

WHO TobLabNet  
Official Method  
**SOP 05**

# STANDARD OPERATING PROCEDURE FOR DETERMINATION OF BENZO[A]PYRENE IN MAINSTREAM CIGARETTE SMOKE UNDER ISO AND INTENSE SMOKING CONDITIONS

Tobacco Free Initiative  
Tobacco Laboratory Network (TobLabNet)



**World Health  
Organization**





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Official Method  
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for determination of benzo[*a*]pyrene  
in mainstream cigarette smoke under  
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WHO Library Cataloguing-in-Publication Data

Standard operating procedure for determination of benzo[a]pyrene in mainstream cigarette smoke under ISO and intense smoking conditions.

1.Benzo[a]pyrene – analysis. 2.Tobacco – chemistry. 3.Smoke – analysis. 4.Consumer Product Safety.  
5.Materials Testing – methods. I.World Health Organization.

ISBN 978 92 4 150832 2

(NLM classification: QV 137)

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Printed in Switzerland



**No.:** SOP 05

**Date:** February 2015



World Health  
Organization

**World Health Organization  
Tobacco Laboratory Network**

**Standard operating procedure for method**

**Determination of benzo[*a*]pyrene  
in mainstream cigarette smoke under ISO  
and intense smoking conditions**

<b>Method:</b>	Determination of benzo[ <i>a</i> ]pyrene in mainstream cigarette smoke under ISO and intense smoking conditions
<b>Analytes:</b>	Benzo[ <i>a</i> ]pyrene (CAS # 50-32-8)
<b>Matrix:</b>	Tobacco cigarette mainstream smoke particulate matter
<b>Last update:</b>	February 2015



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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 risk. Representing differences in machine measurements as differences in exposure or risk is a misuse of testing with WHO TobLabNet-recommended methods.



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## 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 benzo[a]pyrene (B[a]P) in mainstream cigarette smoke under International Organization for Standardization (ISO) and intense smoking conditions.

## 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-nitrosornicotine (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 B[a]P, 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.

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

This SOP was prepared to describe the procedure for the determination of B[a]P in mainstream cigarette smoke under ISO and intense smoking conditions.

## 1 SCOPE

This method is suitable for the quantitative determination of B[a]P in mainstream cigarette smoke by gas chromatography coupled with mass spectrometry (GC-MS).





B[a]P is a polycyclic aromatic hydrocarbon. It was classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) in 2012. It is formed during incomplete combustion of organic matter. Inhalation, oral ingestion and dermal absorption are important routes of entry.

## 2 REFERENCES

- 2.1 *ISO 3402: Tobacco and tobacco products—Atmosphere for conditioning and testing.*
- 2.2 *ISO 4387: Cigarettes—Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine.*
- 2.3 World Health Organization. *Standard operating procedure for intense smoking of cigarettes.* Geneva: Tobacco Laboratory Network (WHO TobLabNet SOP 01).
- 2.4 *ISO 3308: Routine analytical cigarette-smoking machine—Definitions and standard conditions.*
- 2.5 United Nations Office on Drugs and Crime. *Guidelines on representative drug sampling.* Vienna: Laboratory and Scientific Section; 2009 ([http://www.unodc.org/documents/scientific/Drug\\_Sampling.pdf](http://www.unodc.org/documents/scientific/Drug_Sampling.pdf)).
- 2.6 *ISO 8243: Cigarettes—Sampling.*
- 2.7 *ISO 5725-1: Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions.*
- 2.8 *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.*
- 2.9 World Health Organization. *Method validation report on World Health Organization TobLabNet official method: Determination of benzo[a]pyrene in mainstream cigarette smoke under ISO and Intense smoking conditions.* Geneva: Tobacco Laboratory Network, forthcoming

## 3 TERMS AND DEFINITIONS

- 3.1 *TPM*: total particulate matter
- 3.2 *B[a]P*: benzo[a]pyrene
- 3.3 *B[a]P-d12*: deuterium-labelled B[a]P
- 3.4 *Tobacco products*: Products made entirely or partly of leaf tobacco as the raw material that are manufactured for smoking, sucking, chewing or snuffing (Article 1(f) of the WHO FCTC)



