

## CORESTA RECOMMENDED METHOD N° 62

### DETERMINATION OF NICOTINE IN TOBACCO AND TOBACCO PRODUCTS BY GAS CHROMATOGRAPHIC ANALYSIS

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#### 0. INTRODUCTION

During the development of this CORESTA Recommended method, inter-laboratory tests have been conducted on two different principles for the determination of the nicotine content of raw tobacco and tobacco taken from finished products:

- The gas-chromatographic procedure using MTBE as a solvent, and
- The gas-chromatographic procedure using n-hexane as a solvent.

In the analysis of cut filler and leaf samples, no significant difference was noted between the results obtained by the two different methods, though for smokeless tobacco products, there was a small bias [2.2].

#### 1. FIELD OF APPLICATION

The method is applicable to raw tobacco as well as tobacco taken from finished products. The method is applicable for nicotine contents ranging at least from a mass fraction of 8 mg/g to 41 mg/g by dry weight (2.2). The method is applicable to any type of tobacco sample whose particle size has been reduced to totally pass through a 4 mm screen. Cut filler from cigarettes need not be reduced further in size.

#### 2. NORMATIVE REFERENCES

- 2.1. CORESTA Recommended Method N° 39:1994: *Determination of the purity of nicotine and nicotine salts by gravimetric analysis -Tungstosilicic acid method.*
- 2.2. Franke, J.E., Bennett, C.B., Davis, R.E., Thomsen, H.V., Johnston, K.S., and Shanmugan, S.M.: *Determination of Nicotine in Tobacco: Collaborative Study*; Beitr. Tabakforsch 19 (2001), 251-265.
- 2.3. DIN 10373:2003 Untersuchung von Tabak und Tabakerzeugnissen – Bestimmung des Nikotingehalts – Gaschromatographisches Verfahren.
- 2.4. ISO 6488 Tobacco and tobacco products – Determination of water content – 1. Karl Fischer method, - 2. Gas-chromatographic method

### 3. PRINCIPLE

The nicotine content of a sample of tobacco or a tobacco product is determined by liquid/liquid extraction into an organic extraction solvent containing an internal standard, followed by gas chromatographic (GC) analysis with flame ionization detection (FID).

### 4. APPARATUS

Normal laboratory apparatus and in particular, the following items.

- 4.1. Volumetric flasks, of capacities 50 ml, 100 ml, and 250 ml.
- 4.2. Extraction vessels, several different styles of can be utilized, including but not limited to: 100 ml Pyrex bottles (51,7 x 94,5 mm) with crimp seals and septa, 100 – 250 ml Erlenmeyer flasks with stoppers, and 25 mm x 200 mm culture tubes with teflon lined caps.
- 4.3. Linear shaker (configured to hold the extraction vessels in a horizontal position), or equivalent shaker.  
**Note:** If linear shaker is not available, a wrist action shaker using 250 ml stoppered Erlenmeyer flasks can be substituted. Values for nicotine are equivalent to those obtained from the linear shaker.
- 4.4. Capillary gas chromatograph (GC), equipped with a flame ionization detector, a split inlet system, and a data station or integrator for data collection.
- 4.5. Capillary gas chromatography column capable of distinct separation of the peaks for the solvent, internal standard, nicotine and other tobacco components, typically a (5% phenyl)-methylpolysiloxane (*MTBE method*) or a (50% phenyl)-methylpolysiloxane column (*hexane method*). Use of an inlet liner packed with glass wool may aid in extending the useful lifetime of the capillary column.

### 5. REAGENTS

Use only reagents of recognized analytical grade. Reagents specific to each analytical approach are identified as either (*MTBE method*) or (*Hexane method*).

- 5.1. Carrier gas: helium, nitrogen or high purity hydrogen
- 5.2. (-)-Nicotine >99% purity
- 5.3. Nicotine salicylate >99% purity, possible alternative to the use of Nicotine (*Hexane method*)
- 5.4. 5N Sodium hydroxide solution, mass concentration  $\rho$  (NaOH) = 200 g/L, (*MTBE method*)
- 5.5. 8N Sodium hydroxide solution, mass concentration  $\rho$  (NaOH) = 320 g/L, (*Hexane method*)
- 5.6. Methyl-t-butyl ether (MTBE), (*MTBE method*)
- 5.7. Quinoline >98% purity, internal standard (*MTBE method*)
- 5.8. n-hexane, with a maximum water content of 1,0 mg/ml, (*Hexane method*)
- 5.9. n-heptadecane or quinaldine (purity at least 99% of mass fraction), internal standard (*Hexane method*)

## 6. STANDARDS

Prepare a series of at least four nicotine standard solutions whose concentrations cover the range expected to be found in the test portion, as in the example given below. The standard solutions shall be made up fresh each time extraction solution is prepared. Transfer to injection vials and cap immediately.

