FDA/OC/OCS/OARL

Laboratory Information Bulletin

Update to LIB No. 4550 Quantitation of Nicotine in Tobacco Products

Julio J. Arrecis, John A. Mathis, Rayman D. Stanelle and Ronald E. Hunter, Jr.
Food and Drug Administration
Office of Chief Scientist
Tobacco Products Laboratory
Atlanta, GA

ABSTRACT

A method for the determination of nicotine in select tobacco products is presented. The method uses a GC-MSD running in Selective Ion Monitoring (SIM) mode using a 25m x 0.32 mm x 0.53 µm Ultra-2® column. The column flow rate is 2.0 mL/min and uses a simple temperature ramp starting at 175°C and ending at 240°C. Samples are extracted using 2N sodium hydroxide and methyl tert-butyl ether and analyzed by direct injection. The range of the method is from 0.1 mg/g to 2 mg/g, providing a bridge to lower levels from previously reported methods for nicotine.

A. Introduction:

Nicotine extraction and analysis methodologies for tobacco products are well documented in the literature and in tobacco scientific work groups such as the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA). All these methodologies address the quantitation of nicotine at historical market product levels, which ranges from 2 mg/g to over 20 mg/g, wet weight. However, there were few methods available to address nicotine levels below the 2 mg/g level. The method presented here uses modification of a previously reported rapid GC-MS technique to provide quantitation range from 0.1 mg/g to 2 mg/g. In addition, the presented method uses less solvent per analysis, thereby reducing the cost of purchasing and disposal of reagents.

B. Experimental:

1. Equipment

NOTE: Equivalent equipment can be substituted.

A. GC/MS, Agilent Gas Chromatograph and Mass Spectrometer or Agilent GC Triple quadrupole MS operated in single quad selected ion monitoring (SIM) mode.
B. Agilent GC column, Ultra-2, 25m x 0.32 mm x 0.5 µm (P/N: 19091B-112).
C. Agilent split liners with Glass wool (P/N: 5183-4647).
D. Adjustable Pipettes, ranges: 10-100 µl, 100-1000 µl, 1-10 mL.
E. Analytical Balance.
F. 50 mL plastic centrifuge tubes with leak resistant caps compatible with methyl *tert*-Butyl Ether (MTBE).
G. Coffee or spice mills for grinding tobacco filler.
H. Laboratory shaker capable of at least 200 rotations per minute (rpm).
I. 10 mL bottle-top dispenser attached to a 1-liter bottle or larger.
J. Centrifuge with 50 mL conical tube carrier inserts.

2. Reagents:

A. Nicotine, 99%, CAS 54-11-5, from two different sources.
B. Methanol, HPLC grade or higher
C. Quinoline (internal standard), 97%, CAS 91-22-5.
D. 2N sodium hydroxide solution.
E. MTBE, HPLC grade or higher.

C. Procedure:

1. Stock Solution Preparation:

NOTE: Third party commercially prepared nicotine standards can be substituted.

A. **2 mg/mL Nicotine Calibration Spiking Standard:** Accurately weigh out 100 mg of the neat nicotine into a 50.0 mL volumetric flask. Dilute to volume with methanol. Mix well, and transfer to an amber bottle. Store at -10°C or lower.

B. **Initial Calibration Verification (ICV) stock:** This solution is prepared fresh with each batch of samples and discarded after preparation. Accurately weigh out 50 mg of the neat nicotine from a second source into a 25 mL volumetric flask. Dilute to volume with methanol. This solution is approximately 2 mg/mL nicotine.

C. **Continuing Calibration Verification (CCV) Solution:** This solution is the same as a standard prepared in Step 4.G. The level is selected to match a mid-level standard, either the same standard vial or an additional vial can be prepared. The CCV injections are used to bracket samples during the chromatographic sequence before and after unknown samples. For system

suitability, the calibration accuracy is verified for each CCV and at least five replicates are required to evaluate precision post analysis.

D. **Quinoline Internal Standard Solution:** Add approximately 1250 mg of neat Quinoline to a 50.0 mL volumetric flask, note that the accurate weight stated is not necessary to be within any error interval and the final concentration may be adjusted in the next dilution step, including the purity correction. Dilute to volume with MTBE. Mix well, and transfer to an amber bottle and label. Store at -4°C or lower. The solution concentration is approximately 25 mg/mL.
E. **Extraction Solution:** Pipette 1.0 mL of the approximately 0.025 mg/mL Quinoline Internal Standard Solution into a 1.0 L volumetric flask. Dilute to 1.0 L with MTBE, and mix well. Record the quinoline concentration with purity correction. This will accommodate 80-90 samples.
NOTE: This solution can be scaled to accommodate the number of samples to be analyzed.

