STANDARD OPERATING PROCEDURE FOR VALIDATION OF ANALYTICAL METHODS OF TOBACCO PRODUCT CONTENTS AND EMISSIONS

Tobacco Free Initiative
Tobacco Laboratory Network (TobLabNet)
Standard operating procedure for method

Determination of tobacco-specific nitrosamines in mainstream cigarette smoke under ISO and intense smoking conditions

Method: Determination of tobacco-specific nitrosamines in mainstream cigarette smoke under ISO and intense smoking conditions

Analytes: 3-(1-Nitrosopyrrolidin-2-yl)pyridine (CAS# 16543-55-8)

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (CAS# 64091-91-4)

N-Nitrosoanatabine (CAS# 71267-22-6)

N-Nitrosoanabasine (CAS# 37620-20-5)

Matrix: Tobacco cigarette mainstream smoke particulate matter

Last update: June 2014
WHO TobLabNet
Official Method

SOP 02

Standard operating procedure for validation of analytical methods of tobacco product contents and emissions
Standard operating procedure for validation of analytical methods of tobacco product contents and emissions, WHO TobLabNet Official Method SOP02
ISBN 978-92-4-151206-0

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Printed in Switzerland
No.: SOP 02
Date: November 2016

World Health Organization
Tobacco Laboratory Network

Standard operating procedure for method

Validation of analytical methods of tobacco product contents and emissions

<table>
<thead>
<tr>
<th>Method:</th>
<th>Standard operating procedures for validation of analytical methods of tobacco contents and emissions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytes:</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Matrix:</td>
<td>Tobacco cigarette mainstream smoke and tobacco filler contents</td>
</tr>
<tr>
<td>Last update:</td>
<td>November 2016</td>
</tr>
</tbody>
</table>
No machine smoking regimen can represent all human smoking behaviour: machine smoking testing is useful for characterizing cigarette emissions for design and regulatory purposes, but communication of machine measurements to smokers can result in misunderstanding about differences between brands in exposure and risk. Data on smoke emissions from machine measurements may be used as inputs for product hazard assessment, but they are not intended to be nor are they valid as measures of human exposure or risks. Representing differences in machine measurements as differences in exposure or risk is a misuse of testing with WHO TobLabNet standards.
FOREWORD
This document was prepared by members of the World Health Organization (WHO) Tobacco Laboratory Network (TobLabNet) as a standard operating procedure (SOP) for the validation of analytical methods of tobacco cigarette mainstream smoke contents and cigarette tobacco filler contents.

INTRODUCTION
In order to establish comparable measurements for testing tobacco products globally, consensus methods are required for measuring specific contents and emissions of cigarettes. The Conference of the Parties (COP) to the WHO Framework Convention on Tobacco Control (WHO FCTC) at its third session in Durban, South Africa, in November 2008, recalling decisions FCTC/COP1(15) and FCTC/COP2(14) on the elaboration of guidelines for implementation of Articles 9 (Regulation of the contents of tobacco products) and 10 (Regulation of tobacco product disclosures) of the WHO FCTC, noting the information contained in the report of the working group to the third session of the Conference of the Parties on the progress of its work ... requested the Convention Secretariat to invite WHO’s Tobacco Free Initiative to ... validate, within five years, the analytical chemical methods for testing and measuring cigarette contents and emissions (FCTC/COP/3/REC/1).

Using the criteria for prioritization set at its third meeting in Ottawa, Canada, in October 2006, the working group on Articles 9 and 10 identified the following contents for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- nicotine
- ammonia
- humectants (propane-1,2-diol, glycerol (propane-1,2,3-triol) and triethylene glycol (2,2-ethylenedioxybis(ethanol))).

Measurement of these contents will require validation of three methods: one for nicotine, one for ammonia and one for humectants.

Using the criteria for prioritization set at the meeting in Ottawa mentioned above, the working group identified the following emissions in mainstream smoke for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
- N-nitrosonornicotine (NNN)
• acetaldehyde
• acrylaldehyde (acrolein)
• benzene
• benzo[a]pyrene
• 1,3-butadiene
• carbon monoxide
• formaldehyde

Measurement of these emissions with the two smoking regimens described below will require validation of five methods: one for tobacco-specific nitrosamines (NNK and NNN), one for benzo[a]pyrene, one for aldehydes (acetaldehyde, acrolein and formaldehyde), one for volatile organic compounds (benzene, 1,3-butadiene) and one for carbon monoxide).

The table below sets out the two smoking regimens for validation of the test methods referred to above.

<table>
<thead>
<tr>
<th>Smoking regimen</th>
<th>Puff volume (mL)</th>
<th>Puff frequency</th>
<th>Filter ventilation holes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO regimen: ISO 3308; Routine analytical cigarette smoking machine — definitions and standard conditions</td>
<td>35</td>
<td>Once every 60 s</td>
<td>No modifications</td>
</tr>
<tr>
<td>Intense regimen: Same as ISO 3308, but modified as indicated</td>
<td>55</td>
<td>Once every 30 s</td>
<td>All ventilation holes must be blocked 100% as described in WHO TobLabNet SOP 01.</td>
</tr>
</tbody>
</table>

This method SOP was prepared to describe the procedure for the determination of nicotine and carbon monoxide in mainstream cigarette smoke under intense smoking conditions.

1. **SCOPE**

This document describes the standard procedures for validating analytical methods used for analysing tobacco cigarette mainstream smoke contents and cigarette tobacco filler contents.
2. REFERENCES

2.1 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 Tobacco products: Products entirely or partly made of leaf tobacco as the raw material that are manufactured to be used for smoking, sucking, chewing or snuffing (Article 1(f) of the WHO FCTC).

3.2 Cigarette tobacco filler: Tobacco-containing part of a cigarette, including reconstituted tobacco, stems, expanded tobacco and additives.

3.3 ISO Regimen: Parameters used to smoke tobacco products which include 35-mL puff volume, 60-second puff interval, 2-second puff duration and no blocking of the filter ventilation holes.

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 from the test sample to be used for a single determination. The number of products taken shall be representative of the test sample.

3.7 Repeatability: All measurements carried out on each individual test sample shall be carried out under repeatability conditions, i.e. within a short interval, using the same instrument and by the same operator. Measurements of different test samples may be carried out on different days, if needed.

3.8 Reproducibility: All measurements are carried out under reproducibility conditions, i.e. allowing for differences between days, operators, laboratories, instruments, reagents and environmental conditions. Measurements are carried out on the same or identical materials using the same methodology.

4. METHOD SUMMARY

4.1 For each individual analytical method to be validated, a leading laboratory will be appointed by WHO's TobLabNet.

4.2 A Standard Operating Procedure and validation study protocol are written for each analytical method to be validated and sent to all participating laboratories.
4.3 For the purposes of validation of analytical methods, products are chosen whenever possible to cover the range of design properties to be found worldwide, while using specific product varieties that are intended to be consistent from pack to pack. In general, products to be analysed include three reference cigarettes and two commercially available brand varieties. The specific commercial brand varieties change with the specific test(s) to be performed.

4.4 Testing of analytical methods for validation purposes consists of an initial evaluation of a single cigarette variety, followed by a full evaluation of four additional varieties. After confirmation of the initial evaluation results, the other samples are sent for analysis. The leading laboratory has the discretion to include, or not to include, an initial evaluation.

4.5 Data analysis and quality control are carried out according to the Standard Operating Procedure to be validated, or the validation study protocol, taking into account procedures as defined at each laboratory.