### MTBE method

- 6.1. Internal standard stock solution (40 mg/ml) Weigh approximately 10 g of quinoline (5.7) to 0,01 g accuracy into a 250 ml dark volumetric flask and dilute to volume with MTBE. This solution will be used for preparation of the extraction solution.
- 6.2. Extraction solution (0,40 mg/ml) To a 2000 ml volumetric flask pipette 20,0 ml of internal standard (6.1) and dilute to volume with MTBE. This solution will be used for preparation of the nicotine standards.
- 6.3. Nicotine stock solution (10,00 mg/ml) Weigh approximately 1 g of nicotine (5.2) to 0,0001 g accuracy into a 100 ml volumetric flask and dilute to volume with the MTBE extraction solution (6.2).
- 6.4. Nicotine standards To prepare a nicotine standard corresponding to concentration of 0,8 mg/ml, pipette 4,0 ml of the nicotine stock solution (6.3) to a 50 ml volumetric flask and dilute to volume with extraction solution (6.2). To obtain subsequent nicotine concentrations equivalent to 0,60 mg/ml, 0,40 mg/ml, 0,20 mg/ml and 0,10 mg/ml, pipette 3,0, 2,0, 1,0 and 0,50 ml of the nicotine stock solution into a 50 ml volumetric flask each and dilute to volume with extraction solution (6.2).

### Hexane method

- 6.5. Extraction solution (0,500 mg/ml) 1000 ml of *n*-hexane (5.8) mixed with approximately 0,5 g of *n*-heptadecane or other internal standard (5.9) to 0,001 g accuracy.
- 6.6. Nicotine stock solution (4,500 mg/ml) Weigh approximately 0,450 g of nicotine salicylate (5.3) or 0,240 g of nicotine (5.2) to 0,0001 g accuracy in a 200 ml Erlenmeyer flask and dissolve in 50 ml of water. Subsequently add 100 ml of extraction solution (6.5) and 25 ml of sodium hydroxide solution (5.5). Shake the two-phase mixture obtained vigorously for  $60 \pm 2$  min. using a shaker (4.3). Care should be taken to mix the phases well. Subsequently separate the supernatant organic phase and store the parent solution obtained in a dark glass bottle at 4°C to 8°C.
- 6.7. Nicotine standards Dilute 1,00 ml, 2,00 ml, 3,00 ml, 5,00 ml and 8,00 ml of the parent solution respectively to 20 ml with extraction solution (6.5). With a content of 0,450 g of nicotine salicylate, the standard solutions cover a range of approx. 0,5% to 3% of the mass fraction of nicotine in the tobacco. The standard solutions are to be stored at 4°C to 8°C with exclusion of light.

Solvents and solutions that are stored at low temperatures must be adjusted to room temperature before use.

## 7. PROCEDURES

### 7.1. *Sample Handling*

Combine and mix sufficient tobacco to constitute at least 100 g for each test subsample. If size reduction is employed, the sample should be reduced enough to pass through a 4 mm screen.

### 7.2. *Sample Preparation – MTBE Method*

**7.2.1** Using an analytical balance, accurately weigh  $1,000 \pm 0,020$  grams of prepared tobacco sample into extraction vessel (4.2) and record weight. Pipette 7 ml of 5N NaOH into the vessel using a non-glass 10 ml volumetric dispenser, swirl to wet sample and allow to stand 15 minutes. Pipette 50,0 ml of extraction solution into the vessel using a 50 ml volumetric dispenser, cap vessel and tighten. Place vessels in linear shaker in horizontal position and shake for two hours. Remove vessels from shaker and place in vertical position to allow the phases to separate. Allow the solvent and sample to separate (maximum 24h), transfer aliquot from extraction vessel to a GC vial and cap.

**Note:** Steps 6.4 and 7.2.1 should be performed at the same temperature. For instance, if the extraction solution is dispensed cold (4 °C) in step 6.4, then the phase separation in 7.2.1 should also be performed cold (4 °C).

### 7.3. *Sample Preparation - Hexane Method*

**7.3.1** Depending on the expected nicotine content, 1 g to 2 g of the well mixed and ground test sample is weighed to 0,001 g accuracy into a 100 ml Erlenmeyer flask. The test sample is mixed with 20 ml of water, 40 ml of extraction medium (6.5) and 10 ml of sodium hydroxide solution (5.5) and shaken for 60 min. on a shaker (4.3) and good mixing of the phases should be ensured. After separating the supernatant organic phase in the Erlenmeyer flask, this phase is to be analyzed as rapidly as possible by gas chromatography. The Erlenmeyer flask may be placed in an ultrasonic bath to assist separation of the phases. If the sample needs to be stored, this is performed at 4°C to 8°C with exclusion of light.

