

## 10 PREPARATION OF STANDARDS

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

### 10.1 Nicotine standard stock solution (2 g/L)

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

**10.1.2** Dissolve the measured nicotine in 50 ml of water.

**10.1.3** Pipette 100 ml of extraction solution (9.2.2), and add 25 ml of 2 mol/l sodium hydroxide solution.

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

**10.1.5** Separate the supernatant organic phase, and store this solution, protected from light, at 4–8 °C.

### 10.2 Nicotine standard solutions

**10.2.1** Pipette 0.5 ml, 2.5 ml, 5.0 ml, 7.5 ml and 10.0 ml of the standard stock solution prepared in 10.1.5 into 20-ml volumetric flasks.

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

**10.2.3** Store the standard solutions, protected from light, at 4–8 °C.

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

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

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

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

**Table 1. Concentrations of nicotine in standard solutions**

Standard	Volume of nicotine standard solution (2g/l) (ml)	Volume of internal standard solution (µl)	Total volume (ml)	Approximate nicotine concentration in final mixed standard solution (mg/L)	Approximate level equivalent to unknown levels in cigarette tobacco filler (mg/g)
1	0.5		20	50	1.4
2	2.5	Not applicable, included in extraction solution	20	250	7
3	5.0		20	500	14
4	7.5		20	750	21
5	10.0		20	1000	28



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

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

## **11 SAMPLING**

**11.1** Sample cigarettes according to ISO 8243 [2.3] or as required for specific application of the method. Alternative approaches may be used to obtain a representative laboratory sample in accordance with individual laboratory practice, or when required by specific regulation or availability of samples.

### **11.2 Constitution of test sample**

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

**11.2.2** Take an equal amount of product for each test sample from at least  $\sqrt{n}$  [2.4] of the individual units (e.g. packet, container).

## **12 CIGARETTE PREPARATION**

**12.1** Remove the tobacco filler from the cigarettes and quality control samples (when applicable) in one packet (e.g. containing 20 cigarettes), or use at least 15 g of processed cigarette tobacco filler.

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

**12.3** Mix and grind the cigarette tobacco filler until it is fine enough to pass through a 4-mm screen.

**12.4** Condition the ground cigarette tobacco filler as required for the tobacco product according to ISO 3402 [2.1].

## **13 PREPARATION OF THE SMOKING MACHINE**

Not applicable.

## **14 SAMPLE GENERATION**

Not applicable.

## **15 SAMPLE PREPARATION**

**15.1** Take 1.5 g of the well-mixed, ground, conditioned test sample, and weigh it to 0.001-g accuracy into an extraction vessel.

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

**15.3** Shake the flask for  $60 \pm 2$  min on a shaker.

**15.4** Leave the sample flask to stand for another 20 min to allow visible, clear separation of the phases. After separation of the phases, analyse the organic (upper) phase as rapidly as possible by GC. To facilitate separation of the phases, the Erlenmeyer flask can be placed in an ultrasonic bath.



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

## **16 SAMPLE ANALYSIS**

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

### **16.1 GC operating conditions: example**

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

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

### **16.2 Expected retention times**

- 16.2.1** For the conditions described here, the expected sequence of elution will be *n*-heptadecane, nicotine.
- 16.2.2** Differences in e.g. temperature, gas flow rate and age of the column may alter retention times.
- 16.2.3** The elution order and retention times must be verified before analysis is begun.
- 16.2.4** Under the above conditions, the expected total analysis time will be about 10 min. The analysis time may be extended to optimize performance.

### **16.3 Determination of nicotine**

The sequence of determination will be in accordance with individual laboratory practices. This section illustrates an example of a sequence of determination for nicotine in cigarette tobacco filler.

- 16.3.1** Inject two replicate aliquots of the standard solutions and sample extracts under identical conditions.
- 16.3.2** Condition the system just before use by injecting two 1-µl aliquots of a sample solution as a primer.
- 16.3.3** Inject a check standard (blank with labelled internal standard) under the same conditions as the samples to verify the performance of the GC system.