4.6 Individual sample results are reported to WHO’s Tobacco Free Initiative. After assigning a code to each laboratory and removing all information that can lead to identification of a laboratory, the results are sent to at least two entities. These entities will evaluate the data for outliers and calculate repeatability and reproducibility independently from each other, according to ISO procedures. After completing the evaluation of the data, the results from the entities will be compared.

4.7 A report of the validation study will be written and the results will be included in the analytical Standard Operating Procedure.

5. SAFETY AND ENVIRONMENTAL PRECAUTIONS

5.1 Follow routine safety and environmental precautions, as in any chemical laboratory activity, taking into account the specific safety precautions as described in the analytical SOP to be validated.

5.2 The testing and evaluation of certain products with the test method to be validated may require the use of materials or equipment that could be hazardous or harmful to the environment and this document does not purport to address all the safety aspects associated with its use. All persons using this method, or the analytical method to be validated, have a responsibility to consult with 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. LEADING LABORATORY

For each analytical method to be validated, WHO’s TobLabNet will appoint a laboratory from its members to act as leading laboratory for that specific validation study.

The leading laboratory will:

- Develop or select the appropriate analytical procedure, write the SOP and validation protocol
- Lead the validation study and track progress
- Provide support to participating laboratories if needed
- Generate a validation study report

7. STANDARD OPERATING PROCEDURE

A detailed description of all operations, including reagents, supplies and any equipment needed are documented in the SOP. The SOP also contains any special precautions that are required and a summary of method performance specifications, including repeatability and reproducibility.

The lay-out of the SOP will be based on a standardized template – see Annex 1.

Training in the use of the smoking machine and other analytical equipment is important for successful performance of the analytical SOP. For those not experienced in operating smoking machines or analysis using the analytical method(s) for measuring tobacco product emissions and contents, training is available through the WHO TobLabNet.

8. VALIDATION STUDY PROTOCOL

A study protocol is sent to all participants of the validation study of an analytical method. The study protocol should always contain details of the following:

- Timeline of the validation study
- Number and type of samples to be analysed
- Number of replicates per sample
- Instruction on how to report the results, preferably using an Excel template
- Detailed schedule of smoke plans (if applicable)

Examples of study protocols are given in Annex 2 (cigarette tobacco filler contents) and Annex 3 (mainstream cigarette smoke contents).

9. SAMPLES

9.1 Description

9.1.1 Cigarettes are sampled randomly from the products available, mixed, and repackaged before shipping for analysis.
9.1.2 For the purposes of method validation only, products are checked for physical anomalies. All products having visual physical anomalies are excluded from despatch to participants.

9.2 Shipment and Receipt

9.2.1 TobLabNet will ship cigarettes of each cigarette variety to each participating laboratory. Sufficient samples will be sent to meet the measurement requirements plus additional spare samples.

9.2.2 Additional samples, beyond those required for the testing, are intended to provide extras in case of problems encountered during the testing.

9.2.3 TobLabNet will inform all participating laboratories that samples have been sent.

9.2.4 Upon receipt, each laboratory will notify TobLabNet by e-mail of the receipt of samples and any problems with or damage to the materials.

9.2.5 If problems were encountered during shipment, TobLabNet will send replacement samples as soon as practicable after notification.

9.2.6 After receipt of samples, each laboratory will complete analysis and report the data within the study protocol specified time limit.

9.2.7 Cigarettes should be carefully handled to avoid causing damage.

9.2.8 Cigarettes that are obviously bent, torn, crushed, or missing significant amounts of tobacco should be discarded.

9.3 Storage

Samples can be stored at room temperature for up to three months. Beyond this period, samples are to be stored in the original packaging or in airtight containers in a freezer at −20 °C or lower.

10. DATA REPORTING

10.1 Report results as specified in the SOP or study protocol to WHO’s Prevention of Noncommunicable Diseases. For consistency and efficient statistical evaluation of the results, a Microsoft Excel template is recommended for reporting. If Microsoft Excel or compatible spreadsheet software is not available, laboratories can also report data electronically in an ASCII format, or if this is not possible, by hard copy. If available, an Excel file template will be sent to each laboratory by e-mail.

10.2 To maintain confidentiality, a laboratory code will be issued to each laboratory and all information that can lead to identification of a participating laboratory will be deleted by WHO’s Tobacco Free Initiative (TFI). The assigned laboratory code should accompany each data record when results are reported.
10.3 Results below the limit of reporting (LOR) should be reported as < LOR. Do not leave data fields blank when results are below the LOR.

10.4 Each data record will contain the information as required in the study protocol, with a minimum of:

10.4.1 Laboratory code
10.4.2 Sample ID
10.4.3 Analyte 1 result
10.4.4 Analyte 2 result
10.4.5 Analyte n result
10.4.6 Specific analytical method
10.4.7 Comments

Do not report data with quality control or quality assurance parameters out of control.

11. DATA ANALYSIS
The coded results are sent to at least two entities by WHO’s TFI for statistical evaluation. The entities will independently evaluate the data according to ISO procedures.

11.1 Data analysis will follow procedures as described in ISO 5725-2 [2.1].

11.2 Identification of outliers will be performed as described in ISO 5725-2. Results identified as outliers will not be included in the final statistical determination of method repeatability and reproducibility.

11.3 If there is any inconsistency in data, or if data has been identified as an outlier, the laboratory reporting this data will be requested to check for irregularities via WHO’s TFI to maintain confidentiality.

11.4 Repeatability and reproducibility will be calculated independently by the entities according to ISO procedures ISO 5725-1 [13.2] and ISO 5725-2 [13.3] to give the precision data. After completing the evaluation of the data, the results of the entities will be compared.

11.5 The results of the statistical evaluation will be sent to WHO and the lead laboratory.

12. VALIDATION REPORT
An extended report will be made of the validation study, including:
- Outline of the study
- Overview of participants and equipment used
- Description of data analysis methods used
- Summary of data analysis;
  - anomalous data
  - missing data
  - mean values and standard deviations
  - consistency data (Mandel’s h and k statistics)
  - outlier tests (Cochran’s and Grubbs’ test)
  - exploratory data analysis
  - precision analysis (repeatability/reproducibility variance and precision limits)

The report will be submitted to WHO’s TFI for publication.

13. **BIBLIOGRAPHY**


13.2 ISO 5725-1. Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions.

13.3 ISO 5725-2. Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.
Annex 1. Example / Template Standard Operating Procedure

WHO TobLabNet
Official Method
SOP 03

Standard operating procedure for determination of **NAME CONTENT(S)** in **Mainstream Cigarette Smoke under ISO and intense smoking conditions** / **Cigarette Tobacco Filler**
World Health Organization
Tobacco Laboratory Network

Standard operating procedure for method

Determination of NAME CONTENT(S) in mainstream cigarette smoke under ISO and intense smoking conditions / cigarette tobacco filler

Method: Determination of NAME CONTENT(S) in mainstream cigarette smoke using ISO and intense smoking conditions / cigarette tobacco filler

Analytes: CONTENT (CAS # )
CONTENT (CAS # )

Matrix: Tobacco cigarette mainstream smoke particulate matter / cigarette tobacco filler

Last update: Month yyyy
No machine smoking regimen can represent all human smoking behaviour: machine smoking testing is useful for characterizing cigarette emissions for design and regulatory purposes, but communication of machine measurements to smokers can result in misunderstanding about differences between brands in exposure and risk. Data on smoke emissions from machine measurements may be used as inputs for product hazard assessment, but they are not intended to be nor are they valid as measures of human exposure or risks. Representing differences in machine measurements as differences in exposure or risk is a misuse of testing with WHO TobLabNet standards.
FOREWORD

This document was prepared by members of the World Health Organization (WHO) Tobacco Laboratory Network (TobLabNet) as an analytical method standard operating procedure (SOP) for measuring CONTENTS in mainstream cigarette smoke under ISO and intense smoking conditions / cigarette tobacco filler.