2. Standard Preparation:

A. Choose six (6) 50 mL centrifuge tubes to prepare a solvent based standard curve and ICV. Add 10.0 mL of extraction solution to each tube, and prepare the standard curve as follows:

Calibration curve standards preparation and concentration ranges

Standard Level	Nominal concentration in tube	Spike Volume of nicotine stock solution	Corresponding nicotine in 1 gram of tobacco
Std 1	10 µg/mL	50 µL	0.1 mg/g
Std 2	20 µg/mL	100 µL	0.2 mg/g
Std 3	50 µg/mL	250 µL	0.5 mg/g
Std 4	100 µg/mL	500 µL	1.0 mg/g
Std 5	200 µg/mL	1000 µL	2.0 mg/g
ICV	150 µg/mL	750 µL	1.5 mg/g

B. Cap tightly, shake each tube vigorously, and set aside until analysis.

3. Sample Preparation:

A. The sample consists of material in the product that contains tobacco. Items that are excluded from the sample are the filters and cigarette papers.
B. Place the filler or entire tobacco product as appropriate into a centrifugal coffee/spice grinder. Approximately 10 cigarette sized products are needed get a uniform grind in the spice mill/grinder.
C. Homogenize the tobacco by pulsing the grinder on and off to obtain a rough powder that appears consistent. Finer grinds can be obtained but are not necessary for analysis. Avoid heating the sample during the grinding process.
D. Remove the homogenized tobacco and place into an airtight container. Store the ground product in a refrigerator until analysis. Freeze the homogenized samples at -10°C or lower for longer term storage.

E. Thoroughly clean the grinder between each sample by removing all visible tobacco residues followed by a wiping or rising with methanol. Allow equipment to dry before processing the next sample.
F. Products containing reconstituted sheet or paper-like tobacco do not homogenize well in a spice or coffee mill and should be separated from the filler prior to grinding. To reduce the particle size, cut the contents into strips approximately 1- 2 mm in width. Collect the pieces in an airtight container, and store in refrigerator until analysis. Freeze at -10°C or lower for longer term storage.

4. Sample Extraction:

A. If homogenized samples have been frozen or refrigerated, allow them to come to room temperature before weighing.
B. Weigh out 1.0 ± 0.05 grams of homogenized tobacco product into a 50 mL tube. Record the weight to nearest 0.001 g.
C. Select one of the following:
    1. Weigh out 1.0 grams of a suitable low level nicotine reference or previously analyzed product.
    2. Weigh out two (2) 1.0 g portions of suitable blank sample or low level nicotine matrix for spiking. Add an appropriate amount of the nicotine stock to one replicate to serve as a matrix spike in the range of 0.5 – 1.5 mg/g nicotine.
D. Reserve one empty 50 mL centrifuge tube to serve as a method blank that is carried through remaining extraction steps.
E. Add 5.0 mL of 2N sodium hydroxide solution to tubes. Swirl, vortex, or tap the tubes to wet as much of the tobacco sample as possible.
F. Wait at least 15 minutes.
G. Add 10.0 mL of extraction solution to all samples.
H. Cap the tubes tightly, and place on a shaker. Shake for 2 hours.
I. Remove vials from shaker, and centrifuge the extracts at 1500-2000 rpm for 3-5 minutes.
J. Remove the MTBE upper portion of the extract, and place in a 2 mL autosampler vial for analysis.
    NOTE: If necessary, reserve the MTBE extracts for analysis for up to 10 days. After analysis, the results of a stability study demonstrated that punctured vials can be left on the instrument and reinjected up to 4 days after initial injection.

5. GC/MS Analysis

A. **Instrument Conditions:**
    Injector: 25:1 spilt ratio at 250°C. Note: Adjust split ratio as needed.
    Column Flow Rate: 2.0 mL/min (Helium).
    Injection volume: 1 µL

GC Oven Program:
Initial 175°C, hold for 1 minute
Ramp 5 °C/min to 180°C, hold for 0 minutes
Fast Ramp to 240°C, hold for 1 minute
Total time: 4.1 minutes.

Mass Spectrometer Settings:
Auxiliary Temperature (MS transfer line) 250°C
MS Source Temperature: 230°C
MS Quad Temperature: 150°C
Scan Parameters: Selective Ion Monitoring (SIM) Mode
Solvent delay: 1.0 – 1.3 minutes, recommended.

Mass Spectrometer SIM Parameters

Quinoline	Dwell Time	Nicotine	Dwell Time
Target: 102 amu	50 msec	Target: 133 amu	50 msec
Qualifier: 129 amu	50 msec	Qualifier: 162 amu	50 msec

Additional settings for Triple Quad systems:
MS collection: MS1 SIM or MS2 SIM
Collision gas: off
(Agilent specific) Quench gas: On 2.25 mL/min

A. **Sample Analysis:**

Using the system software, prepare a calibration curve in µg/mL using quantitation ions 102 amu for the internal standard and 133 amu for nicotine. Use inverse concentration weighting and a linear curve fit.

