WHO TobLabNet
Official Method
SOP 12

Standard Operating Procedure for Determination of Nicotine Content in Smokeless Tobacco Products

No Tobacco Unit (Tobacco Free Initiative)
Tobacco Laboratory Network (TobLabNet)

World Health Organization

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Standard operating procedure for

Determination of nicotine content in smokeless tobacco products

Method:	Determination of nicotine content in smokeless tobacco products
Analytes:	Nicotine (3-[(2S)-1-methylpyrrolidin-2-yl]pyridine) (CAS # 54-11-5)
Matrix:	Smokeless tobacco products
Last update:	December 2021

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FOREWORD

Smokeless tobacco products are gradually attracting the interest of public health organizations. A request was made by the WHO Framework Convention on Tobacco Control (WHO FCTC) Conference of the Parties (COP) at its fifth session (Seoul, 2012) to identify options to regulate chemicals in smokeless tobacco products. This document is prepared in response to the request made by the COP at its seventh session (Delhi, 2016) to the WHO FCTC Secretariat to invite WHO to finalize the standard operating procedures (SOPs) for measuring nicotine and tobacco-specific nitrosamines as requested by decision FCTC/COP6(12) 2b.ii. In pursuance of this request, WHO organized a collaborative study involving its Tobacco Laboratory Network (TobLabNet) testing laboratories, which tested materials for which there was some chemical characterization, represented a range of common forms of smokeless tobacco products and differed in physical and chemical properties. The assessment of the applicability and adaptability of validated WHO SOPs to smokeless tobacco products and the recommended methods are presented in this SOP.

This document was prepared by members of the WHO TobLabNet as an analytical method SOP for measuring nicotine in smokeless tobacco products.

INTRODUCTION

In order to establish comparable measurements for testing tobacco products globally, consensus methods are required to measure specific parameters. WHO TobLabNet reviewed commonly used procedures for the determination of nicotine in smokeless tobacco products in order to prepare a procedure for a WHO TobLabNet SOP.

This SOP was adapted from WHO TobLabNet SOP 04 [**2.1**] to describe the procedure for determination of nicotine in smokeless tobacco products.

1. SCOPE

This method is suitable for quantitative determination of nicotine in smokeless tobacco products by gas chromatography (GC)–flame ionization detection.

2. REFERENCES

2.1 World Health Organization. Standard operating procedure for determination of nicotine in cigarette tobacco filler. Geneva, Tobacco Laboratory Network, 2017 (WHO TobLabNet SOP 04).

2.2 United Nations Office on Drugs and Crime. Guidelines on representative drug sampling. Vienna, Laboratory and Scientific Section, 2009 (http://www.unodc.org/documents/scientific/Drug_Sampling.pdf).

2.3 World Health Organization. Standard operating procedure for validation of analytical methods of tobacco product contents and emissions. Geneva, Tobacco Laboratory Network, 2017 (WHO TobLabNet SOP 02).

2.4 ISO5725-1. Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions.

2.5 ISO 5725-2: Accuracy (trueness and precision) or measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

3 TERMS AND DEFINITIONS

3.1 *Nicotine content*: Total amount of nicotine in smokeless tobacco products, expressed as milligrams per gram dry weight.

3.2 *Smokeless tobacco*: Tobacco-containing part of a smokeless tobacco product.

3.3 Smokeless *tobacco products*: Products made entirely or partly of leaf tobacco as the raw material that are manufactured to be used by sucking, chewing or snuffing (Article 1(f) of the WHO FCTC), including snus (dry and wet), chewing tobacco or a mixture of material originating from a tobacco plant.

3.4 *Laboratory sample*: Sample intended for testing in a laboratory, consisting of a single type of product delivered to the laboratory at one time or within a specified period.

3.5 *Test sample*: Product to be tested, taken at random from the laboratory sample. The number of products taken shall be representative of the laboratory sample.

3.6 *Test portion:* Random portion of the test sample to be used for a single determination. The number of products taken shall be representative of the test sample.

4. METHOD SUMMARY

4.1 Nicotine is extracted from the smokeless tobacco with a mixture of *n*-hexane, sodium hydroxide solution and water.

4.2 The organic layer is analysed by GC with a flame ionization detector.

4.3 The ratio of nicotine peak area to internal standard is compared on a calibration curve created by analysis of standards with known concentrations of nicotine to determine the nicotine content of each test portion.

