Cooperation Centre for Scientific Research Relative to Tobacco

Routine Analytical Chemistry Sub-Group &
Tobacco and Tobacco Products Analytes Sub-Group

CORESTA Recommended Method No. 87

DETERMINATION OF NICOTINE IN TOBACCO PRODUCTS BY GC–MS

April 2020
CORESTA RECOMMENDED METHOD Nº 87

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DETERMINATION OF NICOTINE IN TOBACCO PRODUCTS BY GC−MS

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<th>Date of review</th>
<th>Information</th>
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<tr>
<td>April 2018</td>
<td>Version 1</td>
</tr>
<tr>
<td>April 2020</td>
<td>Version 2– Extension of scope to include very low nicotine (VLN) content</td>
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CORESTA RECOMMENDED METHOD N° 87

DETERMINATION OF NICOTINE IN TOBACCO PRODUCTS BY GC-MS

(April 2020)

0. INTRODUCTION

In 2016, the CORESTA Smokeless Tobacco Sub-Group (STS), now named Tobacco and Tobacco Products Analytes Sub-Group (TTPA), and Routine Analytical Chemistry Sub-Group (RAC) conducted a collaborative study for the determination of nicotine in cigarette filler, cigar filler, ground cigars, and smokeless tobacco products (STP) using gas chromatography with mass spectrometric detection (GC-MS) [1]. Nineteen laboratories participated in the study.

In 2019, TTPA conducted a collaborative study for the determination of nicotine in very low nicotine (VLN) moist smokeless tobacco, VLN cut and ground cigarette filler and traditional tobacco and tobacco products [2]. The intent of this study was to lower the calibration range for this Recommended Method for the analysis of VLN tobaccos and tobacco products. Eighteen laboratories provided data for CRM No. 87. This Recommended Method has been shown to be fit for the analysis of the aforementioned matrices. The repeatability and reproducibility values of this method have been assessed in general accordance with ISO 5725-2:1994.

1. FIELD OF APPLICATION

This Recommended Method is used to quantitatively determine the concentration of nicotine in traditional and VLN content tobacco and tobacco products using Gas Chromatography-Mass Spectrometry (GC-MS). The method is applicable to ground tobacco, cigarette filler, cigar filler, and smokeless tobacco products (e.g. snus, moist snuff, dry snuff, and chewing tobacco). The calibration range specified in the method is from 0,4 µg/ml to 400,0 µg/ml. This range corresponds to 0,064 mg/g to 64,0 mg/g when 0,25 g of tobacco is extracted in 40 ml of methanol.

2. NORMATIVE REFERENCES

2.1 CORESTA Smokeless Tobacco Sub-Group. Smokeless Tobacco Glossary

2.2 CORESTA Guide No. 11 - Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products

2.3 ISO 3696, Water for analytical laboratory use – Specification and test methods

3. PRINCIPLE

The nicotine content of tobacco products is determined by extracting the tobacco with sodium hydroxide and methanol prior to gas chromatography/mass spectrometric (GC-MS) analysis in the selective ion monitoring (SIM) mode. The results are reported as milligrams of nicotine per gram of tobacco as is, wet weight.
4. APPARATUS

Normal laboratory equipment is required, in particular, the following items:

4.1 Analytical balance, accurate to 0,0001 g
4.2 Syringe (5 ml) and syringe filter, 0,45 µm nylon or equivalent
4.3 Volumetric flasks of various capacities
4.4 Mechanical pipettes with disposable plastic tips 10 µl - 1000 µl
4.5 GC column: A polar, base-deactivated, polyethylene glycol (PEG) column (30 m × 0,25 mm id × 0,25 µm df)
4.6 GC-MS with data acquisition system and autosampler
4.7 Glass 4,0 mm I.D. deactivated split liner with glass wool
4.8 50-ml polypropylene centrifuge tube with screw-cap, or equivalent
4.9 Orbital shaker or wrist action shaker
4.10 Amber autosampler vials with PTFE-lined septa

5. REAGENTS

All reagents must be of recognized analytical grade or better.