INTRODUCTION

In order to establish comparable measurements for testing tobacco products globally, consensus methods are required for measuring specific contents and emissions of cigarettes. The Conference of the Parties (COP) to the WHO Framework Convention on Tobacco Control (WHO FCTC) at its third session in Durban, South Africa, in November 2008, recalling its decisions FCTC/COP1(15) and FCTC/COP2(14) on the elaboration of guidelines for implementation of Articles 9 (Regulation of the contents of tobacco products) and 10 (Regulation of tobacco product disclosures) of the WHO FCTC, noting the information contained in the report of the working group to the third session of the Conference of the Parties on the progress of its work ... requested the Convention Secretariat to invite WHO’s Tobacco Free Initiative to ... validate, within five years, the analytical chemical methods for testing and measuring cigarette contents and emissions (FCTC/COP/3/REC/1).

Using the criteria for prioritization set at its third meeting in Ottawa, Canada, in October 2006, the working group on Articles 9 and 10 identified the following contents for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- nicotine
- ammonia
- humectants (propane-1,2-diol, glycerol (propane-1,2,3-triol) and triethylene glycol (2,2-ethylenedioxybis(ethanol))).

Measurement of these contents will require validation of three methods: one for nicotine, one for ammonia and one for humectants.

Using the criteria for prioritization set at the meeting in Ottawa mentioned above, the working group identified the following emissions in mainstream smoke for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

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This SOP was prepared to describe the procedure for the determination of NAME CONTENT(S) in mainstream cigarette smoke / cigarette tobacco filler.

1. SCOPE

This Standard Operating Procedure is suitable for the quantitative determination of NAME CONTENT(S) in mainstream (MS) cigarette smoke / cigarette tobacco filler: NAME ALL CONTENT(S) TO BE ANALYSED SEPARATELY by SPECIFY TECHNIQUE TO BE USED. When more contents (as requested by COP) are determined using this method: ADD “The COP has recommended measurements for …. and …. only …. The inclusion of information on …. and …. in this method is intended for those who choose to include these measurements. IF APPLICABLE.”
2. REFERENCES

2.1 ISO 3308: Routine analytical cigarette-smoking machine – Definitions and standard conditions

2.2 ISO 4387: Cigarettes – Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine

2.3 ISO 3402: Tobacco and tobacco products – Atmosphere for conditioning and testing

2.4 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

2.5 World Health Organization Tobacco Laboratory Network, Standard Operating Procedure for Intense Smoking of cigarettes (WHO TobLabNet SOP_01). IF APPLICABLE

2.6 World Health Organization Tobacco Laboratory Network, Standard Operating Procedure for validation of analytical methods of tobacco product contents and emissions (WHO TobLabNet SOP_02).

3. TERMS AND DEFINITIONS

3.1 AS NEEDED

3.2

3.3

3.4

3.5 Tobacco products: Products entirely or partly made of leaf tobacco as the raw material that are manufactured for smoking, sucking, chewing or snuffing (Article 1(f) of the WHO FCTC).

3.6 Intense Regimen: Parameters used to smoke tobacco products which include 55-mL puff volume, 30-second puff interval, 2-second puff duration and 100% blocking of the filter ventilation holes. IF APPLICABLE

3.7 ISO Regimen: Parameters used to smoke tobacco products which include 35-mL puff volume, 60-second puff interval, 2-second puff duration and no blocking of the filter ventilation holes. IF APPLICABLE

3.8 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.

3.9 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.
3.10 **Test portion**: Random portion from 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 **SUMMARY OF SELECTED ANALYTICAL APPROACH**

4.2

4.3

4.4

5. **SAFETY AND ENVIRONMENTAL PRECAUTIONS**

5.1 Follow 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 the safety aspects associated with its use. All persons using this method have a responsibility to consult with 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.

5.4 Include specific safety and / or environmental precautions if applicable.

6. **APPARATUS AND EQUIPMENT**

6.1 Equipment needed to condition *cigarette tobacco filler / cigarettes* as specified in ISO 3402 [2.3].

6.2 Equipment needed to perform marking for butt length as specified in ISO 4387 [2.2]. **ONLY FOR SMOKING METHOD**

6.3 Equipment needed to cover ventilation holes for the intense regimen as specified in World Health Organization Tobacco Laboratory Network, *Standard Operating Procedure for Intense Smoking of cigarettes (WHO TobLabNet SOP_01)* [2.5]. **ONLY FOR SMOKING METHOD**
6.4 Equipment needed to perform smoking of tobacco products as specified in ISO 3308 [2.1]

6.5 Calibrated analytical balance capable of measuring to at least X decimal places

6.6

6.7

6.8 Capillary Gas Chromatograph (GC), equipped with a flame ionization detector (FID)

   = EXAMPLE

6.9 Capillary Gas Chromatography column capable of distinct separation of solvent peaks, the peaks for the internal standard, nicotine and other tobacco contents (e.g. Varian WCOT FUSED SILICA, 25 m x 0.25 mm ID, Coating: CP-WAX 51).

   = EXAMPLE

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

7.2

7.3

7.4

7.5

8. PREPARATION OF GLASSWARE

   Clean and dry glassware so as to avoid contamination.

9. PREPARATION OF SOLUTIONS

9.1 NAME OF SOLUTION

   9.1.1

   9.1.2

   9.1.3

   9.1.4
9.2 NAME OF SOLUTION

9.2.1

9.2.2

9.2.3

9.2.4

9.3 NAME OF SOLUTION

9.3.1

9.3.2

9.3.3

9.3.4

10. PREPARATION OF STANDARDS

Preparation of the standard solutions as described below is for reference purposes. The preparation of the standard solutions can be adjusted if necessary.

10.1 COMPONENT internal standard solution (if applicable)

10.1.1 Component internal standard stock solution (X.X mg/mL)

10.1.1.1

10.1.1.2

10.1.1.3

10.1.2 COMPONENT(S) internal standard (mixed) solution (X.X mg/mL)

10.1.2.1

10.1.2.2

10.1.2.3

10.2 COMPONENT(S) standard solution (x g/L)

10.2.1 COMPONENT(S) stock solution (X.X mg/mL)

10.2.1.1

10.2.1.2

10.2.2 COMPONENT(S) diluted (mixed) standard solution (X.X mg/mL)

10.2.2.1

10.2.2.2
10.2.3 COMPONENT(s) final (mixed) standard solutions

10.2.3.1 Prepare final standard solutions according to Table X.

10.2.3.2

Final concentrations of the internal standards can be determined using the following equation:

\[
\text{Final concentration (IS) (ng/mL)} = \frac{x \times \text{XXX}}{y}
\]

Where \(x\) is the original weight (in g) of standard as determined in ...

Final concentrations of standards are determined using the following equation:

\[
\text{Final concentration (ng/mL)} = \frac{x \times y}{\text{XXX}}
\]

Where \(x\) is the original weight (in g) of standard as determined in ... \(y\) is the volume (in μl) of the spiking solution as described in ...

The final COMPONENT concentrations in the standard solutions are shown in Table X.

