STANDARD OPERATING PROCEDURE FOR DETERMINATION OF HUMECTANTS IN CIGARETTE TOBACCO FILLER

Tobacco Free Initiative
Tobacco Laboratory Network (TobLabNet)

World Health Organization
Tobacco Free Initiative Tobacco Laboratory Network (TobLabNet)

No.: SOP 03  
Date: June 2014

World Health Organization Tobacco Laboratory Network

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
Standard operating procedure for determination of humectants in cigarette tobacco filler
WHO Library Cataloguing-in-Publication Data

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ISBN 978 92 4 151606 8 (NLM classification: QV 137)

This publication was originally published under ISBN 978 92 4 151047 9

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Printed in Switzerland
World Health Organization
Tobacco Laboratory Network

Standard operating procedure for method

Determination of humectants in cigarette tobacco filler

Method: Determination of humectants in cigarette tobacco filler

Analytes: Propylene glycol (propane-1,2-diol) (CAS # 57-55-6)
Glycerol (propane-1,2,3-triol) (CAS # 56-81-5)
Triethylene glycol (2,2’-ethylenedioxybis(ethanol)) (CAS # 112-27-6)

Matrix: Cigarette tobacco filler

Last update: June 2016
No.: SOP 06  
Date: June 2016  

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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 the results of testing with WHO TobLabNet standards.
FOREWORD
This document was prepared by members of the WHO Tobacco Laboratory Network (TobLabNet) as an analytical method standard operating procedure (SOP) for measuring humectants in 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 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
- propylene glycol (propane-1,2-diol)
- glycerol (propane-1,2,3-triol)
- 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 (B[a]P)
• 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 B[a]P, one for aldehydes (acetaldehyde, acrolein and formaldehyde), one for volatile organic compounds (benzene and 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 modification</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 SOP was prepared to guide participating laboratories in analysing humectants in cigarette tobacco filler.

1 SCOPE
   This standard operating procedure is suitable for quantitative determination of the humectants propylene glycol, glycerol (propane-1,2,3-triol) and triethylene glycol in cigarette tobacco filler by gas chromatography.
2 REFERENCES

2.1 ISO 8243: Cigarettes – Sampling.


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

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

2.6 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.

3 TERMS AND DEFINITIONS

3.1 Humectant content: Individual amounts of glycerol, propylene glycol and triethylene glycol in cigarette tobacco filler, expressed as milligrams per gram of cigarette tobacco filler.

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

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

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 portions taken shall be representative of the test sample.

4 METHOD SUMMARY

4.1 Humectants are extracted from the cigarette tobacco filler with a mixture of methanol and 1,3-butaneediol solution.
4.2 The extract is analysed with a flame ionization or mass spectrometer detector.

4.3 The ratio of the peak area of each humectant to that of the internal standard is compared on a calibration curve created by analysing standards containing known concentrations of each humectant to determine the humectant content in each test sample.

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 address all the safety aspects associated with use of the method. All persons using the method are responsible for consulting the appropriate authorities and establishing 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 Analytical balance capable of measurement to at least four decimal places

6.2 Extraction vessels: flasks with stoppers or equivalent glassware

6.3 Mechanical wrist-action shaker or equivalent

6.4 Gas chromatograph equipped with a flame ionization detector

6.5 Capillary gas chromatography column capable of distinct separation of solvent peaks, the peaks for the internal standard, propylene glycol, glycerol, triethylene glycol and other tobacco components (e.g. DB-Wax fused silica column 30 m × 0.32 mm × 1 µm, or equivalent)

6.7 Centrifuge

7 REAGENTS AND SUPPLIES

All reagents shall be of at least analytical reagent grade, unless otherwise noted. Reagents are identified by their Chemical Abstract Service [CAS] registry numbers, when possible.
7.1 Methanol, chromatographic purity [67-56-1]
7.2 Glycerol [56-81-5]
7.3 Triethylene glycol [112-27-6]
7.4 Propylene glycol [57-55-6]
7.5 1,3-Butanediol [107-88-0] (used as an internal standard)
7.6 Carrier gas: Helium [7440-59-7] of adequate purity
7.7 Auxiliary gases: Hydrogen [1333-74-0] and air of adequate purity for flame ionization

8 PREPARATION OF GLASSWARE
Clean and dry glassware in a manner to avoid contamination from residues.

9 PREPARATION OF SOLUTIONS
1.3-Butanediol primary stock (approximately 200 mg/mL):

9.1 Weigh approximately 20 g (± 0.05 g) of 1,3-butandiol.
9.2 Place measured 1,3-butandiol into a 100-mL volumetric flask, and make up to volume with methanol.