- 16.3.4** Inject 1 µl of a blank solution (extraction solution minus internal standard) to check for any contamination of the system or reagents.
- 16.3.5** Inject an aliquot of each nicotine standard solution into the GC.
- 16.3.6** Assess the retention times and responses (area counts) of the standards. If the retention times are similar ( $\pm 0.2$  min) to the retention times in previous injections, and the responses are within 20% of typical responses in previous injections, the system is ready to perform the analysis. If the responses are outside specifications, seek corrective action according to your laboratory policy.
- 16.3.7** Record the peak areas of nicotine and the internal standard.
- 16.3.8** Calculate the relative response ratio ( $RF$ ) of the nicotine peak to the internal standard peak ( $RF = A_{\text{nicotine}} / A_{\text{IS}}$ ) for each of the nicotine standard solutions, including the solvent blanks.
- 16.3.9** Plot a graph of the concentration of nicotine (X axis) against the area ratios (Y axis).
- 16.3.10** The intercept should not be statistically significantly different from zero.
- 16.3.11** The standard curve should be linear over the entire standard range.
- 16.3.12** 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 coefficient  $R^2$  is less than 0.99, 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.
- 16.3.13** Inject 1 µl of each of the quality control samples and the samples, and determine the peak areas with the appropriate software.
- 16.3.14** The signal (peak area) obtained for all test portions must fall within the working range of the calibration curve; otherwise, solutions should be adjusted.

See Appendix 1 for representative chromatograms.

## **17 DATA ANALYSIS AND CALCULATIONS**

- 17.1** For each test portion, calculate the ratio of the nicotine response to the internal standard response ( $Y_t$ ) from the peak area.
- 17.2** Calculate the nicotine concentration in mg/L for each test portion aliquot using the coefficients of the linear regression ( $m_t = (Y_t - a) / b$ ).



- 17.3** Calculate the nicotine content,  $m_n$ , of the tobacco sample expressed in mg/g using the following equation:

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

where  $m_t$  is the concentration of nicotine in the test solution, in mg/L;  $V_e$  is the volume of the extraction solution used, in ml; and  $m_o$  is the mass of the test portion (conditioned weight), in g.

## **18 SPECIAL PRECAUTIONS**

- 18.1** After installing a new column, condition it by injecting a tobacco sample extract under the GC conditions described. Injections should be repeated until the peak areas (or heights) of both the nicotine and the internal standard are reproducible. This will require approximately four injections.
- 18.2** It is recommended to purge high-boiling-point components from the GC column after each sample set (series) by raising the column temperature to 220 °C for 30 min.
- 18.3** When the peak areas (or heights) for the internal standard are significantly higher than expected, it is recommended that the tobacco sample be extracted without internal standard in the extraction solution. This makes it possible to determine whether any component co-elutes with the internal standard, which would cause artificially lower values for nicotine.

## **19 DATA REPORTING**

- 19.1** Report individual measurements for each sample evaluated.
- 19.2** Report results as specified in the overall project specifications.
- 19.3** For more information, see WHO TobLabNet SOP 02 [2.5].

## **20 QUALITY CONTROL**

### **20.1 Control parameters**

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

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

### **20.2 Laboratory reagent blank**

To detect potential contamination during sample preparation and analysis, include a laboratory reagent blank, as described in **16.3.4**. The blank consists of all reagents and materials used in preparing test samples and is analysed like 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 analytical process, analyse a reference cigarette in accordance with the practices of individual laboratories.



## 21 METHOD PERFORMANCE SPECIFICATIONS

### 21.1 Limit of reporting

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

### 21.2 Laboratory-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 (before extraction) into an Erlenmeyer flask with tobacco and extracting the nicotine by the same method as for samples. Unspiked tobacco is also analysed. The recovery is calculated from the following equation and as shown in Table 2:

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

**Table 2. Mean and recovery of nicotine spiked onto the matrix**

Spiked amount (mg/g)	Nicotine (mg/g)	
	Mean (mg/g)	Recovery (%)
4.92	4.81	97.8
5.34	5.48	102.7
8.01	7.75	96.8
9.84	9.72	98.8
13.37	12.72	95.1
19.68	19.60	99.6

### 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 nicotine calibration curves established are linear over the standard concentration range of 50–1000 mg/L (1.4–27 mg/g).