Table X. Concentrations of COMPONENT(s) in standard solutions

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume of Component(s) in Standard Solution (X g/L) (mL)</th>
<th>Volume of IS Solution (μl)</th>
<th>Total Volume (mL)</th>
<th>Approximate Component(s) concentration in Final Mixed Standard Solution (mg/L)</th>
<th>Approximate Level Equivalent to Unknown Levels in Mainstream Cigarette Smoke (mg/cig) or Cigarette Tobacco Filler (mg/g)</th>
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<tbody>
<tr>
<td>1</td>
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</tbody>
</table>

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

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

11. SAMPLING

11.1 Sample cigarettes according to ISO 8243 [23.4] or as required for specific application of the method. Alternative methods may be used to obtain a representative laboratory sample when required by specific regulation or availability of samples.

11.2 Constitution of test sample
11.2.1 Divide the laboratory sample into separate sales units, if applicable.

11.2.2 Take an equal amount of product for each test sample from at least \( \sqrt{n} \) \( [23.5] \) of the individual sales units.

11.2.3 If no individual sales units are available, combine the entire laboratory sample into one unit.

11.2.4 Take the amount of required product randomly from the combined unit.

12. CIGARETTE PREPARATION

12.1 Condition all cigarettes to be smoked in accordance to ISO 3402 \([2.3]\)

12.2 Mark cigarettes at a butt length in accordance with ISO 4387 \([2.2]\) and World Health Organization Tobacco Laboratory Network, Standard Operating Procedure for Intense Smoking of cigarettes (WHO TobLabNet SOP_01) \([2.5]\).

12.3 Prepare test samples to be smoked under intense smoking conditions as specified in World Health Organization Tobacco Laboratory Network, Standard Operating Procedure for Intense Smoking of cigarettes (WHO TobLabNet SOP_01) \([2.5]\).

OR

12.1 Remove the cigarette tobacco filler from the cigarettes and QC samples (where applicable) of at least one packet (e.g. containing 20 cigarettes) or use at least XX grams of processed cigarette tobacco filler.

12.2 Combine and mix sufficient cigarette tobacco filler to constitute at least XX g for each test sample.

12.3 Mix and grind the cigarette tobacco filler until the cigarette tobacco filler is sufficiently reduced to pass through a 4 mm screen.

12.4 Condition the ground cigarette tobacco filler (for non-volatile constituents) as required for the tobacco product according to ISO 3402 \([2.3]\). Note to editor: Conditioning is recommended unless detrimental to the analytical method.

13. PREPARATION OF THE SMOKING MACHINE

(IF NOT APPLICABLE KEEP PARAGRAPH TITLE AND NOTE “Not applicable for this method”)

13.1 Ambient conditions

The ambient conditions for smoking are specified in ISO 3308 \([2.1]\).

13.2 Machine specifications
Follow ISO 3308 [2.1] machine specifications, except for intense smoking as described in World Health Organization Tobacco Laboratory Network, Standard Operating Procedure for Intense Smoking of cigarettes (WHO TobLabNet SOP_01) [2.5].

14. SAMPLE GENERATION

(IF NOT APPLICABLE KEEP PARAGRAPH TITLE AND NOTE “Not applicable for this method”)

Note: Smoke a sufficient number of cigarettes on the specified smoking machine such that breakthrough does not occur and the concentrations of the NAME CONTENT(S) fall within the calibration range prepared for the analysis.

14.1 Smoke the cigarette test samples and collect the TPM as specified in ISO 4387 [2.2] or in WHO TobLabNet SOP_01 [2.5].

14.2 Include one reference test sample to be used for quality control, if applicable.

14.3 When testing sample types for the first time, an evaluation of breakthrough should be carried out. The number of cigarettes may need to be adjusted to prevent breakthrough of the filter pad. If the TPM exceeds 600 mg for a 92-mm filter pad or 150 mg for a 44-mm filter pad, the number of cigarettes smoked onto each pad must be decreased.

14.4 Number of cigarettes per result for linear and rotary smoking machines at ISO and intense smoking regimens are shown in Table X.

<table>
<thead>
<tr>
<th>ISO smoking regimen</th>
<th>Intense smoking regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>Rotary</td>
</tr>
<tr>
<td>No. of cigarettes per pad</td>
<td>5</td>
</tr>
<tr>
<td>No. of pads per result</td>
<td>4</td>
</tr>
</tbody>
</table>

14.5 Record the number of cigarettes and total puffs for each filter pad.

14.6 After smoking the required number of test samples, perform five clearing puffs and remove the pad holder from the smoking machine.

15. SAMPLE PREPARATION

FOR METHOD INCLUDING SMOKING:

15.1 Extraction of Filter Pads
Remove the pads from the holders. Fold each pad loosely in half, and then in half again, with the TPM on the inside. Use the side opposite where TPM is collected to wipe the inner surface of the pad holder, thus including any particulate matter that may have been left in the holder. Place each filter pad ............

15.2

15.3

15.4

FOR METHOD WITHOUT SMOKING:

15.1 Take X.X g of the well mixed, ground and conditioned test sample and weigh it to X.XXX g accuracy into an extraction vessel.

15.2

15.3

15.4

16. SAMPLE ANALYSIS

SPECIFY TECHNIQUE is used to quantify NAME GROUP CONTENTS in MAINSTREAM CIGARETTE SMOKE / CIGARETTE TOBACCO FILLER. The analytes are resolved from other potential interferences on the column used. Comparison of the area ratio of the unknowns with the area ratio of known standard concentrations yields the concentration of individual analytes.

16.1 NAME INSTRUMENT TYPE Operating Conditions: example

This section illustrates an example of HPLC or GC equipment and operating conditions. Sufficient information should be provided so results could be duplicated by a different laboratory.

HPLC Column: NAME BRAND AND TYPE OF COLUMN (XX x XXX mm, XXX µm particle size) or equivalent

Injection Volume: X µl
Column oven temperature: XX ±X°C (Alter as appropriate for the specific column used)
Degasser: On/Off
Binary pump flow: X.X mL/min
Mobile phase A:
Mobile phase B:
Gradient: Shown in Table X below
Total Analysis Time: XX min
Table X. HPLC gradient for NAME CONTENT(S) separation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (µl/min)</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Adjustment of the operating parameters may be required, depending on the instrument and column conditions as well as the resolution of the chromatographic peaks.

OR

GC Column: NAME BRAND AND TYPE OF COLUMN, XX m x X.XX mm ID
Coating: NAME TYPE OF COATING or equivalent
Column Temperature: XXX °C (isothermal, or include a table with temperature program)
Injection Temperature: XXX °C
Detector Temperature: XXX °C
Carrier Gas: NAME GAS TYPE at a flow rate of X,X mL/min
Injection Volume: X.X µl
Injection Mode: Split X:XX

Note: Adjustment of the operating parameters may be required, depending on the instrument and column conditions as well as the resolution of the chromatographic peaks.

16.2 Expected Retention Times

16.2.1 For the conditions described here, the expected sequence of elution will be NAME CONTENT(S) IN ORDER OF EXPECTED RT

16.2.2 Differences in e.g. FOR HPLC: flow rate, mobile phase concentration and age of the column may alter retention times. FOR GC: temperature, gas flow rate and age of the column may alter retention times.

16.2.3 Elution order and retention times must be verified before analysis is begun.

16.2.4 Using the above conditions, the expected total analysis time will be about XX minutes. (The analysis time may be extended to optimize performance)

16.2.5 IF NEEDED SPECIFIC INSTRUMENT SETTINGS HAVING SPECIFIC INFLUENCE ON THE RETENTION TIMES CAN BE INCLUDED HERE
16.3 NAME CONTENT (GROUP) Determination

The sequence of determination will be in accordance with individual laboratory practices. This section illustrates an example of a sequence of determination for Name Content (Group).