5. SAFETY AND ENVIRONMENTAL PRECAUTIONS

5.1 Take routine safety and environmental precautions, as in any chemical laboratory activity.

5.2 The testing and evaluation of certain products with this test method may require the use of materials or equipment that could be hazardous or harmful to the environment; this document does not purport to address all safety aspects associated with its use. All persons using this method have the responsibility to consult the appropriate authorities and to establish health and safety practices as well as environmental precautions in conjunction with any existing applicable regulatory requirements prior to its use.

5.3 Special care should be taken to avoid inhalation or dermal exposure to harmful chemicals. Use a chemical fume hood, and wear an appropriate laboratory coat, gloves and safety goggles when preparing or handling undiluted materials, standard solutions, extraction solutions or collected samples.

6. APPARATUS AND EQUIPMENT

Usual laboratory apparatus, in particular:

6.1 Extraction vessels: Erlenmeyer flasks (100 mL) with stoppers, 100 mL Pyrex bottles with crimp seals and septa, 100 mL culture tubes with Teflon-lined caps or other suitable flasks.

6.2 Shaker (linear type) configured to hold the extraction vessels in position.

6.3 Capillary GC equipped with a flame ionization detector.

6.4 Capillary GC column capable of distinct separation of peaks for the solvent, the internal standard, nicotine and other tobacco components (e.g. Varian WCOT Fused Silica, 25 m × 0.25 mm ID; coating: CP-WAX 51).

6.5 Ultrasonic bath.

7. REAGENTS AND SUPPLIES

All reagents shall be of at least analytical reagent grade unless otherwise noted. When possible, reagents are identified by their Chemical Abstract Service (CAS) registry numbers.

7.1 Carrier gas: Helium [7440-59-7] of high purity (> 99.999%).

7.2 Auxiliary gases: Air and hydrogen [1333-74-0] of high purity (> 99.999%) for the flame ionization detector.

7.3 *n*-Hexane [110-54-3], GC grade, with a maximum water content of 1.0 g/L.

7.4 –(–)Nicotine [54-11-5] of known purity not less than 98%. Nicotine salicylate [29790-52-1] of known purity not less than 98% may be used.

7.5 Sodium hydroxide [1310-73-2] pellets.

7.6 Internal standard: *n*-heptadecane (purity > 98% of mass fraction) [629-78-7]. Quinaldine [91-63-4], isoquinoline [119-65-3], quinoline [91-22-5] or other suitable alternatives may be used.

8. PREPARATION OF GLASSWARE

8.1 Clean and dry glassware in a manner to avoid contamination.

9. PREPARATION OF SOLUTIONS

9.1 Sodium hydroxide solution (2 mol/L)

9.1.1 Weigh approximately 80 g of sodium hydroxide.

9.1.2 Dissolve measured sodium hydroxide in water, and dilute with water to 1 L.

9.2 Extraction solution (0.5 mg/mL)

9.2.1 Weigh approximately 0.5 g (to 0.001 g accuracy) of *n*-heptadecane or alternative internal standard.

9.2.2 Dissolve measured *n*-heptadecane or alternative internal standard in *n*-hexane, and dilute to 1 L with *n*-hexane.

10. PREPARATION OF STANDARDS

The method for preparing standard solutions described below is for reference purposes and can be adjusted if necessary.

10.1 Nicotine standard stock solution (2 g/L)

10.1.1 Weigh approximately 200 mg nicotine or 370 mg nicotine salicylate to 0.0001 g accuracy into a 200-mL (or 250-mL) Erlenmeyer flask.

10.1.2 Dissolve the measured nicotine in 50 mL of water.

10.1.3 Pipette 100 mL of extraction solution (**9.2.2**) and add 25 mL of 2 mol/L sodium hydroxide solution.

10.1.4 Shake the two-phase mixture obtained vigorously for 60 ± 2 min in a shaker. Take care to mix the phases well.

10.1.5 Draw out the supernatant organic phase for standard solution preparation. If necessary, store this solution, protected from light, at 4–8 °C.

10.2 Nicotine standard solutions

10.2.1 Prepare the calibration solutions using the standard stock solution prepared in **10.1.4**, according to the scheme given in Table 1.

10.2.2 Fill the volumetric flasks to the mark with extraction solution (**9.2.2**).

10.2.3 The standard solutions may be stored at 4–8 °C, protected from light.

10.2.4 Determine the final nicotine concentrations in the standard solutions from:

$$\text{Final concentration (mg/L)} = x * y * \frac{1000}{100 * 20}$$

Where *x* is the original weight (in mg) of nicotine as weighed in **10.1.1**, and *y* is the volume of the stock standard solution as pipetted in **10.2.1.**

The final nicotine concentrations in the standard solutions are shown in Table 1.