### 21.5 Possible interference

The presence of eugenol can cause interference, as its retention time is similar to that of nicotine. This interference is most likely to occur with samples containing clove. The laboratory may need to resolve the interference by adjusting the analytical instrument parameters.

## 22 REPEATABILITY AND REPRODUCIBILITY

An international collaborative study [2.6] conducted in 2010, involving 18 laboratories and five samples (three reference cigarettes and two commercial brands), performed according to WHO TobLabNet SOP 02 [2.5] gave the following values for this method.

The difference between two single results found on 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 not 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 more than the reproducibility,  $R$ , on average no more than once in 20 cases with normal, correct application of the method.

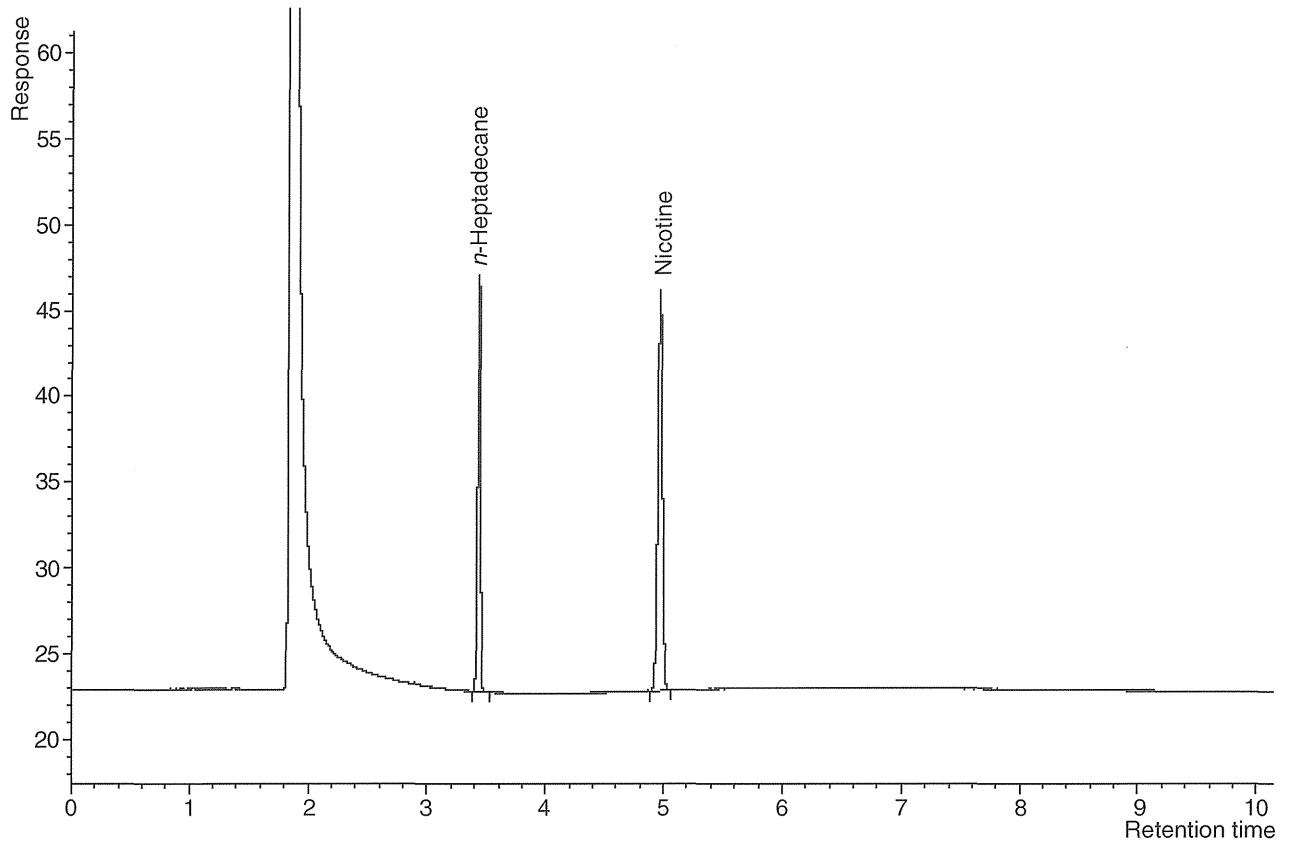
The test results were analysed statistically in accordance with ISO 5725-1 [2.7] and ISO 5725-2 [2.8] to give the precision data shown in Table 3.