16.3.1 Inject two (IF NEEDED) replicate aliquots of the standard solutions and sample extracts under identical conditions.

16.3.2 Condition the system just prior to use by injecting two X µl aliquots of a sample solution as a primer. (IF NEEDED)

16.3.3 Inject a check standard (blank with labelled internal standards) under the same conditions as the samples to verify the performance of the HPLC-MS/MS / GC system.

16.3.4 Inject a blank solution (extraction solution minus internal standard(s)) to check for any contamination in the system or reagents.

16.3.5 Inject an aliquot of each standard solution into the HPLC-MS/MS / GC.

16.3.6 Record the peak areas of NAME CONTENTS and the internal standard(s).

16.3.7 Calculate the relative response ratio (RF) of the NAME CONTENTS peak to the internal standard peak (RF = \( \frac{A_{\text{NAME CONTENTS}}}{A_{\text{IS}}} \)) for each of the content standard solutions including the solvent blanks.

16.3.8 Plot the graph of the concentration of NAME CONTENTS (X axis) in accordance with the area ratios (Y axis).

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

16.3.10 Linearity of the standard curve should extend over the entire standard range.

16.3.11 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. If the linear regression is less than 0.99, then the calibration should be repeated. If an individual calibration point differs by more than 10% from the expected value (estimated by linear regression), this point should be omitted.

16.3.12 Inject the QCs and samples and determine peak areas using the appropriate software.

16.3.13 The signal (peak area) obtained for all test portions must fall within the working range of the calibration curve, otherwise solutions should be adjusted as necessary.

Note: See Appendix X for representative chromatograms.
17. DATA ANALYSIS AND CALCULATIONS

17.1 Calculate the relative response ratio from the peak areas for each of the TSNAs for each of the calibration standards [10.2.3]:

$$RF = \frac{A_a}{A_{is}}$$

where:

$RF$ = relative response ratio

$A_a$ = area of the target analyte

$A_{is}$ = area of the corresponding internal standard.

Plot a graph for each of the NAME CONTENTS (GROUP), of the relative response factor, $A_a / A_{is}$ (Y axis) versus concentration (X axis). Calculate a linear regression equation ($RF = kX + m$) from these data, and use both the slope ($k$) and the intercept ($m$) of the linear regression.

17.2 The linearity of the standard curve should cover the entire standard range.

17.3 The content $M_{is}$ of a given COMPONENT of the target analyte (ng/cigarette) is determined from the calculated relative response ratio for the test portion, the slope and intercept obtained from the appropriate calibration curve, and the equation:

(EXAMPLE)

$$M_{is} = \frac{Y - m}{k} \times \frac{V}{N}$$

where:

$M_{is}$ = calculated content, in ng/cigarette

$V$ = volume of extraction solution (40 mL)

$N$ = number of cigarettes smoked onto each pad.

ALTERNATIVE CALCULATION PROCEDURES MAY ALSO BE USED IF APPLICABLE!

18. SPECIAL PRECAUTIONS

18.1 After installing a new column, condition it as specified by the manufacturer followed by injecting a tobacco sample extract under the specified instrument conditions. Injections should be repeated until the peak areas (or heights) of NAME CONTENTS and internal standards are reproducible.
19. DATA REPORTING

19.1 Report individual measurements for each of the samples evaluated.

19.2 Results should be reported as specified by method specifications.

19.3 For more information, see World Health Organization Tobacco Laboratory Network, Standard operating procedure for validation of analytical methods of tobacco product contents and emissions (WHO TobLabNet SOP_02 [2.6]).

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 in compliance with the policies of the individual laboratory.

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 like a test sample. The assessment of the blank should be in accordance with the practices of the individual laboratory.

20.3 Quality Control Sample

To verify consistency of the entire analytical process, analyse a reference cigarette in accordance with the practices of the individual laboratory.

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 / mg/cig (X.X mg/g / mg/cig with XX mg/L as lowest calibration solution). Data outside the calibration range should be reported as below limit of quantification (BLOQ) or above limit of Quantification (ALOQ) as necessary.

21.2 Lab fortified matrix recovery.
Recovery of analyte spiked onto the matrix is used as a surrogate measure of accuracy. Recovery is determined by spiking known amounts of standards...... **ADD PROCEDURE FOR PERFORMING RECOVERY DETERMINATION.** The recovery is calculated from the following formula, as shown in Table X:

\[ \text{Recovery} = \frac{\text{analytical result-unsiked result}}{\text{spiked amount}} \times 100\% \]

<table>
<thead>
<tr>
<th>Spiked amount (ng)</th>
<th>COMPONENT</th>
<th>Recovery (%)</th>
<th>COMPONENT</th>
<th>Recovery (%)</th>
<th>COMPONENT</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
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<td>XX.X</td>
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<td>XX.X</td>
</tr>
<tr>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
<td>XX.X</td>
</tr>
</tbody>
</table>

21.3 **Analytical Specificity**

**DESCRIBE ANALYTICAL SPECIFICITY OF THE METHOD / TECHNIQUE USED, e.g. The retention time of the analyte of interest is used to verify the analytical specificity. An established range of ratios of the response of the component to that of the internal standard component of QC mainstream cigarette smoke/cigarette tobacco filler is used to verify the specificity of the results from an unknown sample.**

21.5 **Linearity**

The CONTENT(S) calibration curves established is/are linear over the standard concentration range of XX ng/mL to XX ng/mL.

21.6 **Possible interferences**

The presence of XXX can cause interference since the retention time of this component can be similar to the retention time of COMPONENT / INTERNAL STANDARD. This interference is most likely to occur with samples containing XXX.

OR (example)

There are no known components that can cause interference by having a similar retention time as COMPONENT(S), or the internal standard(s).

22. **REPEATABILITY AND REPRODUCIBILITY**

An international collaborative study conducted in 2012, performed according to World Health Organization Tobacco Laboratory Network, *Standard operating procedure for validation of analytical methods of tobacco product contents and emissions (WHO TobLabNet SOP_02)* [2.6] gave the following precision limits for this method:
The difference between two single results found on matched cigarette samples by the same operator using the same apparatus within the shortest feasible time interval will exceed the repeatability, \( r \), on average not more than once in 20 cases in the normal and correct operation of the method.

Single results for matched cigarette samples reported by two laboratories will differ by more than the reproducibility, \( R \), on average not more than once in 20 cases in the normal and correct operation of the method.

The test results were subjected to statistical analysis in accordance with ISO 5725-1 [23.2] and ISO 5725-2 [24.4] to give the precision data shown in Table X.

**FOR METHOD WITH SMOKING**

For the purpose of calculating \( r \) and \( R \), one test result under ISO smoking conditions was defined as the average of seven individual replicates of the mean yield of four Cambridge Filter Pads (CFPs) (five cigarettes smoked per pad) from linear smoking machines and one CFP (20 cigarettes smoked per pad) for rotary smoking machines. Under intense smoking conditions, one test result was defined as the average of seven individual replicates of the mean yield of seven CFPs (three cigarettes smoked per pad) from linear smoking machines and two CFPs (10 cigarettes smoked per pad) for rotary smoking machines.

**Note:** The levels of the commercial brand were inside the range of the reference pieces and are therefore not reported in this table.