- 3.5 *Intense regimen*: Parameters for smoking tobacco products that include a 55-mL puff volume, a 30-s puff interval, a 2-s puff duration and 100% blocking of the filter ventilation holes
- 3.6 *ISO regimen*: Parameters for smoking tobacco products that include a 35-mL puff volume, a 60-s puff interval, a 2-s puff duration and no blocking of the filter ventilation holes
- 3.7 *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
- 3.8 *Test sample*: Product to be tested, taken at random from the laboratory sample
- 3.9 *Test portion*: Random portion from the test sample to be used for a single determination

## 4 METHOD SUMMARY

- 4.1 Mainstream smoke total particulate matter from the cigarette test sample is trapped onto a glass-fibre filter pad (commonly referred to as a Cambridge filter pad).
- 4.2 The number of cigarettes may have 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.
- 4.3 A solution containing an isotope-labelled internal standard is spiked onto the pad, which is extracted with cyclohexane.
- 4.4 The cyclohexane extract is eluted through a silica solid-phase extraction cartridge. The eluent collected is analysed by gas chromatography–mass spectrometry (GC–MS) with electron ionization detection.
- 4.5 A calibration curve is created by plotting the peak area ratio of B[a]P to B[a]P-d12 from the reconstructed ion chromatogram against known concentrations of B[a]P in standards. The B[a]P concentration in the sample is obtained from the calibration curve.

## 5 SAFETY AND ENVIRONMENTAL PRECAUTIONS

**CAUTION: Benzo[a]pyrene is a human carcinogen. Precautions shall be taken to avoid human exposure.**

B[a]P and its solutions should be handled in an adequately ventilated fume hood, glove box or equivalent.

The laboratory shall establish procedures for disposal of solutions containing B[a]P.



- 5.1 Take routine safety and environmental precautions, as in any chemical laboratory activity.
- 5.2 The testing and evaluation of certain products with this test method may require the use of materials and 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 this method have the responsibility to consult the appropriate authorities and to establish health and safety practices as well as environmental precautions in conjunction with any existing applicable regulatory requirements prior to its use.
- 5.3 Special care should be taken to avoid inhalation or oral 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 Equipment required to condition cigarettes as specified in ISO 3402 [2.1]
- 6.2 Equipment required to mark butt length as specified in ISO 4387 [2.2]
- 6.3 Equipment required to cover filter ventilation holes for the intense regimen as specified in WHO TobLabNet SOP 01 [2.3]
- 6.4 Equipment required to perform smoking of tobacco products as specified in ISO 3308 [2.4]
- 6.5 Analytical balance capable of measurement to at least four decimal places
- 6.6 Vortexer or wrist-action shaker or equivalent
- 6.7 Pipettes and tips capable of accurately dispensing volumes of 10–1000 µL
- 6.8 Volumetric pipette(s) or equivalent, 10 mL, 40 mL and 100 mL
- 6.9 Volumetric flasks, 10 mL, 25 mL and 100 mL
- 6.10 Erlenmeyer flasks, 100 mL, 200 mL or suitable flask
- 6.11 Silica solid-phase extraction manifold and cartridges (500 mg): pure silica unbound phase  
  
**Note:** e.g. Sep-pak Vac silica cartridge (Waters), Bond Elut Jr SI cartridge (Agilent), Supelclean LC-Si cartridge (Supelco), Strata SI-1 Silica (Phenomenex) or equivalent
- 6.12 Glass transfer pipettes or equivalent



- 6.13** Rotary evaporator with suitable flasks, or Turbovap with test tubes or equivalent
- 6.14** GC–MS system equipped with a computerized control, data acquisition and processing system. The system must be capable of operating the mass spectrometer in order to obtain chromatographic data in single-ion monitoring detection mode. The gas chromatograph must be configured to perform splitless injections on a capillary column. It is recommended that the gas chromatograph be equipped with an autosampler for sample injection.
- 6.15** Column: Fused silica capillary column with a methylphenyl (5%) polysiloxane stationary phase, a 30-m column with 0.25-mm internal diameter and 0.25- $\mu$ m film thickness is suitable for this analysis.
- Note:** e.g. DB-5ms (Agilent) or equivalent
- 6.16** Autosampler vials, 2 mL or equivalent

## **7 REAGENTS AND SUPPLIES**

- 7.1** 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 available.
- 7.2** Cyclohexane (110-82-7)
- 7.3** B[a]P (50-32-8)
- 7.4** B[a]P-d12 (63466-71-7)

**Note:** B[a]P and B[a]P-d12 are carcinogenic to humans. Appropriate safety precautions shall be taken when manipulating these compounds or any solution containing these compounds.