5.1 (−)-Nicotine [54-11-5] ≥ 99 % purity
5.2 Quinoline [91-22-5] ≥ 98 % purity
5.3 Water, complying with grade 2 of ISO 3696, or better
5.4 Methanol, HPLC grade or better
5.5 Sodium Hydroxide (NaOH), 2 mol/l solution or pellets (97 % purity)

WARNING — The use of this method can involve hazardous materials, operations and equipment. This method does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this method to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

6. PREPARATION OF SOLUTIONS

6.1 2 mol/l Sodium Hydroxide:

6.1.1 Add approximately 500 ml water (5.3) to a 1-litre volumetric flask.
6.1.2 Carefully add 80 grams of solid sodium hydroxide pellets (5.5).
6.1.3 Mix carefully until dissolved.
6.1.4 Allow the solution to cool before bringing to volume with water (5.3).
6.1.5 Close the flask and mix well.
6.1.6 Store at ambient conditions.
7. STANDARDS

All standards should be prepared in amber, or light protected glassware.

Each laboratory should establish the most suitable calibration range depending on the equipment used and the type of samples to be analyzed. The standard preparation procedure is given as an example and is applicable for the range of the products in a collaborative study.

For each standard, calculate the exact concentration based on actual amount weighed.

7.1 Primary Nicotine Stock Solution: Purchase or prepare a 50 mg/ml nicotine stock solution in methanol. Add 2.5 g of (-)-Nicotine (5.1) to a 50-ml volumetric flask. Dilute to volume with methanol (5.4) and mix.

7.2 Secondary Nicotine Stock Solution (1 mg/ml Nicotine): Transfer 1.0 ml of the Primary Nicotine Stock Solution (7.1) to a 50-ml volumetric flask. Dilute to volume with methanol (5.4) and mix.

7.3 Internal Standard Stock Solution: Purchase or prepare a 50 mg/ml quinoline stock solution in methanol. Add 2.5 g of quinoline (5.2) to a 50-ml volumetric flask. Dilute to volume with methanol (5.4) and mix.

7.4 Working Internal Standard Solution (WISS, 4 mg/ml Quinoline): Transfer 2 ml of the Internal Standard Stock Solution (7.3) to a 25-ml volumetric flask. Dilute to volume with methanol (5.4) and mix.

7.5 Solvent Blank with Internal Standard (Methanol with 40 µg/ml of quinoline): This solution is used as a calibration level zero and also as a diluent for samples above the calibration range. Add approximately 15 ml methanol (5.4) to a 25-ml volumetric flask. Next, add 0.25 ml of WISS (7.4) to the 25-ml volumetric flask. Bring to volume with methanol (5.4) and mix well.

7.6 Working Standards: Prepare at least five calibration standard concentrations. Transfer the specified volumes of Secondary Nicotine Stock Solution (7.2) and Working Internal Standard Solution (7.4) according to the table below into 25-ml volumetric flasks. Bring to a final volume with methanol (5.4) and mix.

Table 1. Preparation of Working Calibration Standards

<table>
<thead>
<tr>
<th>Calibration Standards</th>
<th>Volume of Secondary Nicotine Stock (ml)</th>
<th>Volume of WISS (ml)</th>
<th>Final Conc. of Nicotine (µg/ml)</th>
<th>Final Conc. of Quinoline (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0,250</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>0,010</td>
<td>0,250</td>
<td>0,4</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>0,025</td>
<td>0,250</td>
<td>1,0</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>0,100</td>
<td>0,250</td>
<td>4,0</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>0,200</td>
<td>0,250</td>
<td>8,0</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>0,500</td>
<td>0,250</td>
<td>20,0</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>1,00</td>
<td>0,250</td>
<td>40,0</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>3,00</td>
<td>0,250</td>
<td>120,0</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>5,00</td>
<td>0,250</td>
<td>200,0</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>10,00</td>
<td>0,250</td>
<td>400,0</td>
<td>40</td>
</tr>
</tbody>
</table>
**Note:** This calibration range is suitable for products ranging from 0.064 mg to 64 mg of nicotine per gram of tobacco (mg/g). Prepare calibration level 0, i.e. solvent blank with internal standard, for use as a quality control sample to gauge possible carry over.