**OR FOR METHOD WITHOUT SMOKING**

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>ISO smoking regimen</th>
<th>Reference product</th>
<th>Tobacco Filler</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference cigarette</td>
<td>ISO smoking regimen</td>
<td>Reference product</td>
<td>Tobacco Filler</td>
</tr>
<tr>
<td>1R5F</td>
<td>X</td>
<td>XX.XX</td>
<td>XX.XX</td>
</tr>
<tr>
<td>3R4F</td>
<td>X</td>
<td>XX.XX</td>
<td>XX.XX</td>
</tr>
<tr>
<td>CM6</td>
<td>X</td>
<td>XX.XX</td>
<td>XX.XX</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>ISO smoking regimen</th>
<th>Reference product</th>
<th>Tobacco Filler</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R5F</td>
<td>X</td>
<td>XX.XX</td>
<td>XX.XX</td>
</tr>
<tr>
<td>3R4F</td>
<td>X</td>
<td>XX.XX</td>
<td>XX.XX</td>
</tr>
<tr>
<td>CM6</td>
<td>X</td>
<td>XX.XX</td>
<td>XX.XX</td>
</tr>
</tbody>
</table>

**Note:** The levels of the commercial brand were inside the range of the reference pieces and are therefore not reported in this table.
23. BIBLIOGRAPHY


23.2 ISO 5725-1. Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions.

23.3 Reference to basic method of which the method has been deducted (if applicable).

23.4 ISO 8243: Cigarettes—Sampling


23.6 ISO Standards – Products by TC (Website for ordering methods); ISO/TC 126.

23.7
Validation Protocol for the analytical method for determining contents(s) of tobacco
World Health Organization
Tobacco Laboratory Network

Validation Protocol for the analytical method for determining content(s) in tobacco

<table>
<thead>
<tr>
<th>Method:</th>
<th>Protocol for the validation of analytical method for the determination of content(s) in cigarette tobacco filler</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytes:</td>
<td>Target analyte(s)</td>
</tr>
<tr>
<td>Matrix:</td>
<td>Tobacco</td>
</tr>
<tr>
<td>Last update:</td>
<td>Month yyyy</td>
</tr>
</tbody>
</table>
FOREWORD

This document was prepared by members of the World Health Organization (WHO) Tobacco Laboratory Network (TobLabNet) as a standard operating procedure (SOP) for validation of analytical methods for determining tobacco contents and cigarette tobacco filler contents.

INTRODUCTION

In order to establish comparable measurements for testing tobacco products globally, consensus methods are required for measuring specific contents and emissions of cigarettes. The Conference of the Parties (COP) to the WHO Framework Convention on Tobacco Control (WHO FCTC) at its third session in Durban, South Africa, in November 2008, recalling its decisions FCTC/COP1(15) and FCTC/COP2(14) on the elaboration of guidelines for implementation of Articles 9 (Regulation of the contents of tobacco products) and 10 (Regulation of tobacco product disclosures) of the WHO FCTC, noting the information contained in the report of the working group to the third session of the Conference of the Parties on the progress of its work … requested the Convention Secretariat to invite WHO’s Tobacco Free Initiative to … validate, within five years, the analytical chemical methods for testing and measuring cigarette contents and emissions (FCTC/COP/3/REC/1).

Using the criteria for prioritization set at its third meeting in Ottawa, Canada, in October 2006, the working group on Articles 9 and 10 identified the following contents for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- nicotine
- ammonia
- humectants (propane-1,2-diol, glycerol (propane-1,2,3-triol) and triethylene glycol (2,2-ethylenedioxybis(ethanol))).

Measurement of these contents will require validation of three methods: one for nicotine, one for ammonia and one for humectants.

Using the criteria for prioritization set at the meeting in Ottawa mentioned above, the working group identified the following emissions in mainstream smoke for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
- N-nitrosonornicotine (NNN)
- acetaldehyde
- acrylaldehyde (acrolein)
• benzene
• benzo[a]pyrene
• 1,3-butadiene
• carbon monoxide
• formaldehyde

Measurement of these emissions with the two smoking regimens described below will require validation of five methods: one for tobacco-specific nitrosamines (NNK and NNN), one for benzo[a]pyrene, one for aldehydes (acetaldehyde, acrolein and formaldehyde) and one for volatile organic compounds (benzene, 1,3-butadiene) and one for carbon monoxide.

1. **SCOPE**

   To introduce a standardized method to determine contents(s) in tobacco and cigarette filler in non-tobacco affiliated industry laboratories and to determine the degree of agreement on the content among the participants.

2. **SCHEDULES**

   **Introduction of standardized method**

   A standardized method is described, based on the source of the method.

   **2.1** The standardized method will be sent to all TobLabNet members, together with this protocol, and an invitation to participate in this study, which will be closed on dd Month yyyy.

   **2.2** **Test cigarettes**

   Test cigarettes or test articles will be procured and sent to all participants by a TobLabNet member. The total number of cigarettes needed for this study will be determined after the call for participants is closed. dd Month yyyy the samples will be distributed to all participants, together with the SOP, study protocol and a Microsoft Excel data sheet for the test results. Please DO NOT modify any data sheet for the convenience of data analysis.

   **2.3** **Scope and time for measurements**

   The content measurements shall be executed between dd Month yyyy and dd Month yyyy. Participants should carry out each of the seven determinations per sample on a separate day (more than one sample per day can be measured).

   **2.4** **Reporting of results**

   Test results of the study shall be reported to WHO by using the designated data sheet no later than dd Month yyyy to:
WHO TobLabNet Focal Person  
Geneva, Switzerland

WHO will assign code numbers to each participating laboratory in confidence.

2.5 Data analysis and statistical evaluation

The reported data will be statistically evaluated by TobLabNet, as described in World Health Organization Tobacco Laboratory Network, Standard operating procedure for validation of analytical method of tobacco product contents and emissions (WHO TobLabNet SOP_02) in combination with ISO 5725 part 2 – Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

A summary report of the results and the statistical evaluation will be sent to all participants by TobLabNet before dd Month yyyy. The statistical evaluation will be discussed at the first TobLabNet meeting after the report has been sent to the participants.

3. TEST ITEMS

3.1 Description

For the validation study, the number of samples has been set to five, divided into three so-called reference cigarettes and two commercially available brands. For efficiency reasons, the samples to be used for the validation study of all content determinations will be sent to all participants.

Table 1: Example test items for tobacco specific nitrosamines study

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Product type</th>
<th>Sample name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Reference cigarette</td>
<td>1R5F</td>
</tr>
<tr>
<td>B</td>
<td>Reference cigarette</td>
<td>2R4F</td>
</tr>
<tr>
<td>C</td>
<td>Reference cigarette</td>
<td>CM6</td>
</tr>
<tr>
<td>D</td>
<td>Cigarette</td>
<td>Commercial brand name</td>
</tr>
<tr>
<td>E</td>
<td>Cigarette</td>
<td>Commercial brand name</td>
</tr>
</tbody>
</table>

3.2 Shipment and receipt

a) TobLabNet will send "cigarettes or tobacco samples of each sample to each participating laboratory.

b) TobLabNet will inform all participating laboratories that samples have been sent.

c) Upon receipt, each participating laboratory will notify TobLabNet by email of the receipt of the samples and any problems with or damage to the cigarettes.
d) If problems were encountered during shipment, TobLabNet will send replacement samples as soon as practicable after the notification.

e) After receipt of the samples, each laboratory will complete the analyses and report the data within the designated time schedule.

f) Handle the samples carefully to avoid damage. Discard cigarettes that are obviously damaged, or missing significant amounts of tobacco.

3.3 Storage

Samples can be stored at room temperature for up to three months. For longer periods, the samples are to be stored in the original packaging or airtight containers in a freezer at –20 °C or lower.

3.4 Sample preparation

Sample preparation shall be done according to the SOP (grinding and sieving as required).