Table 1. Concentrations of nicotine in standard solutions

Standard	Volume of nicotine standard solution (2 g/L) (mL) (y)	Volume of internal standard solution (µL)	Total volume (mL)	Nicotine concentration in final mixed standard solution (mg/L)	Approximate level equivalent to unknown levels in smokeless tobacco (mg/g) when 1.5 g of sample taken
1	0.5	Not applicable, included in extraction solution	20	50	1.3
2	2.5		20	250	6.7
3	5.0		20	500	13.3
4	7.5		20	750	20.0
5	10.0		20	1000	26.7
6	15.0		20	1500	40.0

The range of the standard solutions may be adjusted, within the calibration range, depending on the equipment used and the samples to be tested, keeping in mind the possible effect on the sensitivity of the method.

All solvents and solutions must be adjusted to room temperature before use.

11. SAMPLING

11.1 Sample smokeless tobacco product according to laboratory sampling procedure. Alternative approaches may be used to obtain a representative laboratory sample in accordance with individual laboratory practice or when required by specific regulation or availability of samples.

11.2 Constitution of test sample

11.2.1 Divide the laboratory sample into separate units (e.g. packet, container), if applicable.

11.2.2 Take an equal amount of products for each test sample from at least √n[**2.2**] of the individual units (e.g. packet, container).

12. PRODUCT PREPARATION

12.1 Remove the smokeless tobacco product from the pack or container. Include quality control samples (when applicable).

12.2 Take an appropriate representative portion of the smokeless tobacco product according to individual laboratory practice (e.g., food analysis sampling approach may be applied).

12.3 Extract the smokeless tobacco from the product.

12.4 Combine and mix sufficient amounts of smokeless tobacco product samples to constitute about 0.5–2 g of homogeneous smokeless tobacco for each test sample.

13. PREPARATION OF THE SMOKING MACHINE

Not applicable

14. SAMPLE GENERATION

Not applicable

15. SAMPLE PREPARATION

15.1 Take 0.5–2 g of the sample and weigh it to 0.001-g accuracy into a 100-mL extraction vessel.

15.2 Mix the test sample with 20 mL of water, 40 mL of extraction solution (**9.2.2**) and 10 mL of 2 mol/L sodium hydroxide solution.

15.3 Shake the flask for 60 ± 2 min on a shaker.

15.4 Leave the sample flask to stand for another 20 min to allow visible, clear separation of the phases. After separation of the phases, analyse an aliquot of the organic (upper) phase as rapidly as possible by GC. If the phases do not separate clearly, place the Erlenmeyer flask in an ultrasonic bath until the phases are clearly separated.

15.5 Additional steps if solutions are found to be cloudy or murky:
(Option 1) To filter the sample through Whatman paper No. 41.
(Option 2) To centrifuge samples at 10 000 rpm or at a suitable relative centrifugal force (> 500) to ensure clear solutions.
(RCF > 500) to ensure clear solutions are obtained

15.6 If the sample is to be stored, keep it protected from light at 4–8 °C.

16. SAMPLE ANALYSIS

GC coupled with a flame ionization detector is used to quantify nicotine in smokeless tobacco products. The analytes are resolved from other potential interference on the GC column. Comparison of the area ratio of the unknowns with the area ratio of the known standard concentrations yields individual analyte concentrations.

16.1 GC operating conditions

GC column:	Varian WCOT fused silica, 25 m × 0.25 mm ID
Coating:	CP-WAX 51 or equivalent
Column temperature:	170 °C (isothermal)
Injection temperature:	270 °C
Detector temperature:	270 °C
Carrier gas:	Helium at a flow rate of 1.5 mL/min
Injection volume:	1.0 µL
Injection mode:	Split 1:10

Note: The operating parameters might have to be adjusted, depending on the instrument and column conditions and the resolution of chromatographic peaks.

16.2 Expected retention times

16.2.1 For the conditions described here, the expected sequence of elution will be *n*-heptadecane, nicotine.

16.2.2 Differences in, e.g., temperature, gas flow rate and age of the column, may alter retention times.

16.2.3 The elution order and retention times must be verified before the analysis of samples.

16.2.4 Under the above conditions, the expected total analysis time will be about 6 min. The analysis time may be extended to optimize performance.

16.3 Determination of nicotine

The sequence of determination will be in accordance with individual laboratory practice. This section gives an example of a sequence of operations for the determination of nicotine in smokeless tobacco products.