7.7 **Storage**

All standard solutions should be stored in the refrigerator at approximately 4 ºC. Standards have been shown to be stable for at least one month at these conditions. Each laboratory should determine the shelf life of the standards and internal standards under their storage conditions.

8. **SAMPLE PROCEDURE**

8.1 **Sample Handling**

Due to the small sample aliquot size (0.25 g), samples must be ground and homogenized prior to removing aliquots for analysis. Refer to CORESTA Guide No. 11, *Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products* for sample handling guidelines.

8.2 **Sample Preparation**

8.2.1 Loose tobacco: Weigh 0.25 g ± 0.05 g of the ground tobacco sample into a suitable extraction vessel (4.8). Record the weight to the nearest 0.0001 g.

8.2.2 Portioned Products: The recommended procedure for portioned products such as snus is to analyze the entire portion by cutting the pouch in half and adding the tobacco and pouch material to the extraction vessel.

8.2.3 Add 400 µl Working Internal Standard Solution (WISS, 7.4) to each sample vial followed by 4 ml of 2 mol/l NaOH.

8.2.4 Swirl to wet sample and allow to stand for approximately 30 minutes to allow the NaOH to completely wet the tobacco.

8.2.5 Add 40 ml methanol (5.4).

8.2.6 Shake samples on an orbital shaker (set to approximately 200 rpm, or a speed suitable for vigorous shaking) for approximately 30 minutes. Once sample extraction is complete, allow any solids to settle to the bottom of tubes (approximately 15 min).

8.2.7 Decant the sample extract into a 5-ml disposable syringe fitted with a 0.45 µm syringe filter taking care not to add the tobacco to the syringe. Typically, 4 ml to 5 ml of sample extract is decanted. Filter the sample extracts into one or more labelled amber autosampler vials.

**Note:** Samples with lower levels of nicotine may be analyzed by extracting more tobacco to bring the nicotine concentration of the test portion within the calibration range.

**Note:** Samples with higher levels of nicotine or samples that exceed the sample aliquot of 0.25 g tobacco (i.e. portioned products) may be analyzed, post extraction, by diluting test portions using the Solvent Blank with Internal Standard (7.5).
9. SAMPLE ANALYSIS

9.1 GC-MS Operating Conditions

Set up and operate the GC-MS system in accordance with the manufacturer’s instructions. The following conditions are suitable for analysis:

9.1.1 Injection Parameters

Mode: constant flow
Carrier Gas: Helium
Flow rate: 1,0 ml/min
Injection Mode: Split (60:1, ratio recommended)
Inlet temp: 230 °C
Injection volume: 1 µl injection

9.1.2 Oven Temperature

Initial 110 °C; hold for 1,0 min
Ramp 10 °C/min to 235 °C hold for 4,5 min
Run time: 18 min

9.1.3 MS Parameters

Transfer line: 230 °C
MS Quad 150 °C, MS Source 230 °C
Solvent delay 5,00 min

Table 2. MSD Quantitation/Qualifier Ions with Approximate Retention Times

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>Quantitative Ion</th>
<th>Qualifier Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoline</td>
<td>8,5</td>
<td>129</td>
<td>NA</td>
</tr>
<tr>
<td>Nicotine</td>
<td>7,8</td>
<td>84 or 162</td>
<td>162 or 84</td>
</tr>
</tbody>
</table>

9.2 System Suitability

The system performance must be evaluated for sensitivity, chromatographic performance, carry over and any other parameters necessary to ensure optimization of the GC-MS system.

9.3 Calibration of the GC-MS

9.3.1 Create an internal standard calibration method in the instrument operating software. A calibration curve is generated by calculating a linear regression model of the area ratios of nicotine to quinoline (y) as a function of the concentration ratios of nicotine to quinoline (x). Use both the slope and the intercept of the linear regression equation to process sample data. 1/x weighting is recommended.