Extraction solution (approximately 2 mg/mL):
9.3 Pipette 20 mL of primary stock into a 2-L volumetric flask, and make up to volume with methanol. The volume of the solution can be scaled up or down as necessary. Mix well. Store at 4–8 °C.

10 PREPARATION OF STANDARDS
10.1 Glycerol primary standard (approximately 100 mg/mL):
Accurately weigh 10 g (± 0.05 g) of glycerol into a 100-mL volumetric flask, and make up to volume with extraction solution [9.3].

10.2 Propylene glycol primary standard (approximately 50 mg/mL):
Accurately weigh 5 g (± 0.05 g) of propylene glycol into a 100-mL volumetric flask, and make up to volume with extraction solution [9.3].

10.3 Triethylene glycol primary standard (approximately 25 mg/mL):
Accurately weigh 2.5 g (± 0.05 g) of triethylene glycol into a 100-mL volumetric flask, and make up to volume with extraction solution [9.3].

10.4 Mixed secondary standard (4 mg/mL glycerol; 3 mg/mL propylene glycol; 1.5 mg/mL triethylene glycol):
Pipette a 4-mL aliquot of glycerol [10.1] and 6-mL aliquots each of the prepared primary stocks of propylene glycol [10.2] and triethylene glycol [10.3] into a 100-mL volumetric flask. Make up to volume with extraction solution [9.3].

Store all standard solutions at 4–8 °C.

10.5 Working standards

All standards are made up in volumetric flasks at the dilutions listed.

### Table 1. Working standard solutions

<table>
<thead>
<tr>
<th>No.</th>
<th>Mixed secondary standard solution (mL)</th>
<th>Final volume (mL)</th>
<th>Approximate glycerol concentration in working standard solution (mg/mL)</th>
<th>Approximate propylene glycol concentration in working standard solution (mg/mL)</th>
<th>Approximate triethylene glycol concentration in working standard solution (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>10</td>
<td>0.20</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>10</td>
<td>0.40</td>
<td>0.30</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>10</td>
<td>0.80</td>
<td>0.60</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>4.0</td>
<td>10</td>
<td>1.60</td>
<td>1.20</td>
<td>0.60</td>
</tr>
<tr>
<td>6</td>
<td>8.0</td>
<td>10</td>
<td>3.20</td>
<td>2.40</td>
<td>1.20</td>
</tr>
</tbody>
</table>

### Table 2. Working standard solutions for low yields of propylene glycol

<table>
<thead>
<tr>
<th>No.</th>
<th>Mixed secondary standard solution (mL)</th>
<th>Final volume (mL)</th>
<th>Approximate propylene glycol concentration in working standard solution (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.017</td>
<td>10</td>
<td>0.005</td>
</tr>
<tr>
<td>2</td>
<td>0.050</td>
<td>10</td>
<td>0.015</td>
</tr>
<tr>
<td>3</td>
<td>0.100</td>
<td>10</td>
<td>0.030</td>
</tr>
<tr>
<td>4</td>
<td>0.250</td>
<td>10</td>
<td>0.075</td>
</tr>
<tr>
<td>5</td>
<td>0.500</td>
<td>10</td>
<td>0.150</td>
</tr>
<tr>
<td>6</td>
<td>1.000</td>
<td>10</td>
<td>0.300</td>
</tr>
</tbody>
</table>

Make up to volume in volumetric flasks with extraction solution [9.3] containing the internal standard.

Note: The range of the standard solutions may be adjusted according to the equipment used and the samples to be tested, keeping in mind a possible effect on the sensitivity of the method.

A lower or higher concentration of standards can be prepared with the required volume of mixed secondary stock to make up to 10 mL in a volumetric flask with extraction solvent to a lower or higher limit of quantification, if necessary. See Table 2 for the example of propylene glycol; it is important to adjust the range of calibration if necessary. All solvents and solutions shall be at room temperature before use.
11 SAMPLING

11.1 Sample cigarettes according to ISO 8243 [2.1] or as required for specific application of the method. Alternative methods may be used to obtain a representative sample when required by specific regulation or the 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 √n [2.2] of the individual sales unit.