## **8 PREPARATION OF GLASSWARE**

Clean and dry glassware in a manner to avoid contamination.

## **9 PREPARATION OF SOLUTIONS**

Not applicable

## **10 PREPARATION OF STANDARDS**

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

## 10.1 Isotope-labelled internal standard

Prepare in accordance with either option A or B.

### Option A

#### 10.1.1 Primary B[a]P-d12 stock solution (200 µg/mL)

**10.1.1.1** Weigh approximately 0.005 g of B[a]P-d12 into a 25-mL volumetric flask on a four-decimal place balance, and record the weight to the nearest 0.0001 g.

**10.1.1.2** Dissolve B[a]P-d12 internal standard in 25 mL of cyclohexane, and mix well.

**10.1.1.3** Label, and store at  $-20 \pm 5$  °C in amber vials.

#### 10.1.2 B[a]P-d12 spiking solution (2 µg/mL)

**10.1.2.1** Pipette 1 mL of the primary B[a]P-d12 stock solution (**10.1.1**) into a 100-mL volumetric flask.

**10.1.2.2** Dilute to the mark with cyclohexane, and shake well.

**10.1.2.3** Label and store at  $-20 \pm 5$  °C.

### Option B

**10.1.3** Obtain primary B[a]P-d12 stock solution of 1000 µg/mL in methylene chloride from a commercial supplier, label, and store at  $-20 \pm 5$  °C.

#### 10.1.4 Prepare B[a]P-d12 spiking solution (2 µg/mL).

**10.1.4.1** Pipette 200 µL of 1000 µg/mL B[a]P-d12 stock solution (**10.1.3**) into a 100-mL volumetric flask.

**10.1.4.2** Dilute to the mark with cyclohexane, and shake well.

**10.1.4.3** Label and store at  $-20 \pm 5$  °C.

## 10.2 Native standards

#### 10.2.1 Primary B[a]P stock solution (200 µg/mL)

**10.2.1.1** Weigh approximately 0.005 g B[a]P into a 25-mL volumetric flask on a four-decimal place balance, and record the weight to the nearest 0.0001 g.

**10.2.1.2** Dissolve the B[a]P standard in 25 mL of cyclohexane, and mix well.

**10.2.1.3** Label and store at  $-20 \pm 5$  °C.

#### 10.2.2 Secondary B[a]P stock solution (1 µg/mL)

**10.2.2.1** Pipette 0.5 mL of the primary B[a]P stock solution (**10.2.1**) into a 100-mL volumetric flask.



**10.2.2.2** Dilute to the mark with cyclohexane, and mix well.

**10.2.2.3** Label and store at  $-20 \pm 5$  °C.

The standard solutions are stable for up to 6 months if stored at  $-20 \pm 5$  °C.

Note: Sonication may be used in the preparation of primary stock solutions to help dissolve B[a]P and B[a]P-d12 standards completely. In this case, first dissolve the standards in about 15 mL of solvent. After sonication, let the solution stand until it cools to working temperature (e.g. room temperature), then dilute to the mark with cyclohexane.

### 10.3 B[a]P working standard solutions

Prepare working standard solutions as stated below and summarized in Table 1.