#### 7.4. Gas Chromatography

- 7.4.1** Set up and operate the gas chromatograph (4.4), data station or integrator and autosampler (if used) according to the manufacturer's instructions. Ensure that the peaks for solvent, internal standard, and nicotine are well resolved. Condition the system just prior to use by injecting two 1,0 µl aliquots of a sample solution or a nicotine standard as a primer. Typical operating conditions are as follows:

*Gradient Temperature Program on a typical 0,32 mm or 0,25 mm ID by 30 m column (MTBE method)*

Carrier gas: helium  
Injection temperature: 250 °C  
Injection mode: split (40:1)  
Injection volume: 1,0 µl  
Column flow rate: 1,7 ml/min  
Initial temperature: 110 °C  
Initial hold time: 0 min  
Temperature ramp A: 10 °C/min  
Final temperature A: 185 °C  
Temperature ramp B: 6 °C  
Final temperature B: 245 °C  
Final hold time B: 10 min  
Detector: 250 °C  
Total analysis time: 28.5 min

*Isothermal Temperature Program on a typical 0,53 mm ID by 15 m column (hexane method)*

Carrier gas: helium, nitrogen, or hydrogen  
Injection temperature: 270 °C  
Injection mode: split 1:10  
Injection volume: 1,0 µl  
Column flow rate: 5 - 6 ml/min  
Column temperature: 170 °C  
Detector: 270 °C  
Total analysis time: 10 min

- 7.4.2** Optimize the GC conditions for analyte separation and sensitivity. Once optimized, the same GC conditions must be used for the analysis of all standards and samples, including the same injection volume of 1,0 µl.

#### 7.5. Calibration of the gas chromatograph

- 7.5.1** Inject an aliquot of each of the nicotine standards (6.4 MTBE method or 6.7 Hexane method) into the gas chromatograph. Record the peak areas (or height) of nicotine and the internal standard. Carry out the determination at least twice, with one series interspersed with the test portion injections.

- 7.5.2** Calculate the ratio of the nicotine peak to the internal standard peak ( $Y = A_{\text{nicotine}}/A_{\text{IS}}$ ) from the peak area (or height) data for each of the nicotine standards including the solvent blanks. Plot the graph of the concentrations of added nicotine (X axis) in accordance with the area ratios (Y axis). Calculate a linear regression equation ( $Y = a + bx$ ) from this data, and use both the slope (b) and the intercept (a) of the linear regression.

- 7.5.3** If the correlation coefficient  $R^2$  is less than 0,99, then the calibration should be repeated. If an individual calibration point differs by 10 % or more from the expected value (estimated by linear regression), it should be omitted. The signal (peak area or height) obtained for all test portions must fall within the working range of the calibration curve.

#### 7.6. Determination of the nicotine content of samples

- 7.6.1** Inject replicate aliquots of the test portion from the sample extracts. A minimum of two replicate determinations should be made under identical conditions.

- 7.6.2** Calculate the test portion ratio of the nicotine response/internal standard response ( $Y_t$ ) from the peak area (or height) data. Calculate the mass of nicotine for each test portion aliquot using the coefficients of the linear regression ( $m_t = (Y_t - a)/b$ )

The nicotine content,  $m_n$ , of the tobacco sample expressed in milligrams per gram, is given by the equation

$$m_n = \frac{m_t}{m_0}$$

where

$m_t$  is the mass of nicotine in the test portion (7.2.1 / 7.3.1), in mg;

$m_0$  is the mass of the test portion (dry weight), in g.

## 8. SPECIAL PRECAUTIONS

- 8.1.** After installing a new column, condition the column by injecting a tobacco sample extract on the column, using the described column conditions. Injections should be repeated until areas of IS and nicotine are reproducible. This will require approximately four injections.
- 8.2.** Recondition the chromatographic column when the instrument has been used infrequently and after replacing the glass liner. Glass liner and septum should be replaced after every 100 injections. (*MTBE method*).
- 8.3.** It is beneficial to purge high boiling point components from the column in between each large sample set. Typically, raising the temperature to 220 °C, for 30 minutes has been found to be sufficient. (*Hexane method*).
- 8.4.** When analyzing new tobacco products, extract product without IS to determine if any components co-elute with the IS. This interference could artificially lower the calculated values for nicotine.