Note: During the development of this method, a linear regression model using 1/x weighting was demonstrated to ensure that the low end of the calibration curve was not excessively biased by the high end of the calibration range. The user shall determine the level of weighting (typically 1/x or 1/x²) required in order to meet the acceptance criteria below.
9.3.2 Inspect the calibration model for the following acceptance criteria:

- The coefficient of determination ($r^2$) shall be greater than or equal to 0.99.
- Evaluate the difference between the measured and the true (expected) concentration of each calibration level used to create the linear regression model with the formula below:

$$%\text{Error} = \frac{x_i - x'_i}{x_i} \times 100$$

where:

- $x'_i$ = Measured concentration of analyte at calibration level $i$
- $x_i$ = True (expected) concentration of analyte at calibration level $i$

- If the difference, % Error, for calibration level 1 is greater than 20 % or other calibration levels are greater than 10 % from the expected concentration (measured by linear regression model), the problem shall be investigated and corrected.
- The responses from the calibration levels used to create the linear regression model (calibration curve) shall bracket the responses from all test portions.

9.4 Determination of the concentration of Nicotine

Inject each sample and calculate the area ratio of nicotine to quinoline for each sample and obtain the concentration ratio by comparing the area ratio with the calibration curve. The amount of nicotine in the tobacco samples is quantified by the internal standard method. The concentration of nicotine in the samples is reported in µg/ml by the chromatography software. Examples of chromatograms are shown in Appendix 1.

9.5 Determination of the nicotine content of samples

The concentration of nicotine expressed in milligrams per gram of tobacco is calculated with the formula below:

$$\text{Nicotine (mg/g)} = \frac{C}{M} \times Vol \times \frac{1\text{mg}}{1000\text{µg}}$$

where:

- $C$ = the concentration obtained from the calibration curve (µg/ml)
- $M$ = the mass of tobacco extracted (g)
- $Vol$ = the volume of methanol added to the sample (40 ml)

Note: A correction factor does not need to be applied to account for the NaOH added; the internal standard corrects for this small dilution effect.
10. REPEATABILITY AND REPRODUCIBILITY

In 2016, an international collaborative study was conducted using cigarette filler, cigar filler, ground cigars (filler, wrapper, and binder) and smokeless tobacco products\(^1\). A statistical analysis of the results from 18 laboratories was conducted in accordance with ISO 5725-2:1994 and ISO/TR 22971:2005. In 2019, the TTPA conducted a collaborative study involving 18 laboratories using an expanded calibration range to bracket VLN concentrations in order to expand the scope of the Recommended Method beyond traditional nicotine content tobacco to include very low nicotine (VLN) content ground tobacco, cigarette filler and moist smokeless tobacco\(^2\). Results were analyzed in basic conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. The mean values, %r, and %R are presented in Table 3. The value of ‘N’ is the number of the laboratories used to determine the statistics after the removal of outliers.

Table 3. Results of 2016\(^1\) and 2019\(^2\) Collaborative Study for Nicotine “as-is”