3.5 Number of test items to be analysed

For each individual sample seven replicate measurements are to be executed. Each of the seven determinations per sample shall be executed on a separate day (more than one sample per day can be measured).

4. REPORTING TEST RESULTS

Test results shall be reported using the Microsoft Excel template file provided by email. Please DO NOT modify any data sheet for the convenience of data analysis.

If Microsoft Excel is not available, laboratories can report data electronically by using an ASCII format or if this is not possible, by hard copy.

Test results shall be given according to the details given in Table 2.

| Parameter               | Unit       | Report to the nearest ...
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of tobacco used</td>
<td>gram</td>
<td>#.###1 gram</td>
</tr>
<tr>
<td>Content</td>
<td>mg/gram · mg/gram</td>
<td>#.##1 mg/gram</td>
</tr>
</tbody>
</table>

Participants shall also report an overview of the major equipment used in the study (type, model, manufacturer). Please refer to the data sheet for further details.

If, for any reason, there has been a deviation from the SOP or the study protocol, please note the deviation and the reason for this in the Microsoft Excel data sheet or individual participating laboratory report.
5. PROJECTED TIMELINE

<table>
<thead>
<tr>
<th>Content(s) method validation schedule</th>
<th>Timeline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sending of SOP, Study protocol and call for participants</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Returning comments and participation inquiry</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Shipping samples to participants</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Carry out analyses and report results</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Statistical evaluation of results</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Report evaluation summary to participants</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Report summary results to COP Working Group Articles 9 &amp; 10 via TFI</td>
<td>After discussion at TobLabNet meeting</td>
</tr>
</tbody>
</table>
Protocol for the validation of analytical method for the determination of contents(s) of mainstream cigarette smoke
World Health Organization
Tobacco Laboratory Network

Validation protocol for analytical methodology for determination of contents(s) of mainstream cigarette smoke under ISO and intense smoking conditions

<table>
<thead>
<tr>
<th>Method:</th>
<th>Protocol for the validation of analytical method for the determination of contents(s) of mainstream cigarette smoke under ISO and intense smoking conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytes:</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Matrix:</td>
<td>Mainstream cigarette smoke</td>
</tr>
<tr>
<td>Last update:</td>
<td>Month yyyy</td>
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</table>
FOREWORD

This document was prepared by members of the World Health Organization (WHO) Tobacco Laboratory Network (TobLabNet) as a standard operating procedure (SOP) for validation of analytical methods of tobacco cigarette mainstream smoke contents and cigarette tobacco filler contents.

INTRODUCTION

In order to establish comparable measurements for testing tobacco products globally, consensus methods are required for measuring specific contents and emissions of cigarettes. The Conference of the Parties (COP) to the WHO Framework Convention on Tobacco Control (WHO FCTC) at its third session in Durban, South Africa, in November 2008, recalling its decisions FCTC/COP1(15) and FCTC/COP2(14) on the elaboration of guidelines for implementation of Articles 9 (Regulation of the contents of tobacco products) and 10 (Regulation of tobacco product disclosures) of the WHO FCTC, noting the information contained in the report of the working group to the third session of the Conference of the Parties on the progress of its work ... requested the Convention Secretariat to invite WHO’s Tobacco Free Initiative to ... validate, within five years, the analytical chemical methods for testing and measuring cigarette contents and emissions (FCTC/COP/3/REC/1).

Using the criteria for prioritization set at its third meeting in Ottawa, Canada, in October 2006, the working group on Articles 9 and 10 identified the following contents for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- nicotine
- ammonia
- humectants (propane-1,2-diol, glycerol (propane-1,2,3-triol) and triethylene glycol (2,2-ethylenedioxycybis(ethanol))).

Measurement of these contents will require validation of three methods: one for nicotine, one for ammonia and one for humectants.

Using the criteria for prioritization set at the meeting in Ottawa mentioned above, the working group identified the following emissions in mainstream smoke for which methods for testing and measurement (analytical chemistry) should be validated as a priority:

- 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
- N-nitrosonornicotine (NNN)
- acetaldehyde
- acrylaldehyde (acrolein)
• benzene
• benzo[a]pyrene
• 1,3-butadiene
• carbon monoxide
• formaldehyde

Measurement of these emissions with the two smoking regimens described below will require validation of five methods: one for tobacco-specific nitrosamines (NNK and NNN), one for benzo[a]pyrene, one for aldehydes (acetaldehyde, acrolein and formaldehyde), one for volatile organic compounds (benzene, 1,3-butadiene) and one for carbon monoxide.

The table below sets out the two smoking regimens for validation of the test methods referred above.

<table>
<thead>
<tr>
<th>Smoking regimen</th>
<th>Puff volume (mL)</th>
<th>Puff frequency</th>
<th>Filter ventilation holes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO regimen: ISO 3308; Routine analytical cigarette smoking machine – definitions and standard conditions</td>
<td>35</td>
<td>Once every 60 s</td>
<td>No modifications</td>
</tr>
<tr>
<td>Intense regimen: Same as ISO 3308, but modified as indicated</td>
<td>55</td>
<td>Once every 30 s</td>
<td>All ventilation holes must be blocked 100% as described in WHO TobLabNet SOP 01.</td>
</tr>
</tbody>
</table>

1. **SCOPE**

To introduce a standardized method for the determination of content(s) of mainstream cigarette smoke in non-tobacco industry laboratories and on the content.

2. **SCHEDULES**

2.1 **Introduction of standardized method.**

A standardized method is described, based on the source of the method.

The standardized method will be sent to all TobLabNet members, together with this protocol, including a call for participants in this study, which will be closed on **dd Month yyyy**.

2.2 **Test cigarettes.**

Test cigarettes will be purchased and sent to all participants by TobLabNet. The total number of cigarettes needed for this study will depend on the number of participants and will be determined after the call for participants is closed. **dd Month yyyy** the samples will be distributed to all participants, together with
the SOP, study protocol and a Microsoft Excel data sheet for the test results. Please DO NOT modify any data sheet for the convenience of data analysis.

2.3 **Scope and time for measurements.**

The **content group** to be determined by the study participants shall be **contents** in mainstream tobacco smoke. A smoking machine, using both ISO and intense regimen, shall be used to generate mainstream smoke. More details related to the type of smoking machines, including smoking plans, are given in Tables 3 to 6.

The **content** measurements shall be executed between dd Month yyyy and dd Month yyyy. Participants are kindly asked to carry out each of the seven determinations per sample on a separate day (more than one sample per day can be measured).

2.4 **Reporting of results.**

Test results of the study shall be reported to WHO using the designated data sheet no later than dd Month yyyy to:

WHO TobLabNet Focal Person  
Geneva, Switzerland

WHO will assign code numbers to each participating laboratory in confidence.

2.5 **Data analysis and statistical evaluation.**

The reported data will be statistically evaluated by TobLabNet, as described in *World Health Organization Tobacco Laboratory Network, Standard operating procedure for validation of analytical methods of tobacco product contents and emissions (WHO TobLabNet SOP_02)*, in combination with ISO 5725-2: *Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.*

A summary report of the results and the statistical evaluation will be sent to all participants by TobLabNet before dd Month yyyy. The statistical evaluation will be discussed at the first TobLabNet meeting after the report has been sent to the participants.