16.3.1 Inject an aliquot of n-hexane (**7.3**) to check for any contamination in the system or reagents.

16.3.2 Condition the system just before use by injecting two 1-µL aliquots of a sample solution as a primer.

16.3.3 Inject 1 µL extraction solution (**9.2**) and a test calibration standard solution under the same conditions as the samples to verify the performance of the GC system.

16.3.4 Inject 1 µL n-hexane (**7.3**) to check for any contamination of the system or reagents.

16.3.5 Inject in random order an aliquot of each nicotine standard solution into the GC.

16.3.6 Assess the retention times and responses (area counts) of the standards. If the retention times are similar (± 0.2 min) to the

retention times in previous injections and the responses are within 20% of typical responses in previous injections, the system is ready to perform the analysis. If the responses are outside specifications, seek corrective action according to your laboratory policy.

16.3.7 Record the peak areas of nicotine and the internal standard.

16.3.8 Calculate the relative response ratio (*RF*) of the nicotine peak to the internal standard peak ($RF = A_{nicotine} / A_{IS}$) for each of the nicotine standard solutions, including the solvent blanks.

16.3.9 Plot a graph of the concentration of nicotine (X axis) against the area ratios (Y axis).

16.3.10 The intercept should not be statistically significantly different from zero.

16.3.11 The standard curve shall be linear over the entire calibration range.

16.3.12 Calculate the calibration curve ($Y = a + bx$) by linear regression from these data and use both the slope (b) and the intercept (a) of the calibration curve for calculation of the results. If the coefficient of determination R^2 is < 0.99, the calibration should be repeated. If an individual calibration point differs by more than 10% from the calculated value (estimated from the calibration curve), the calibration point should be omitted.

16.3.13 Inject 1 µL of each of the quality control sample (**20.3**) and the test sample extracts (**15.4**), and determine the peak areas with the appropriate software.

16.3.14 The signal (peak area ratio) obtained for all test portions must fall within the working range of the calibration curve; otherwise, the test portion size must be adjusted or the test sample extract must be diluted.

See Annex 1 for representative chromatograms.

17 DATA ANALYSIS AND CALCULATIONS

17.1 For each test portion, calculate the ratio (Y_t) of the nicotine peak area to the internal standard peak area.

17.2 Calculate the nicotine concentration in mg/L for each test portion aliquot using the coefficients of the calibration curve ($m_t = (Y_t - a) / b$).

17.3 Calculate the nicotine content, m_n, of the tobacco sample expressed in milligrams per gram from the following equation:

$$m_n = \frac{m_t * V_e}{m_o * 1000}$$

where m_t is the concentration of nicotine in the test solution, in mg/L; V_e is the volume of the extraction solution used, in mL; and m_o is the mass of the test portion, in g.

18. SPECIAL PRECAUTIONS

18.1 After installing a new column, condition it by injecting a tobacco sample extract under the GC conditions described above. Injections should be repeated until the peak areas (or heights) of both the nicotine and the internal standard are reproducible. This will require approximately four injections.

18.2 It is recommended that high-boiling-point components be purged from the GC column after each sample set (series) by raising the column temperature to 220 °C for 30 min.

18.3 When the peak areas (or heights) for the internal standard are significantly higher than expected, it is recommended that the tobacco sample be extracted without internal standard in the extraction solution. This makes it possible to determine whether any component co-elutes with the internal standard, which would cause artificially lower content values for nicotine.

19. DATA REPORTING

19.1 Report individual measurements for each sample evaluated.

19.2 Report results as specified in the overall project specifications.

19.3 For more information, see WHO TobLabNet SOP 02 [**2.3**].

20. QUALITY CONTROL

20.1 Control parameters

Note: If the control measurements are outside the tolerance limits of the expected values, appropriate investigation and action must be taken.

Note: Additional laboratory quality assurance procedures should be carried out if necessary, in order to comply with the policies of individual laboratories.

20.2 Laboratory reagent blank

To detect potential contamination during sample preparation and analysis, include a laboratory reagent blank, as described in **16.3.4**. The blank consists of all reagents and materials used in analysing test samples and is analysed in the same way as a test sample. The content of the blank should be below the limit of detection.

20.3 Quality control sample

To verify the consistency of the entire analytical process, analyse a reference tobacco product such as Coresta Reference Products (CRP), when available, in accordance with the practices of individual laboratories.

21. METHOD PERFORMANCE SPECIFICATIONS

21.1 Limit of reporting

The limit of reporting is set to the lowest concentration of the calibration standards used, recalculated to mg/g (e.g., 1.3 mg/g with 50 mg/L as the lowest calibration standard concentration).