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

12 CIGARETTE PREPARATION

12.1 Remove the tobacco filler from the cigarettes or quality control samples (when applicable) in two packs, or use at least 10 g of processed cigarette tobacco filler.

12.2 Combine and mix sufficient cigarette tobacco filler to constitute at least 10 g for each test portion.

12.3 Homogenize the cigarette tobacco filler by grinding, and keep in an airtight amber bottle or container. Divide the sample into portions.

13 PREPARATION OF THE SMOKING MACHINE

Not applicable

14 SAMPLE GENERATION

Not applicable

15 SAMPLE PREPARATION

15.1 Take 4 g of well-mixed, ground test sample, and weigh it to 0.001 g accuracy into a suitable extraction flask.

15.2 Mix the test sample with 50 mL of extraction solution [9.3].

Note: Sample weight and dilution factor may be adjusted according to the concentrations of humectants or availability of sample.
15.3 Stop the flasks, and place them on a wrist-action shaker or equivalent at a minimum rate of 210 rpm for at least 60 min.

15.4 Remove the samples from the shaker, and swirl the flasks to dissolve all the tobacco.

15.5 Let the samples stand undisturbed until the supernatant is clear. Alternatively, use a centrifuge.

15.6 Transfer the supernatant to an autosampler vial, and analyse on the gas chromatograph.

16 SAMPLE ANALYSIS

This method involves gas chromatography coupled with a flame ionization or mass spectrometry detector to quantify humectants in cigarette tobacco filler. The analytes are resolved from potential interference on the column. Comparison of the area of the unknowns with the area of the known standard concentrations yields the concentrations of individual analytes.

16.1 Gas chromatography operating conditions: example

- Gas chromatograph column: DB-Wax fused silica, 30 m × 0.32 mm interior diameter × 25.0 μm
- Coating: DB-Wax or equivalent
- Carrier gas: Helium at a flow rate of 1.8 mL/min
- Injection volume: 1 μL
- Injection mode: split 25:1

Temperature programme:
- Injection temperature: 220 °C
- Detector temperature: 260 °C
- Start temperature: 120 °C, hold for 3 min
- Rate: 10 °C/min to 180 °C, hold for 11 min
- Total run time: 20 min

Note: The operating parameters might have to be adjusted according to the instrument and column conditions and 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 propylene glycol, 1,3-butanediol (internal standard), glycerol and triethylene glycol.

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

16.2.3 The elution order and retention times must be verified before analysis is begun.
16.3 Determination of humectants

The sequence of determination steps will be in accordance with individual laboratory practices. The following are provided as a guide:

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

16.3.2 Inject a standard (extraction solution [9.3]) under the same conditions as the samples to verify the performance of the gas chromatography system.

16.3.3 Inject a blank solution (solvent [7.1]) to check for contamination in the system or reagents.

16.3.4 Inject an aliquot of each standard solution [10.5] into the gas chromatograph.

16.3.5 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 the typical responses in previous injections, the system is ready to perform the analysis. If the responses are outside the specifications, use follow-up actions according to individual laboratory policy.

16.3.6 Record the peak area of each humectant and the internal standard.

16.3.7 Calculate the relative response factor (RF) as the ratio between the peak areas of the humectants and that of the internal standard. RF = A<sub>humectants</sub> / A<sub>IS</sub> for each humectant standard solution, including the solvent blanks.

16.3.8 Plot the concentration of added humectants (X axis) in accordance with the area ratios (RF, Y axis).

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

16.3.10 The standard curve should be linear over the entire range.

16.3.11 Calculate a linear regression equation (Y = a + bX) from these data, and use both the slope (b) and the intercept (a). If the linear regression coefficient R<sup>2</sup> is < 0.99, the calibration should be repeated. If an individual calibration point differs by > 10% from the expected value (estimated by linear regression), that point should be omitted. Inject the quality controls and samples, and determine the peak areas with appropriate instrument software.

16.3.12 The signals (peak areas) obtained for all test portions must fall within the working range of the calibration curve; otherwise, solutions should be adjusted.

See Annex 1 for a representative chromatogram.
17 **DATA ANALYSIS AND CALCULATIONS**

17.1 Inject two replicate aliquots of the sample extracts under identical conditions.

17.2 For each test portion, calculate the ratio of the response of each humectant (glycerol, propylene glycol, triethylene glycol) to that of the internal standard response ($Y_i$) from the peak area.