## 9. REPEATABILITY AND REPRODUCIBILITY

An international collaborative study was conducted including sample types of leaf, cigarette cut filler, pipe tobacco, loose leaf chewing tobacco, and moist snuff. Both capillary and packed columns were used in this study. Statistical analysis results from 18 laboratories (MTBE method) and 14 laboratories (hexane method) were used to in accordance with ISO 5725 procedures (detailed in detail elsewhere [2.2]) to calculate mean values and standard deviations for repeatability ( $S_r$ ) and reproducibility ( $S_R$ ) as shown in tables 1 and 2. Although no significant difference between the methods was noted in the analysis of cut filler and leaf samples, there was a slight significant difference in the analysis of moist smokeless products [2.2].

**Table 1 — Results of Inter-laboratory tests - MTBE**

sample type	nicotine (mg/g)	$s_r$ (mg/g)	$s_R$ (mg/g)
Loose leaf chewing tobacco	8,5	0,10	0,31
Oriental leaf	12,7	0,21	0,39
Cigar	13,3	0,11	0,46
Pipe tobacco	14,1	0,34	0,43
Menthol cigarette	18,6	0,13	0,51
Cigarette	18,8	0,20	0,56
1R4F cigarette	20,0	0,17	0,52
Moist snuff wintergreen	22,9	0,12	0,75
Moist snuff 1	25,9	0,24	0,84
Moist snuff long cut	28,4	0,13	0,91
Moist snuff 2	30,7	0,47	1,98
Bright leaf	35,2	0,30	0,86
Burley leaf	41,2	0,27	1,12

**Table 2 — Results of Inter-laboratory tests - Hexane**

sample type	nicotine (mg/g)	$s_r$ (mg/g)	$s_R$ (mg/g)
Loose leaf chewing tobacco	8,8	0,22	0,46
Oriental leaf	12,7	0,51	0,76
Cigar	13,6	0,28	0,56
Pipe tobacco	14,2	0,42	0,64
Menthol cigarette	18,5	0,66	0,77
Cigarette	19,0	0,64	0,83
1R4F cigarette	20,2	0,37	0,93
Moist snuff wintergreen	23,1	0,40	1,05
Moist snuff 1	26,4	0,65	1,01
Moist snuff long cut	29,3	0,55	1,26
Moist snuff 2	31,5	0,57	1,45
Bright leaf	35,7	0,66	1,88
Burley leaf	41,3	0,79	2,28

Figure 1 — Repeatability ( $s_r$ ) and reproducibility ( $s_R$ ) for nicotine by gas-chromatography (MTBE)