**Table 3. Precision limits for determination of nicotine (mg/g) in cigarette tobacco filler from reference cigarettes**

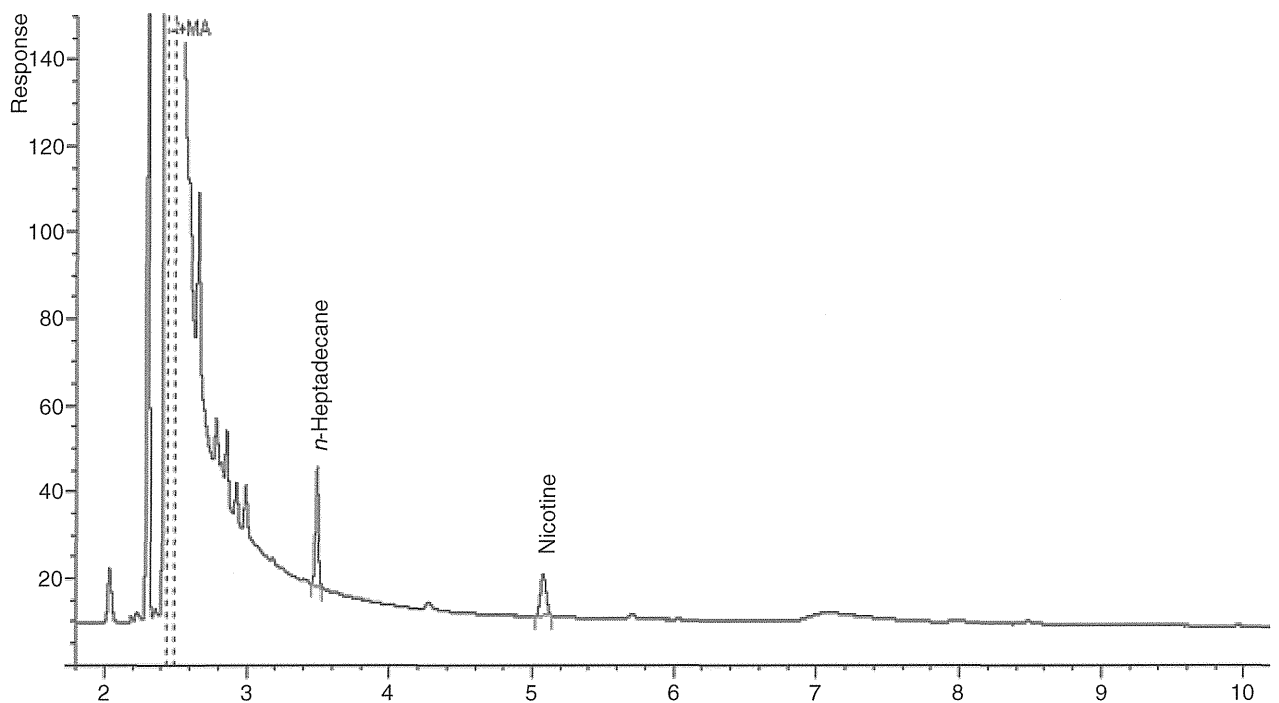
Reference cigarette	n	General mean	Repeatability limit	Reproducibility limit
1R5F	15	15.92	0.977	2.243
3R4F	17	17.16	1.152	2.414
CM6	17	18.77	1.378	2.635

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

**Appendix 1. Typical chromatograms obtained in the analysis of cigarette tobacco filler for nicotine content**



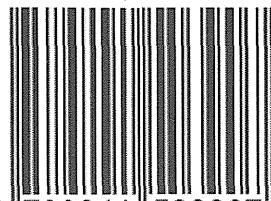
**Figure 1.** Example of a chromatogram of a standard solution with a nicotine concentration of 50 mg/L



**Figure 2.** Example of a chromatogram of a test solution



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