17.3 For each humectant (glycerol, propylene glycol, triethylene glycol), calculate the concentration in mg/mL for each test aliquot from the coefficients of the linear regression ($m_i = (Y_i - a) / b$).

17.4 For each humectant (glycerol, propylene glycol, triethylene glycol), calculate the content $m_h$, of the tobacco sample expressed in milligrams per gram from the following equation:

$$m_h = \frac{m_t * V_e}{m_o}$$

where:

$m_h$ is the content of the humectant (glycerol, propylene glycol, triethylene glycol) in mg/g;

$m_t$ is the concentration of each humectant (glycerol, propylene glycol, triethylene glycol) in the test solution, in mg/mL;

$V_e$ is the volume of the extraction solution, in mL; and

$m_o$ is the mass of the test portion, in g.

18 **SPECIAL PRECAUTIONS**

18.1 After installing a column, condition it as specified by the manufacturer, and then inject a tobacco sample extract under the instrument conditions described above. Injections should be repeated until the peak areas (or heights) of both the humectant (propylene glycol, glycerol, triethylene glycol) 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 gas chromatography column after each sample set (series) by raising the column temperature to 220 °C for 30 min.

18.3 When the peak area (or height) of the internal standard is 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 values for humectants.
19 DATA REPORTING

19.1 Report individual measurements for each sample evaluated.

19.2 Report results as milligrams per gram of tobacco or as required.

20 QUALITY CONTROL

20.1 Control parameters

Note 1: If the control measurements are outside the tolerance limits of the expected values, make appropriate investigations and take action.

Note 2: Additional quality assurance procedures should be used in accordance with the practices 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 16.3.2. The blank consists of all reagents and materials used in analysing test samples and is analysed as a test sample. The result should be less than the limit of detection.

20.3 Quality control sample:

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

20.4 Additional quality control samples may be added as required by individual laboratory policy.

21 METHOD PERFORMANCE

21.1 Limit of reporting:

The limit of reporting is set to the lowest concentration of the calibration standards, recalculated to mg/g (with 0.08 mg/mL for glycerol, 0.06 mg/mL for propylene glycol and 0.03 mg/mL for triethylene glycol as the lowest concentrations). The limits of reporting are therefore 1.0 mg/g for glycerol, 0.75 mg/g for propylene glycol and 0.375 mg/g for triethylene glycol.

For the propylene glycol working standard solutions (Table 2), the limit of reporting is set to the lowest concentration of the calibration standards, recalculated to 0.063 mg/g. (At 0.005 mg/mL, the limit of reporting is 0.063 mg/g.)

21.2 Laboratory-fortified matrix recovery:

Recovery of analyte spiked onto the matrix is used as a surrogate measure of accuracy. It is determined by spiking known amounts of standards into tobacco and extracting the tobacco in the same way as for samples. Unspiked tobacco is also analysed. Recovery is calculated from the following formula:

\[
\text{Recovery} = 100 \times \left(\frac{\text{analytical result} - \text{unspiked result}}{\text{spiked amount}}\right)
\]
Table 3. Mean recovery of laboratory-fortified matrix

<table>
<thead>
<tr>
<th>Propylene glycol</th>
<th>Glycerol</th>
<th>Triethylene glycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked amount</td>
<td>Mean</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>(mg/g)</td>
<td>(mg/g)</td>
<td>(%)</td>
</tr>
<tr>
<td>5.00</td>
<td>4.94</td>
<td>99</td>
</tr>
<tr>
<td>10.00</td>
<td>9.87</td>
<td>99</td>
</tr>
<tr>
<td>20.00</td>
<td>19.64</td>
<td>98</td>
</tr>
</tbody>
</table>

21.3 Analytical specificity:
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 quality control cigarette tobacco filler is used to verify the specificity of the results for an unknown sample.

21.4 Linearity:
The calibration curves of the humectants are linear over the standard concentration range: 0.08–3.2 mg/mL for glycerol, 0.005–0.30 mg/mL for propylene glycol and 0.03–1.2 mg/mL for triethylene glycol.

21.5 Possible interference:
At the time of validation, there were no known components that would cause interference because of similar retention times as propylene glycol, triethylene glycol, glycerol or the internal standard (1,3-butanediol).