**Table 1. Working standard solutions of B[a]P**

Standard	Volume of secondary B[a]P stock standard solution (1 µg/mL) (µL)	Volume of B[a]P-d12 spiking solution (2 µg/mL from 10.1.2 or 10.1.4) (µL)	Total volume (mL)	Approximate concentration of B[a]P working standard solution (ng/mL)
1	20	100	10	2
2	40	100	10	4
3	80	100	10	8
4	200	100	10	20
5	400	100	10	40
6	600	100	10	60

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

**10.3.1** Spike variable volumes of the secondary B[a]P stock solution from 10.2.2 into a 10-mL volumetric flask.

**10.3.2** Add 100 µL of the B[a]P-d12 spiking solution prepared in 10.1.2 or 10.1.4.

**10.3.3** If option A is chosen, the concentrations of B[a]P-d12 spiking solution (10.1.2) can be determined from the equation:

$$\text{Final concentration (µg/mL)} = x \times 400,$$

where x is the original weight (in g) of standard as determined in 10.1.1.1.

**10.3.4** When option B is chosen, the concentration of B[a]P-d12 spiking solution (10.1.4) is computed according to the value provided by the supplier.

**10.3.5** Fill to the mark with cyclohexane, and mix well.

**10.3.6** The final concentrations of standard are determined from the equation:

$$\text{Final concentration (ng/mL)} = x \times y \times 20.$$

Note: The equation in 10.3.6 is derived as follows:

The concentrations of B[a]P working standard solutions (in ng/mL) can be calculated from:

$$\begin{aligned} \frac{x \text{ (g)}}{25 \text{ mL}} \times \frac{0.5 \text{ mL}}{100 \text{ mL}} \times \frac{y \text{ (}\mu\text{L)}}{10 \text{ mL}} \times \frac{1 \text{ mL}}{1000 \mu\text{L}} \times \frac{1\,000\,000\,000 \text{ ng}}{1 \text{ g}} \\ = \frac{x \times 0.5 \times 1000 \times y \text{ (ng)}}{25 \text{ mL}} \\ = x \times y \times 20 \end{aligned}$$

where x is the original weight (in g) of standard as determined in **10.2.1.1**, and y is the volume (in  $\mu\text{L}$ ) of secondary B[a]P stock solution as described in **10.2.2** and Table 1.

**10.3.7** The range of the standard solutions may be adjusted in accordance with the equipment used and the samples to be tested, keeping in mind a possible effect on the sensitivity limits of the method.

## **11 SAMPLING**

**11.1** Sample cigarettes according to ISO 8243 [2.6]. Alternative approaches may be used to obtain a representative laboratory sample in accordance with individual laboratory practice or 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 units (e.g. packet, container), if applicable.

**11.2.2** Take an equal amount of product for each test sample from at least  $\sqrt{n}$  [2.5] of the individual units (e.g. packet, container).

## **12 CIGARETTE PREPARATION**

**12.1** Condition all cigarettes to be smoked in accordance with ISO 3402 [2.1].

**12.2** Mark cigarettes at a butt length in accordance with ISO 4387 [2.2] and WHO TobLabNet SOP 01 [2.3].





- 12.3** Prepare test samples to be smoked in accordance with either ISO or intense smoking conditions as specified in WHO TobLabNet SOP 01 [2.3].

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

#### **13.1 Ambient conditions**

The ambient conditions for smoking are specified in ISO 3308 [2.4].

#### **13.2 Smoking machine specifications**

Follow ISO 3308 [2.4] smoking machine specifications, except for intense smoking, for which the smoking machine should be prepared as described in WHO TobLabNet SOP 01 [2.3].

### **14 SAMPLE GENERATION**

Smoke a sufficient number of cigarettes on the specified smoking machine such that breakthrough does not occur and the concentrations of the B[a]P 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 WHO TobLabNet SOP 01 [2.3].
- 14.2** Include at least one reference test sample for quality control, if applicable.
- 14.3** When testing sample types for the first time, evaluate breakthrough of the filter pad. The number of cigarettes might have to be adjusted. If the TPM exceeds 600 mg for a 92-mm filter pad or 150 mg for a 44-mm filter pad, decrease the number of cigarettes smoked onto each pad.
- 14.4** The numbers of cigarettes to be smoked per measurement for linear and rotary smoking machines in ISO and intense smoking regimes are shown in Table 2.

