrate concentration for all the calibration solutions.
- 7.2. 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  $\text{cm}^3$ , obtained from the calibration curve (7.1);  
*V* is the volume, in  $\text{cm}^3$ , of extract prepared (6.2) (normally  $25 \text{ cm}^3$ );  
*m* is the mass, in milligrams, of the sample (6.2);  
*M* is the moisture content, expressed as percentage by mass, of the tobacco (6.1).

The test result shall be expressed to two decimal places.

**Note 4:** When using 5% acetic acid extracts the standard nitrate solutions (4.7) must be made up with 5% acetic acid and the wash cycle must be with 5% acetic acid.

**Note 5:** 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.

## 8. REPEATABILITY AND REPRODUCIBILITY

**8.1.** 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 for different replicate extractions conducted 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 that are summarized in Tables 1 and 2.

**TABLE 1: Extraction with Water (1993 Study)**

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 (1993 Study)**

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.

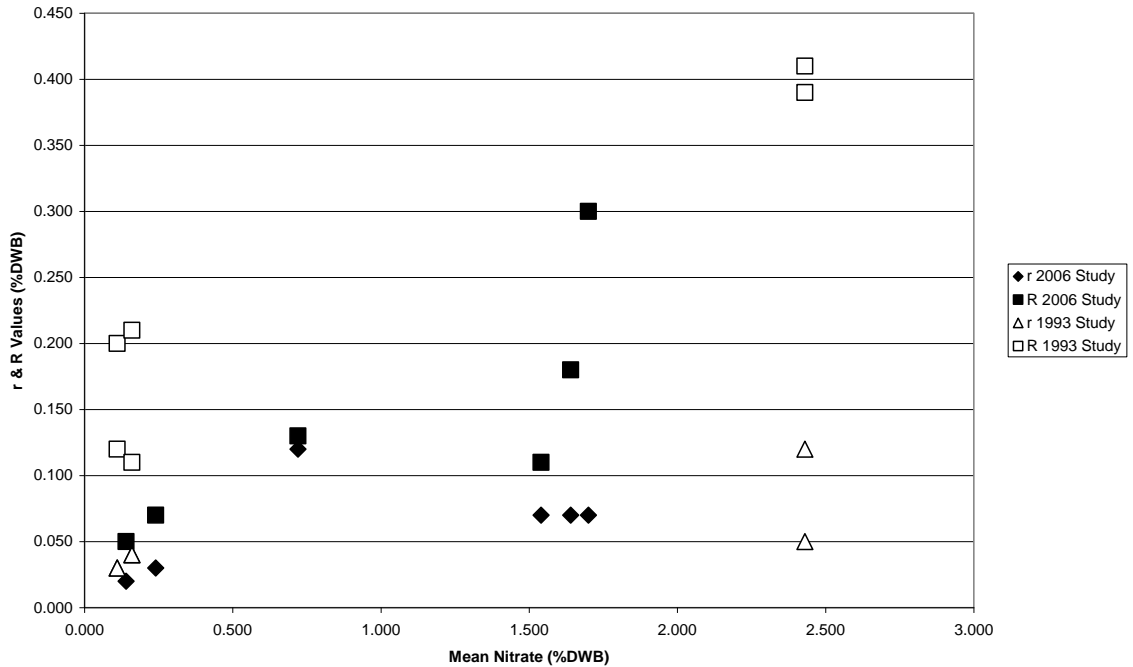
**8.2.** During 2005 the CORESTA Scientific Commission sanctioned the CORESTA Routine Analytical Chemistry Sub-Group to carry out a collaborative study to confirm these r & R values. This international study involved 11 laboratories and 6 samples and was conducted during 2006. The resulting data are to be found in Table 3.

**TABLE 3: Results from the 2006 RAC Collaborative Study**

Tobacco Type	Mean Content of Nitrate % (dwb)	Repeatability Conditions r	Repeatability coefficient of variation r CV	Reproducibility Conditions R	Reproducibility coefficient of variation R CV
Flue-Cured Sample A	0,24	0,03	12,5	0,07	29,2
Flue-Cured Sample B	0,14	0,02	14,3	0,05	35,7
Flue-Cured Sample C	1,70	0,07	4,1	0,30	17,6
Burley Sample D	1,54	0,07	4,5	0,11	7,1
Burley Sample E	0,72	0,12	16,7	0,13	18,1
Flue-Cured Sample F	1,64	0,07	4,3	0,18	11,0

**Note 6:** This CRM recommends that equivalent results are obtained when either water or 5% acetic acid are used as the extraction solvents and therefore the results from this study were not segregated in the subsequent data analysis. A comparison of these 2006 data with the data from the original 1993 study is presented below (Figure 1):

**FIGURE 1: Comparison of r and R Results from the 1993 and 2006 Studies**



**8.3.** During 2010, the CORESTA Smokeless Tobacco Sub-Group (CSTS) conducted a Collaborative Study which included nine types of smokeless tobacco (2.2). In this international study up to 11 laboratories measured nitrate levels in each tobacco sample using Continuous Flow Analysis as described in this CRM. The Collaborative Study results are included in Table 4 and Figure 2.

**Note 7:** The nitrate results are reported on a wet weight basis (wwb). Smokeless tobacco products have a very wide range of moisture content (5 - 60%). Currently there is no agreed method for the determination of moisture content, hence corrected dwb results may not be comparable between laboratories and are not shown here.