22 REPEATABILITY AND REPRODUCIBILITY LIMITS

An international collaborative study with flame ionization detection [2.3], conducted in 2012–2014 by 13 laboratories and with seven samples (five reference cigarettes and two commercial cigarette brands), performed according to WHO TobLabNet SOP 02 [2.4], gave the following values for this method:

The difference between two single results found for matched cigarette tobacco filler samples by the same operator using the same apparatus within the shortest feasible time will exceed the repeatability, r, on average no more than once in 20 cases with normal, correct application of the method.

Single results for matched cigarette tobacco filler samples reported by two laboratories will differ by no more than the reproducibility, R, on average no more than once in 20 cases with normal, correct application of the method.

A collaborative study with mass spectrometry detection, conducted in 2012–2014 by seven laboratories and with seven samples (five reference cigarettes and two commercial cigarette brands) gave equivalent values (Annex 2).
The test results were analysed statistically in accordance with ISO 5725-1 \cite{2.5} and ISO 5725-2 \cite{2.6} to determine the repeatability and reproducibility limits shown in Tables 4–6 for gas chromatography–flame ionization detection analysis. The repeatability and reproducibility limits for gas chromatography in mean standard deviation analyses are shown in Tables A2.2–A2.4 in Annex 2.

**Table 4. Repeatability and reproducibility limits for the determination of propylene glycol in tobacco from reference test pieces and commercial cigarettes by gas chromatography–flame ionization detection**

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>n*</th>
<th>( \bar{m} ) (mg/g)</th>
<th>Repeatability limit (r) (mg/g)</th>
<th>Reproducibility limit (R) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R5F</td>
<td>12</td>
<td>0.231</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>3R4F</td>
<td>12</td>
<td>0.180</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>CM6</td>
<td>12</td>
<td>0.140</td>
<td>0.000</td>
<td>0.004</td>
</tr>
<tr>
<td>Commercial cigarette No. 1</td>
<td>12</td>
<td>5.496</td>
<td>0.244</td>
<td>1.972</td>
</tr>
<tr>
<td>Commercial cigarette No. 2</td>
<td>12</td>
<td>0.078</td>
<td>0.000</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Results in one data set were removed as they were outliers.

**Table 5. Repeatability and reproducibility limits for the determination of glycerol in tobacco from reference test pieces and commercial cigarettes by gas chromatography–flame ionization detection**

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>n*</th>
<th>( \bar{m} ) (mg/g)</th>
<th>Repeatability limit (r) (mg/g)</th>
<th>Reproducibility limit (R) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R5F</td>
<td>11</td>
<td>21.603</td>
<td>1.490</td>
<td>6.082</td>
</tr>
<tr>
<td>3R4F</td>
<td>11</td>
<td>21.203</td>
<td>1.299</td>
<td>7.638</td>
</tr>
<tr>
<td>CM6</td>
<td>11</td>
<td>1.160</td>
<td>0.006</td>
<td>0.063</td>
</tr>
<tr>
<td>Commercial cigarette No. 1</td>
<td>11</td>
<td>14.376</td>
<td>0.648</td>
<td>3.621</td>
</tr>
<tr>
<td>Commercial cigarette No. 2</td>
<td>11</td>
<td>1.222</td>
<td>0.015</td>
<td>0.049</td>
</tr>
</tbody>
</table>

* Results in one data set were removed as they were outliers.

**Table 6. Repeatability and reproducibility limits for the determination of triethylene glycol in tobacco from reference test pieces by gas chromatography–flame ionization detection**

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>n*</th>
<th>( \bar{m} ) (mg/g)</th>
<th>Repeatability limit (r) (mg/g)</th>
<th>Reproducibility limit (R) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample TG No. 1</td>
<td>12</td>
<td>0.891</td>
<td>0.207</td>
<td>0.409</td>
</tr>
<tr>
<td>Sample TG No. 2</td>
<td>11</td>
<td>8.078</td>
<td>1.565</td>
<td>2.621</td>
</tr>
</tbody>
</table>

* Results in one data set were removed for sample TG No. 1, and results in two data sets were removed for sample TG No. 2, as they were outliers.
TEST REPORT

The following information shall be included in the test report:
(a) A reference to this method, i.e. WHO TobLabNet SOP 06
(b) Date of receipt of the sample
(c) The results and its units.
ANNEX 1. Example of a gas chromatography–flame ionization detection chromatogram of a standard solution

Elution order of humectants:
1. Propylene glycol
2. 1,3 Butanediol (internal standard)
3. Glycerol
4. Triethylene glycol
ANNEX 2. Main points to consider when using a mass spectrometer detector instead of a flame ionization detector