<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>N*</th>
<th>Mean (mg/g)</th>
<th>Repeatability</th>
<th>Reproducibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>r r (%)</td>
<td>R R (%)</td>
</tr>
<tr>
<td>1R5F(^1)</td>
<td>American blended cigarette filler, ground</td>
<td>16</td>
<td>15.97</td>
<td>0.65 4.0</td>
<td>2.44 15.3</td>
</tr>
<tr>
<td>1R6F(^1)</td>
<td>American blended cigarette filler</td>
<td>14</td>
<td>18.56</td>
<td>0.90 4.8</td>
<td>2.74 14.7</td>
</tr>
<tr>
<td>CM8(^1)</td>
<td>CORESTA Monitor 8 test piece</td>
<td>18</td>
<td>27.52</td>
<td>1.91 7.0</td>
<td>3.83 13.9</td>
</tr>
<tr>
<td>CRP1(^1)</td>
<td>Swedish-style snus pouch</td>
<td>16</td>
<td>10.36</td>
<td>1.40 13.5</td>
<td>3.18 30.7</td>
</tr>
<tr>
<td>CRP2(^1)</td>
<td>American-style loose moist snuff</td>
<td>15</td>
<td>12.98</td>
<td>0.93 7.2</td>
<td>2.09 16.1</td>
</tr>
<tr>
<td>CRP3(^1)</td>
<td>American-style dry snuff powder</td>
<td>15</td>
<td>22.10</td>
<td>1.66 7.5</td>
<td>2.60 11.8</td>
</tr>
<tr>
<td>Cigar filler #1(^1)</td>
<td>Flavoured ground cigar</td>
<td>15</td>
<td>8.46</td>
<td>0.42 5.0</td>
<td>1.29 15.3</td>
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<tr>
<td>Cigar filler #2(^1)</td>
<td>Dark air-cured ground cigar (wrapper and filler)</td>
<td>15</td>
<td>7.73</td>
<td>0.58 7.4</td>
<td>1.46 18.9</td>
</tr>
<tr>
<td>Mint MST(^1)</td>
<td>Flavoured American-style loose moist snuff</td>
<td>14</td>
<td>12.37</td>
<td>0.50 4.1</td>
<td>1.59 12.8</td>
</tr>
<tr>
<td>NIST SRM 3222(^2)</td>
<td>VLNC cigarette tobacco filler</td>
<td>15</td>
<td>0.207</td>
<td>0.05 25.9</td>
<td>0.11 54.9</td>
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<tr>
<td>VLNCF1(^2)</td>
<td>VLNC American blended cigarette filler - experimental prototype produced in limited quantities</td>
<td>17</td>
<td>0.399</td>
<td>0.04 8.7</td>
<td>0.11 28.0</td>
</tr>
<tr>
<td>VLNCF2(^2)</td>
<td>VLNC American blended cigarette filler - experimental prototype produced in limited quantities</td>
<td>17</td>
<td>0.928</td>
<td>0.05 5.8</td>
<td>0.24 26.0</td>
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<td>CRP1.1(^2)</td>
<td>Swedish-style snus pouch</td>
<td>18</td>
<td>7.48</td>
<td>0.89 11.9</td>
<td>1.49 19.9</td>
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<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>N*</th>
<th>Mean (mg/g)</th>
<th>Repeatability</th>
<th>Reproducibility</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>r</td>
<td>r (%)</td>
</tr>
<tr>
<td>CRP4.1²</td>
<td>American-style chopped loose-leaf chewing tobacco</td>
<td>18</td>
<td>9,13</td>
<td>0,56</td>
<td>6,2</td>
</tr>
<tr>
<td>CRP2.1²</td>
<td>American-style loose moist snuff</td>
<td>18</td>
<td>10,80</td>
<td>0,77</td>
<td>7,1</td>
</tr>
<tr>
<td>RT6²</td>
<td>Cigar filler, flavored, ground</td>
<td>16</td>
<td>11,24</td>
<td>1,42</td>
<td>12,6</td>
</tr>
<tr>
<td>RT8²</td>
<td>Cigar filler, unflavored, ground</td>
<td>16</td>
<td>14,64</td>
<td>0,98</td>
<td>6,7</td>
</tr>
<tr>
<td>CRP3.1²</td>
<td>American-style dry snuff powder</td>
<td>18</td>
<td>17,22</td>
<td>1,26</td>
<td>7,3</td>
</tr>
<tr>
<td>3R4F²</td>
<td>American blended cigarette filler</td>
<td>18</td>
<td>18,16</td>
<td>0,88</td>
<td>4,8</td>
</tr>
<tr>
<td>RT1²</td>
<td>1R6F Filler, American blended cigarette filler, ground</td>
<td>16</td>
<td>18,71</td>
<td>0,722</td>
<td>3,9</td>
</tr>
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</table>

* The number of laboratory data sets after removal of outliers.


11. TEST REPORT

The test report shall state the amount of nicotine in mg per gram tobacco (wet weight) and shall include all conditions which may affect the result. The report shall also give all details necessary for the identification of each sample. Moisture content may be determined on separate tobacco aliquots if it is necessary to present the final results on a dry-weight basis. The determination of moisture is detailed in CORESTA Recommended Method No. 76: Determination of Moisture Content (Oven Volatiles) of Tobacco and Tobacco Products.

12. REFERENCES


Appendix 1A - Example chromatogram of nicotine in calibration standard (100 µg/ml)

Appendix 1B - Example chromatogram of a cigar sample extract

Appendix 1C - Example chromatogram of CRP1 sample extract

Appendix 1D - Example chromatogram of a mint flavored US MST Smokeless tobacco product extract