21.2 Internal quality control

Recovery of reference material is a surrogate measure of accuracy. Recovery is determined by measuring the level of nicotine in reference smokeless tobacco products. The recovery is calculated from the following equation.

$$\text{Recovery (\%)} = 100 \times (\text{analytical result} / \text{certified amount})$$

Table 2. Mean and recovery of nicotine content in smokeless tobacco products

Smokeless tobacco sample	Certified value (mg/g)	Mean nicotine content (mg/g)	Recovery (%)
CRP 1	8.0	7.40	92.4
CRP 2	12.0	10.58	88.2
CRP 3	17.0	16.54	97.3
CRP 4	9.0	9.03	100.4

21.3 Analytical specificity

The retention time of the analyte of interest is used to verify analytical specificity. An established range of ratios of the response of the component to that of the internal standard component of a quality control smokeless tobacco product is used to verify the specificity of the results for an unknown sample.

21.4 Linearity

The nicotine calibration curves established are linear over the standard concentration range of 50–1500 mg/L (1.3–40 mg/g).

21.5 Possible interference

The presence of eugenol or flavours can cause interference, as their retention times are similar to that of nicotine. This interference is most likely to occur with samples containing clove or added flavours. The laboratory can resolve the interference by adjusting the chromatographic parameters.

22. REPEATABILITY AND REPRODUCIBILITY

An international collaborative study conducted between September 2020 and March 2021, involving 13 laboratories and four samples (four CRP smokeless tobacco products), performed according to WHO TobLabNet Method Validation Protocol and this SOP gave the following values for this method.

The test results were analysed statistically in accordance with ISO 5725-1 [**2.4**] and ISO 5725-2 [**2.5**] to give the precision data shown in Table 3.

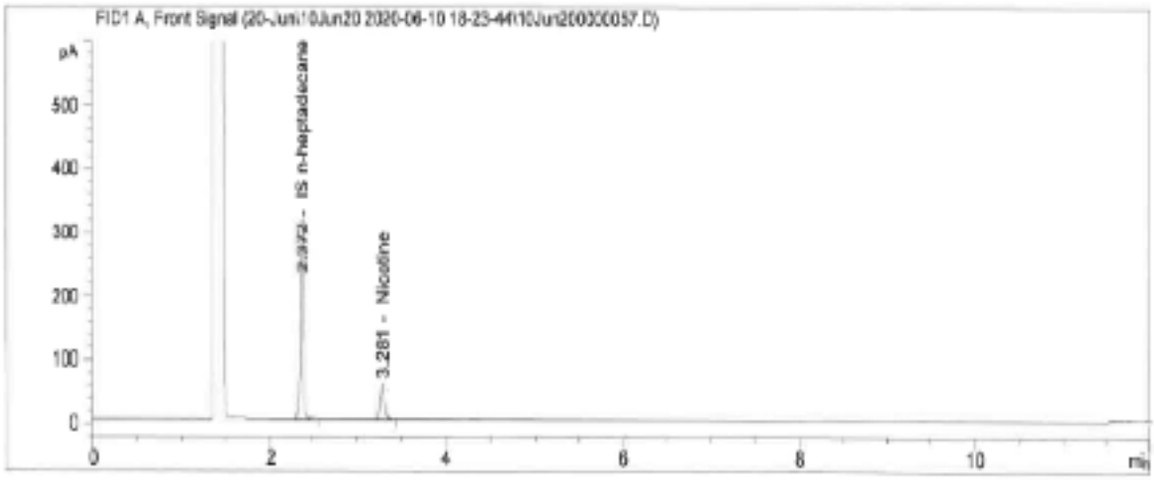
Table 3. Precision limits for determination of nicotine content (mg/g) in smokeless tobacco products

Reference tobacco product	n	$\hat{m}$	Repeatability limit (r)	Reproducibility limit (R)
CRP1	9	7.40	0.49	2.92
CRP2	8	10.58	0.38	2.75
CRP3	7	16.54	0.50	2.77
CRP4	10	9.03	0.49	1.90

APPENDIX 1.

Typical chromatogram obtained in the analysis of smokeless tobacco products for nicotine content

Fig. 1. Example of a chromatogram of a standard solution with a nicotine concentration of 250mg/L



This document was prepared by the No Tobacco Unit of the Health Promotion Department of the World Health Organization (WHO) and members of the WHO Tobacco Laboratory Network (TobLabNet) as an analytical method standard operating procedure (SOP) for measuring nicotine content in smokeless tobacco products.

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