A2.1.1 METHOD SUMMARY

The method is a gas chromatographic method with a silica column and a mass spectrometry detector. Extract 4 g of ground cigarette tobacco filler with 50 mL of a methanol-based extraction solvent on a mechanical shaker for 60 min. Pipette 2 mL of the sample extract onto a dispersive solid phase extraction cartridge containing 150 mg anhydrous MgSO₄ and 25 mg N-propylethylenediamine. Oscillate the cartridge in a vortex oscillator at 2000 rpm for 2 min, and centrifuge it at 10 000 rpm for 10 min if necessary. Transfer the supernatant to an autosampler vial, and analyse on the gas chromatograph.

A2.1.2 APPARATUS AND EQUIPMENT

Analytical balance capable of measurement to at least four decimal places
125-mL Erlenmeyer flasks with stoppers or equivalent
10-mL, 100-mL and 2000-mL volumetric flasks
20-mL pipette for preparation of extraction solvent
Volumetric pipettes for preparation of standard solutions (various sizes)
Disposable transfer pipettes Brinkmann Dispenser, 10–50 mL or equivalent
2-mL autosampler vials and caps with Teflon-faced septa
0.22-μm membrane filter
Vortex oscillator
Centrifuge
High-speed disintegrator
0.45-mm screen
Gas chromatograph equipped with mass spectrometer
Capillary gas chromatography column capable of distinct separation of peaks for the solvent, the internal standard, propylene glycol, glycerol, triethylene glycol and other tobacco components (e.g. DB-Wax fused silica column 30 m × 0.32 mm × 1 μm, or equivalent)

A2.1.3 REAGENTS AND SUPPLIES

Add the following if necessary.
A2.1.3.1 Anhydrous MgSO₄ [7487-88-9]

A2.1.3.2 N-Propylethylenediamine [111-39-7]

Note: **A1.3.1** and **A1.3.2** can be replaced by a commercially dispersive solid-phase extraction cartridge containing 150 mg anhydrous MgSO₄ [7487-88-9] and 25 mg N-propylethylenediamine [111-39-7].

### A2.1.4 Preparation of Standards

As for the gas chromatography-flame ionization detection method [10.1–10.5]

### A2.1.5 Sample Preparation

**A2.1.5.1** Pipette 1–2 mL of the sample extract onto a dispersive solid-phase extraction cartridge containing 150 mg anhydrous MgSO₄ and 25 mg N-propylethylenediamine if needed.

**A2.1.5.2** Oscillate the cartridge in a vortex oscillator at 2000 rpm for 2 min, and centrifuge it at 10 000 rpm for 10 min. Filter the supernatant through a 0.22-μm membrane of a syringe filter until it is clear.

**A2.1.5.3** Transfer the supernatant to an autosampler vial, and analyse on the gas chromatograph.

### A2.1.6 Sample Analysis

**A2.1.6.1** Gas chromatograph operating conditions

- **Injection mode:** Split, ratio 100:1
- **Column:** DB-WAX, 30 m × 0.25 mm × 1.0 μm
- **Detector:** Mass spectrometer
- **Carrier gas:** Helium at a flow rate of 1.0 mL/min
- **Temperature programme:**
  - **Injector:** 250 °C
  - **Start temperature:** 90 °C, hold for 0 min
  - **Rate:** 15 °C/min to 180 °C, hold for 8 min, 50 °C/min to 230 °C, hold at 230 °C for 5 min, post run at 250 °C for 10 min
- **Total run time:** 17 min
- **Autosampler conditions:** Injection volume: 1.0 μL
A2.1.6.2 Mass spectrometer operating conditions

Transfer line temperature: 280 °C
Ionization mode: Electrospray ionization
Ionization voltage: 70 eV
Ion source temperature: 250 °C
Quadrupole temperature: 150 °C
Solvent delay: 3 min

Note: These gas chromatography–mass spectrometry operating conditions should be adapted to obtain correct resolution of the glycerol, triethylene glycol and propylene glycol peaks. A typical chromatogram (total ion chromatogram) is shown in Annex 3.