Quantitate the samples, calibration verifications (ICV, CCV), and reference product or spiked matrix. To convert the extracts into mg/g, use the following formula:

$$\text{Nicotine (mg/g)} = \frac{\text{Instrument reading } \left(\frac{\mu g}{mL}\right) * 10 \text{ mL}}{\text{Sample weight (grams)}} * \frac{1 \text{ mg}}{1000 \text{ µg}}$$

The reporting range is 0.1 mg/g to 2.0 mg/g. Samples that exceed the upper range should be diluted with extraction fluid and re-injected. Samples with values below 0.1 mg/g should be reported as “Below LOQ”.

F. **Quality Control (QC)**

A. System Suitability

A. The standard curve must be linear and have a coefficient of determination (r^2) ≥ 0.995. The quantitated values of the standards used to make up the curve must be within 85-115% of the true value. If there is an obvious problem with a standard, investigate the cause and remove that standard from the curve fitting algorithm.
B. Each CCV must be within 85-115% of the true value. The RSD from the first 5 CCV injections must be less than or equal to 5%.
    1. If a CCV exceeds the 15% range, the samples within the previous acceptable bracketing CCV may be reported, but the samples following the last acceptable CCV cannot be reported.
    2. If a CCV accuracy (15%) or precision (5%) failure occurs, investigate the cause and determine the appropriate corrective action.
    3. ICV recovery must within 90-110%. If a ICV failure occurs, investigate the cause and determine the appropriate corrective action.
C. The reference product or matrix spike recovery must be within 85-115% of target value.
D. Sample injections must be within the calibration range. If dilutions are required, dilute the sample with extraction fluid.
E. If any QC requirements are not met, investigate the problem, and choose appropriate corrective action.

G. **Single Lab Validation Results**

The method selectivity was evaluated using matrix blanks, solvent blank, and spike recoveries. For linearity, seven (7) replicate preparations at each calibration curve point were analyzed to collect data for total error analysis. For accuracy and precision evaluation, the single lab validation was performed using ground tobacco filler from a king-sized VLN brand and NIST 1573a (Solanaceae Family substitute) as a blank spiking matrix. The matrix blank was spiked at 3 different levels. At each level, seven replicates of the matrix blank was spiked with nicotine (spiked NIST 1573a at approximately 0.2 mg/g, VLN tobacco was spiked at approximately 0.5 mg/g and at approximately 1.5 mg/g). For ruggedness, all extracts obtained from linearity and accuracy were injected on two different GCMS systems and one GC-MS-MS system running as a single quadrupole mode. A summary of results is presented below:

System Suitability:

- No peak was observed in the method and solvent blank injections.
- All ICV and CCV included in each analytical run passed the specification of 90%-110% and 85%-115% respectively.
- The coefficients of determination (r^2) for all analysis days on the three systems exceeded 0.995 with results in the range of 0.998 – 1.0.

Total Error Probability Evaluation:

- All five (5) calibration levels passed a total error probability calculation target of greater than 0.95 (>0.95) using an accuracy acceptance range of 85% - 115%.
- The Limit of Quantitation (LOQ) was 10 µg/mL, converted to tobacco filler weight is 0.10 mg/g, and was experimentally verified by replicate injections and acceptable total error probability greater than 0.95 (>0.95).
- All three levels of nicotine in matrix extracts passed a total error target of greater than 0.95 (>0.95) using an accuracy acceptance range of 85% - 115%.

H. Total Error Probability Calculation

Total error may be considered in terms of a probability function instead of individual terms of sensitivity, accuracy, and precision in isolation relative to method validation results. The probability function is the likelihood that the true value of an analyte at a target concentration will pass the acceptance criteria at a defined confidence interval. A 95% confidence interval is recommended for evaluating method performance. This probability is calculated as:

Total Error Probability = 1- t(qU,df) − t(qL,df),
where
t(q,df) = cumulative Student's t distribution with degrees of freedom (df) evaluated at quantile, q
qU = (Upper-Mean) / (RSD√(1 + 1/n))*
qL = (Lower-Mean)) / (RSD√(1 + 1/n))*
df = n − 1
n = number of replicate Standard solutions prepared
Upper = upper limit of the generally accepted range of the analyte (e.g., 102.0%)
Lower = lower limit of the generally accepted range of the analyte (e.g., 98.0%)
*Mean = % recovery = average measured value/known value*100%*
*RSD = Standard deviation of a measurement/value of the measurement*100%*

When calculated in this manner, a total error probability of 0.95 or greater indicates the precision and accuracy are suitable for the intended purposes of this method.

I. References:

1. Stanfill, Jia, Ashley and Watson, *Journal of Chromatographic Science,* Vol. 4, Nov/Dec 2009.

2. CORESTA Recommended Method Nº62, "Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis." February 2005.

3. Arrecis, Julio J. and McLeod, Marcela, LIB #4550, Quantitation of Low Level Nicotine in Combustible Tobacco Products, Food and Drug Administration, 2013.

4. CORESTA Recommended Method Nº87, “Determination of Nicotine in Tobacco Products by Gas Chromatography – Mass Spectrometry.” April 2020.