**Table 2. Suggested numbers of cigarettes to be smoked for one measurement on linear and rotary smoking machines**

	ISO smoking regimen		Intense smoking regimen	
	Linear	Rotary	Linear	Rotary
No. of cigarettes per pad	5	20	3	10
No. of pads per result	4	1	3	1

**Note:** The number of cigarettes to be smoked should be adjusted such that breakthrough does not occur and the concentrations of B[a]P fall within the calibration range prepared for the analysis.





- 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

### 15.1 Extraction of filter pads

**15.1.1** 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 to that on which 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. Transfer the filter pad(s) into a 100-mL Erlenmeyer or suitable flask for a 44-mm pad or a 200-mL Erlenmeyer or suitable flask for a 92-mm pad.

**Note:** In the ISO regimen, four 44-mm pads for a linear smoking machine or one 92-mm pad for a rotary smoking machine are extracted into one flask. In the intense regimen, three 44-mm pads for a linear smoking machine or one 92-mm pad for a rotary smoking machine are extracted into one flask.

**15.1.2** Add cyclohexane (40 mL for 44-mm pads, 100 mL for 92-mm pads) to the flask.

**15.1.3** Spike each flask with B[a]P-d12 spiking solution (**10.1.2** or **10.1.4**) (40 µL for a 44-mm pad, 100 µL for a 92-mm pad)

**15.1.4** Using a vortexer, wrist-action or equivalent device, extract the B[a]P from the pad by shaking at 200 rpm for 60–80 min.

### 15.2 Sample clean-up

**15.2.1** Condition a solid-phase extraction cartridge with 10 mL of cyclohexane, and discard the eluate. Do not let the cartridge run dry.

**15.2.2** Pipette 10 mL of sample extract onto the cartridge, and allow it to pass through the cartridge. Collect the eluate.

**15.2.3** Elute the cartridge with two further 15-mL aliquots of cyclohexane, allowing the cartridge to run dry after the last aliquot is passed through.

**15.2.4** Evaporate the cyclohexane solution almost to dryness.

**Note:** The conditions of the rotary evaporator are to be set at 55 °C with a vacuum pressure of 30 kPa (300 mbar) (approximately 10 min). The vacuum pressure can be adjusted for the equipment. The conditions of the Turbovap are to be set at 30 °C in a steady stream of nitrogen (approximately 15 min).



**15.2.5** Pipette 1 mL of cyclohexane into the flask to dissolve the extract. Cap the flask, and rotate it carefully to rinse the inner surface. Take an aliquot for GC–MS testing.

**Important note:** The final concentration of B[a]P-d12 internal standard in the sample is approximately 20 ng/mL.

## 16 SAMPLE ANALYSIS

This method for quantifying B[a]P in mainstream cigarette smoke involves GC–MS. The analytes are resolved from other potentially interfering substances on a GC column. Comparison of the area ratio (native analyte to isotope-labelled analyte) of the unknowns with the area ratio (native analyte to isotope-labelled analyte) of the known standard concentrations yields the concentrations of the analyte.

### 16.1 GC–MS operating conditions: example

GC column	Fused silica capillary column with a methylphenyl (5%) polysiloxane stationary phase, a 30-m column with a 0.25-mm internal diameter and 0.25-µm film thickness is suitable (DB-5ms Agilent or equivalent).
Injector temperature	280 °C
Mode	Constant flow
Flow rate	1.2 mL/min
Injection	1 µL or 2 µL splitless
Column temperature	150 °C for 0 min; 6 °C/min to 260 °C, hold at 260 °C for 7 min; 50 °C/min to 290 °C, hold at 290 °C for 20 min
Transfer line temperature	280 °C
MS source	230 °C
Ion traces	B[a]P: $m/z$ 252 B[a]P-d12: $m/z$ 264
Dwell time	50 ms
Ionization mode	Electron ionization