Table A2.1. Qualitative and quantitative ions of humectants and internal standard

<table>
<thead>
<tr>
<th>Humectant</th>
<th>Quantitative ions</th>
<th>Secondary quantitative ions</th>
<th>Qualitative ions (abundance ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>61</td>
<td>43</td>
<td>61:43 (100:79)</td>
</tr>
<tr>
<td>Triethylene glycol</td>
<td>45</td>
<td>89</td>
<td>45:89 (100:19)</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>45</td>
<td>43</td>
<td>45:43 (100:20)</td>
</tr>
<tr>
<td>1,3-Butanediol</td>
<td>72</td>
<td>43</td>
<td>43:72 (100:28)</td>
</tr>
</tbody>
</table>

Table A2.2. Repeatability and reproducibility limits for determination of propylene glycol in tobacco in reference test pieces by gas chromatography–mass spectrometry

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>n*</th>
<th>m̄ (mg/g)</th>
<th>Repeatability limit (r) (mg/g)</th>
<th>Reproducibility limit (R) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R5F</td>
<td>6</td>
<td>0.215</td>
<td>0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>3R4F</td>
<td>6</td>
<td>0.179</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>CM6</td>
<td>6</td>
<td>0.113</td>
<td>0.000</td>
<td>0.002</td>
</tr>
<tr>
<td>Commercial cigarette No.1</td>
<td>6</td>
<td>6.310</td>
<td>0.128</td>
<td>0.668</td>
</tr>
<tr>
<td>Commercial cigarette No.2</td>
<td>6</td>
<td>0.037</td>
<td>0.000</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Results in one data set were removed as they were outliers.

Table A2.3. Repeatability and reproducibility limits for determination of glycerol in tobacco in reference test pieces by gas chromatography–mass spectrometry

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>n*</th>
<th>m̄ (mg/g)</th>
<th>Repeatability limit (r) (mg/g)</th>
<th>Reproducibility limit (R) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R5F</td>
<td>5</td>
<td>21.147</td>
<td>0.659</td>
<td>1.941</td>
</tr>
<tr>
<td>3R4F</td>
<td>5</td>
<td>21.510</td>
<td>0.715</td>
<td>1.331</td>
</tr>
<tr>
<td>CM6</td>
<td>5</td>
<td>1.466</td>
<td>0.006</td>
<td>0.106</td>
</tr>
<tr>
<td>Commercial cigarette No.1</td>
<td>5</td>
<td>14.762</td>
<td>0.512</td>
<td>1.164</td>
</tr>
<tr>
<td>Commercial cigarette No.2</td>
<td>5</td>
<td>1.457</td>
<td>0.005</td>
<td>0.110</td>
</tr>
</tbody>
</table>

* Results in two data sets were removed as they were outliers.
Table A2.4. Repeatability and reproducibility limits for determination of triethylene glycol in tobacco in reference test pieces by gas chromatography–mass spectrometry

<table>
<thead>
<tr>
<th>Reference cigarette</th>
<th>$n^*$</th>
<th>$\hat{m}$ (mg/g)</th>
<th>Repeatability limit ($r$) (mg/g)</th>
<th>Reproducibility limit ($R$) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample TG No. 1</td>
<td>6</td>
<td>0.971</td>
<td>0.006</td>
<td>0.010</td>
</tr>
<tr>
<td>Sample TG No. 2</td>
<td>6</td>
<td>8.450</td>
<td>0.189</td>
<td>0.617</td>
</tr>
</tbody>
</table>

* Results in one data set were removed as they were outliers.
Annex 3. Example of chromatographs obtained with mass spectrometry

Fig. A3.1. Chromatogram of a standard solution
World Health Organization Tobacco Laboratory Network SOP 03
Determination of tobacco-specific nitrosamines in mainstream tobacco smoke

Tobacco Free Initiative Tobacco Laboratory Network (TobLabNet)

No.: SOP 03
Date: June 2014

World Health Organization
Tobacco Laboratory Network

Standard operating procedure for 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

Fig. A3.2. Mass spectra of propylene glycol in scan mode

Fig. A3.3. Mass spectra of triethylene glycol in scan mode
Standard operating procedure for determination of humectants in cigarette tobacco filler

1. Nitrosamine – analysis
2. Tobacco – chemistry
3. Smoke – analysis
4. Consumer products safety
5. Materials testing – methods

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Fig. A3.4. Mass spectra of glycerol in scan mode

Fig. A3.5. Mass spectra of 1,3-butanediol (internal standard) in scan mode
Fig. A3.6. Chromatogram of a sample solution
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 determination of humectants in cigarette tobacco filler under International Organization for Standardization (ISO) and intense smoking conditions.