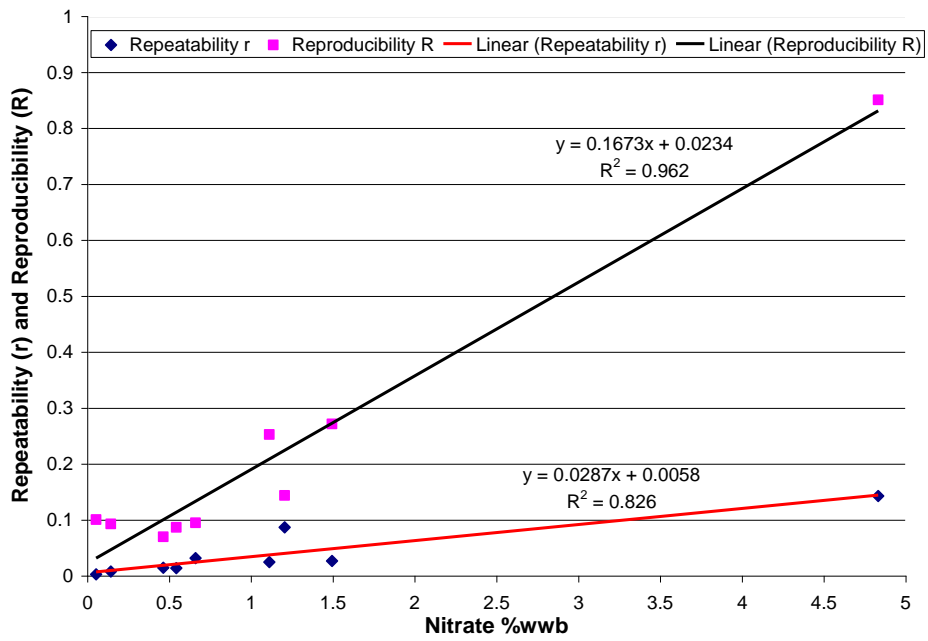
**TABLE 4: Results from the 2010 Collaborative and Proficiency Studies**

<b>Tobacco Type</b>	<b>Mean Content of Nitrate % (wwb)</b>	<b>Repeatability Conditions r</b>	<b>Repeatability coefficient of variation r CV</b>	<b>Reproducibility Conditions R</b>	<b>Reproducibility coefficient of variation R CV</b>	<b>N</b>
Loose snus	0,542	0,014	2,58	0,087	16,05	10
CRP1 pouched snus	0,660	0,032	4,85	0,095	14,39	11
CRP2 moist snuff	1,493	0,027	1,81	0,272	18,22	9
CRP4 loose leaf	1,204	0,087	7,23	0,144	11,96	10
Chewing Tobacco, bits	0,462	0,015	3,25	0,070	15,15	7
Hard Snuff/Pellet	0,142	0,008	5,63	0,093	65,49	7
Nasal Snuff	1,110	0,025	2,25	0,253	22,79	10
Chewing Tobacco, flake	0,052	0,003	5,77	0,101	194,23	6
CRP3 dry snuff	4,831	0,143	2,96	0,851	17,62	10

CRP: CORESTA Reference Product

Supplementary information related to the description of the types of smokeless tobacco can be found in the Smokeless Tobacco Glossary (2.1).

**FIGURE 2: Repeatability (r) and reproducibility (R) of nitrate determination (2010 CSTS Collaborative Study)**



## APPENDIX 1

The concentration of the hydrazinium sulphate - copper sulphate reagent (4.4) must be optimized when initially setting up the instrument and when a fresh batch of hydrazinium sulphate is used.

### **1. Standard Nitrite Solutions**

- 1.1. Stock Solution:** Dissolve 0,900 g sodium nitrite ( $\text{NaNO}_2$ ) in distilled water and dilute to volume in a  $1 \text{ dm}^3$  volumetric flask. This solution contains 0,6 mg nitrite per  $\text{cm}^3$ .
- 1.2. Working Solution:** Pipette a  $25 \text{ cm}^3$  aliquot of the stock solution into a  $100 \text{ cm}^3$  volumetric flask and dilute to volume with distilled water. This solution contains  $150 \mu\text{g}$  nitrite per  $\text{cm}^3$ .

### **2. Optimisation of the Hydrazinium Sulphate Reagent**

- 2.1.** Dilute  $0,75 \text{ cm}^3$  of the copper sulphate stock solution (4.3) to  $1 \text{ dm}^3$  with distilled water.
- 2.2.** Dissolve 0,5 g hydrazinium sulphate in distilled water and dilute to  $100 \text{ cm}^3$  in a volumetric flask.
- 2.3.** From a burette, dispense in  $1,0 \text{ cm}^3$  increments,  $1,0$  to  $10,0 \text{ cm}^3$  aliquots of the hydrazinium sulphate solution (Appendix 1, 2.2) into  $25 \text{ cm}^3$  volumetric flasks and dilute to volume with distilled water. These solutions contain, in  $0,2 \text{ g per dm}^3$  increments,  $0,2$  to  $2,0 \text{ g}$  hydrazinium sulphate per  $\text{dm}^3$ .
- 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 coloured complex 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)