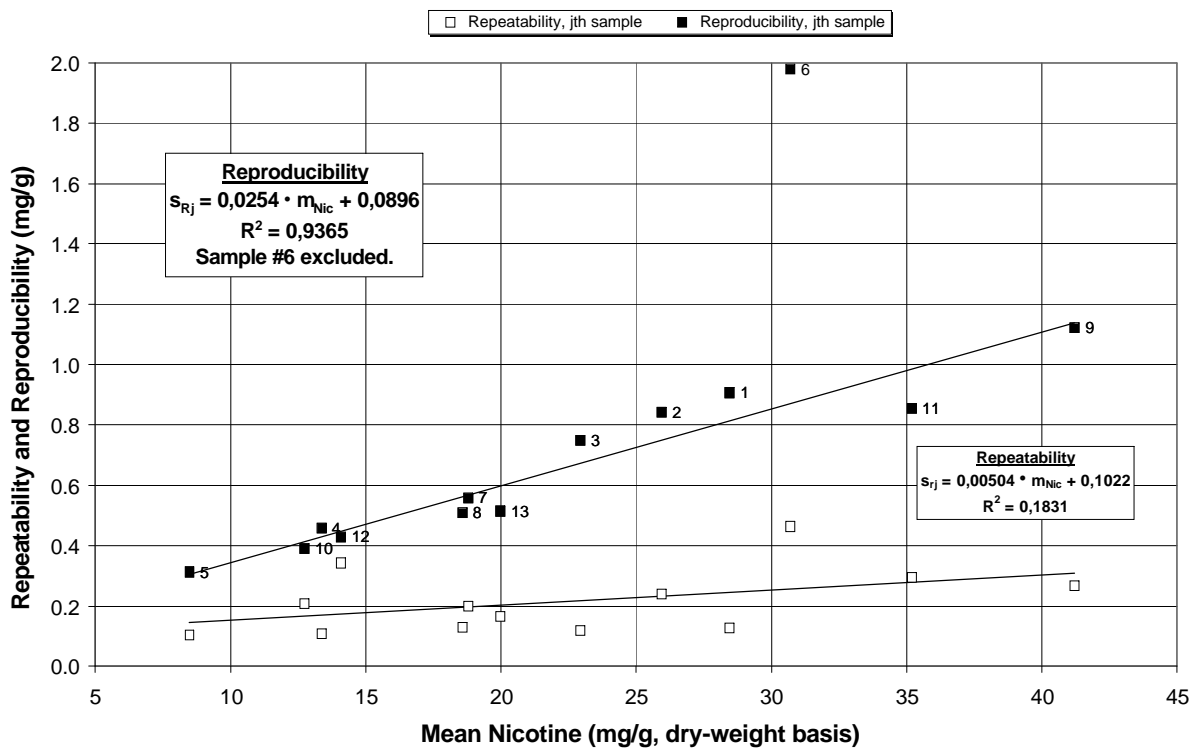
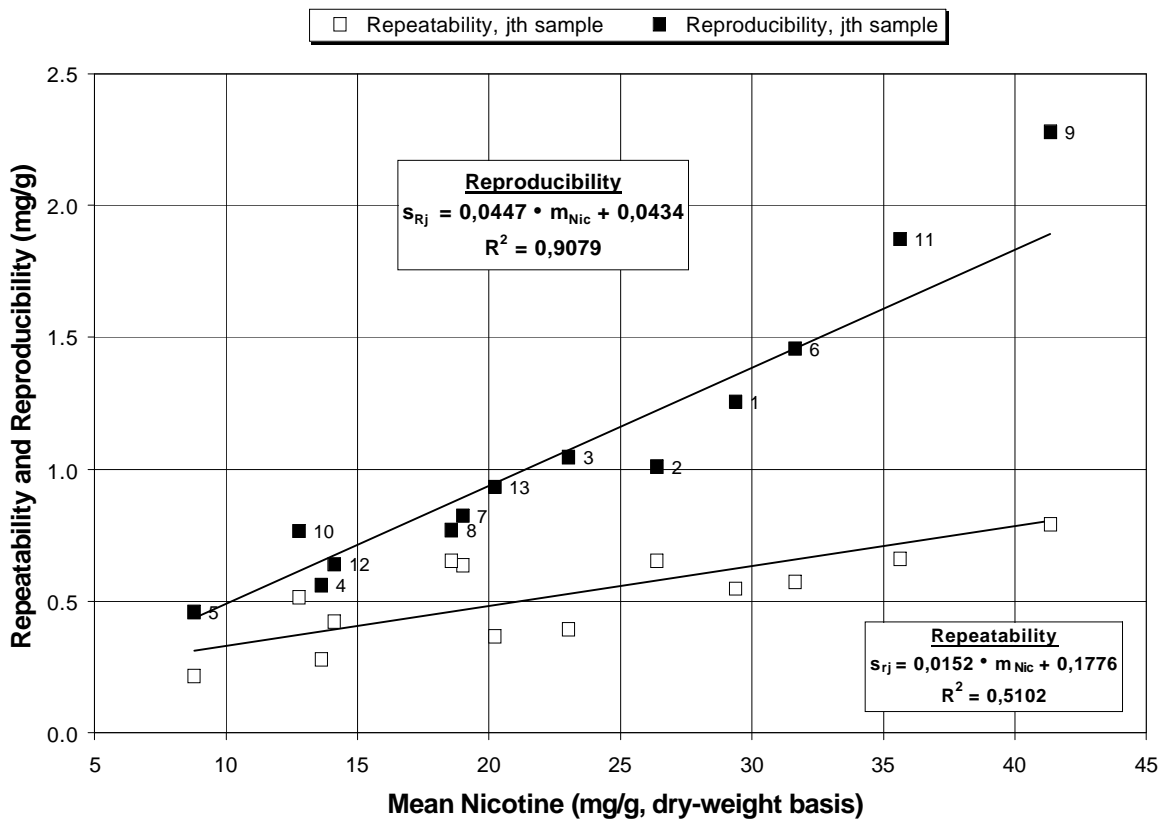


Figure 2 — Repeatability ( $s_r$ ) and reproducibility ( $s_R$ ) for nicotine by gas-chromatography (Hexane)



## **10. TEST REPORT**

The test report shall give the nicotine content of the sample as a mass fraction in percent and the method used. The test report shall also mention all operating conditions not specified in this CORESTA Recommended Method, or regarded as optional, as well as any circumstances that may have affected the result. It shall also include all details required for the identification of the sample.