3. **TEST ITEMS**

3.1 **Description**

For the validation study the number of samples has been set to five, divided into three so-called reference cigarettes and two commercially available brands. For efficiency reasons the samples to be used for the validation study of all emissions determinations will be sent to all participants at once.
Table 1: Test items for tobacco specific nitrosamines study

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Product type</th>
<th>Sample name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Reference cigarette</td>
<td>1R5F</td>
</tr>
<tr>
<td>B</td>
<td>Reference cigarette</td>
<td>2R4F</td>
</tr>
<tr>
<td>C</td>
<td>Reference cigarette</td>
<td>CM6</td>
</tr>
<tr>
<td>D</td>
<td>Cigarette</td>
<td>Commercial brand name</td>
</tr>
<tr>
<td>E</td>
<td>Cigarette</td>
<td>Commercial brand name</td>
</tr>
</tbody>
</table>

3.2 Shipment and receipt

a) TobLabNet will send **## cigarettes** of each sample to each participating laboratory.

b) TobLabNet will inform all participating laboratories that samples have been sent.

c) Upon receipt, each participating laboratory will notify TobLabNet by email of the receipt of the samples and any problems with or damage to the cigarettes.

d) If problems were encountered during shipment, TobLabNet will send replacement samples as soon as practicable after notification.

e) After receipt of the samples, each laboratory will complete the analyses and report the data within the designated time schedule.

f) Handle the samples carefully to avoid damage. Discard cigarettes that are obviously damaged, or missing significant amounts of tobacco.

3.3 Storage

Samples can be stored at room temperature for up to three months. For longer periods, the samples are to be stored in the original packaging or airtight containers in a freezer at –20 °C or lower.

3.4 Sample preparation

Sample preparation shall be done according to the SOP (selection and storage). Mark the samples for further identification. If the tobacco products are removed from the original packages, store them in airtight containers just large enough to contain the sample and keep them at **–20 °C or lower until needed**.

3.5 Number of test items to be analysed

For each individual sample, seven replicate measurements are to be executed. Each of the seven determinations per sample shall be executed on a separate day (more than one sample per day can be measured).
4. REPORTING TEST RESULTS

Test results shall be reported using the Microsoft Excel template file provided by email. Please DO NOT modify any data sheet for the convenience of data analysis.

If Microsoft Excel is not available, laboratories can report data electronically by using an ASCII format or if this is not possible, by hard copy.

Test results shall be given according to the details given in Table 2.

Table 2: Data and formats for reporting

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Report to the nearest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette weight</td>
<td>gram</td>
<td>#,##1 gram</td>
</tr>
<tr>
<td>Component</td>
<td>ng/cigarette</td>
<td>#,##1 ng/cigarette</td>
</tr>
<tr>
<td>Component</td>
<td>ng/cigarette</td>
<td>#,##1 ng/cigarette</td>
</tr>
<tr>
<td>Component</td>
<td>ng/cigarette</td>
<td>#,##1 ng/cigarette</td>
</tr>
</tbody>
</table>

Participants shall also report an overview of the major equipment used in the study (type, model, manufacturer). Please refer to the data sheet for further details.

If, for any reason, there has been a deviation from the SOP or the study protocol, please note the deviation and the reason for this in the Microsoft Excel data sheet.

5. PROJECTED TIME LINE

<table>
<thead>
<tr>
<th>Content(s) method validation schedule</th>
<th>Timeline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sending of SOP, study protocol and call for participants</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Returning comments and participation inquiry</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Shipping samples to participants</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Carry out analyses and report results</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Statistical evaluation of results</td>
<td>Dd Month yyyy</td>
</tr>
<tr>
<td>Report evaluation summary to participants</td>
<td>Dd Month yyyy</td>
</tr>
</tbody>
</table>

- Report summary results to COP Working Group Articles 9 & 10 via TFI After discussion at TobLabNet meeting

6. SMOKING PLANS

The described smoking plans are based on five different samples (cigarettes brands/types) and seven replicates per sample. If a different number of samples or replicates are used, the plans should be adjusted accordingly.

6.1 ISO Regime
Table 3: Smoking plan for rotary smoking machine (20 cigarettes per filter pad) (Example)

<table>
<thead>
<tr>
<th>Day</th>
<th>Run</th>
<th>Sample</th>
<th>Day</th>
<th>Run</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>A</td>
<td>5</td>
<td>21</td>
<td>C</td>
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<td></td>
<td>3</td>
<td>C</td>
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<td>23</td>
<td>E</td>
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<td></td>
<td>4</td>
<td>D</td>
<td></td>
<td>24</td>
<td>A</td>
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<td>20</td>
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</tbody>
</table>

**Note:** Each filter pad should be extracted individually with xx mL extraction solution, which will be further analysed. One result is the result of each individual filter pad / extraction solution (20 cigarettes).

Table 4: Smoking plan for 20-port linear smoking machine (5 cigarettes per filter pad) (Example)

| Port number | Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|-------------|-----|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
|             | 1   | A | A | A | A | B | B | B | B | C | C | C | C | C | D | D | D | D | E | E | E | E |
|             | 2   | D | D | D | D | E | E | E | E | A | A | A | B | B | B | B | B | C | C | C | C | C |
|             | 3   | B | B | B | B | C | C | C | C | D | D | D | D | D | E | E | E | E | A | A | A | A |
|             | 4   | E | E | E | E | A | A | A | A | B | B | B | B | B | C | C | C | C | D | D | D | D |
|             | 5   | C | C | C | C | D | D | D | D | E | E | E | E | A | A | A | B | B | B | B | B |
|             | 6   | D | D | D | D | C | C | C | C | B | B | B | B | B | A | A | A | E | E | E | E | E |
|             | 7   | C | C | C | C | B | B | B | B | A | A | A | E | E | E | E | D | D | D | D | D |

**Note:** Four filter pads should be combined and extracted with xx mL extraction solution, which will be further analysed. One result is the result of one extraction solution combining a set of four filter pads in each extraction solution (20 cigarettes).

6.2 Intense Regime

Table 5: Smoking plan for rotary smoking machine (10 cigarettes per filter pad) (Example)
Table 6: Smoking plan for 20-port linear smoking machine (3 cigarettes per filter pad) (Example)

<table>
<thead>
<tr>
<th>Day</th>
<th>Run</th>
<th>Sample</th>
<th>Day</th>
<th>Run</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>36</td>
<td>A</td>
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<td>75</td>
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</tr>
</tbody>
</table>

Note: Each filter pad should be extracted individually with xx mL extraction solution, which will be further analysed. One result is the average result of two individual filter pads / extraction solutions (20 cigarettes).
Standard operating procedure for determination of tobacco-specific nitrosamines in mainstream cigarette smoke under ISO and intense smoking conditions.

2. Tobacco – chemistry.
5. Materials testing – methods.

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World Health Organization Tobacco Laboratory Network SOP 03

Determination of tobacco-specific nitrosamines in mainstream cigarette smoke under ISO and intense smoking conditions

Method: Determination of tobacco-specific nitrosamines in mainstream cigarette smoke under ISO and intense smoking conditions

Analytes:
- 3-(1-Nitrosopyrrolidin-2-yl)pyridine (CAS# 16543-55-8)
- 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (CAS# 64091-91-4)
- N'-Nitrosoanatabine (CAS# 71267-22-6)
- N'-Nitrosoanabasine (CAS# 37620-20-5)

Matrix: Tobacco cigarette mainstream smoke particulate matter

Last update: June 2014

<table>
<thead>
<tr>
<th>Day</th>
<th>Run</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
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Note: Three filter pads are combined and extracted with xx mL extraction solution, which will be further analysed. One result is the average of two sets of combined filter pads / extraction solutions (18 cigarettes = 2 x 9 cigarettes).
This document was prepared by members of the World Health Organization (WHO) Tobacco Laboratory Network (TobLabNet) as an analytical method standard operating procedure (SOP) for validation of analytical methods of tobacco product contents and emissions.