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

## 16.2 General analytical information

- 16.2.1 For the conditions described here, the expected sequence of elution will be B[a]P-d12, B[a]P.
- 16.2.2 Differences in e.g. temperature, gas flow rate and the age of the column can be expected to alter retention times.
- 16.2.3 The sequence of determination of B[a]P will be in accordance with individual laboratory practice. This section gives an example.
- 16.2.4 Inject a blank solution (extraction solution minus labelled internal standard) to check for any contamination in the system or reagents.
- 16.2.5 Inject a standard blank (blank with labelled internal standard) to verify the performance of the GC–MS system.
- 16.2.6 Inject the calibration standards, the quality control and the samples.
- 16.2.7 Record the peak areas of B[a]P and B[a]P-d12.
- 16.2.8 Calculate the relative response ratio of the B[a]P peak to the B[a]P-d12 peak ( $A_{\text{B[a]P}} / A_{\text{B[a]P-d12}}$ ) for each standard solution, including standard blanks.
- 16.2.9 Plot a graph of the concentration of B[a]P (X axis) against the area ratios (Y axis).
- 16.2.10 The intercept should not be statistically significantly different from zero.
- 16.2.11 The standard curve should be linear over the entire standard range.
- 16.2.12 Calculate the linear regression ( $Y = a + bx$ ) from these data, and use both the slope ( $b$ ) and the intercept ( $a$ ) of the linear regression.

If the linear regression,  $R^2$ , 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), the point should be omitted.
- 16.2.13 Inject the quality controls and samples, and determine the peak areas with the appropriate software.
- 16.2.14 The peak ratio obtained for all test portions must fall within the working range of the calibration curve; otherwise, the concentrations of the standard and standard solutions or test portion solutions should be adjusted.

See Annex 1 for representative chromatograms.



## 17 DATA ANALYSIS AND CALCULATIONS

17.1 The slope and intercept are determined from the relative responses of B[a]P and B[a]P-d12 versus the relative concentrations of B[a]P.

17.2 Calculate the relative response ratio from the peak areas for each of the calibration standards (10.3):

$$RF = A_{B[a]P} / A_{B[a]P-d12},$$

where RF is the relative response ratio,  $A_{B[a]P}$  is the peak area of B[a]P (reconstructed ion chromatogram  $m/z$  252) and  $A_{B[a]P-d12}$  is the peak area of B[a]P-d12 (reconstructed ion chromatogram  $m/z$  264).

17.3 Plot a graph of the relative response factor,  $A_{B[a]P} / A_{B[a]P-d12}$  (Y axis), versus concentration (X axis) for each standard solution. Calculate the linear regression ( $Y = a + bx$ ) from these data, and use both the slope ( $b$ ) and the intercept ( $a$ ) of the linear regression.

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

17.5 The content of B[a]P (ng/cigarette) is determined from the calculated relative response ratio for the test portion, the slope and the intercept obtained from the appropriate calibration curve and the equation:

$$M = \frac{Y - a}{b} \times \frac{V \times V_e}{V_c \times n}$$

where  $M$  is the calculated content of B[a]P in nanograms per cigarette,  $Y$  is the relative response ratio  $A_{B[a]P} / A_{B[a]P-d12}$ ,  $a$  is the intercept of the linear regression obtained from the standard calibration curve,  $b$  is the slope of the linear regression obtained from the standard calibration curve,  $V$  is the volume of the sample solution (1 mL),  $V_e$  is the volume of the extraction solution (40 mL for a 44-mm Cambridge filter pad, 100 mL for a 92-mm Cambridge filter pad),  $V_c$  is the volume of the aliquot of the extraction solution used in clean-up (10 mL), and  $n$  is the number of cigarettes smoked (see Table 2).

Alternative calculation procedures may be used if applicable.

## 18 SPECIAL PRECAUTIONS

After installing a new column, condition it as specified by the manufacturer by injecting a tobacco sample extract under the specified instrument conditions. Injections should be repeated until the peak areas (or heights) of both B[a]P and B[a]P-d12 are reproducible.

## 19 DATA REPORTING

19.1 Report individual measurements for each sample evaluated.



**19.2** Report results as nanograms per cigarette or as required.

## **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 undertaken.

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

### **20.2 Laboratory reagent blank**

To detect any contamination during sample preparation and analysis, include a laboratory reagent blank, as described in **16.2.5** The blank consists of all the reagents and materials used in analysing test samples and is analysed like a test sample. The blank should be assessed in accordance with the practices of individual laboratories.

### **20.3 Quality control sample**

To verify the consistency of the entire analytical process, analyse a reference cigarette (or an appropriate quality control sample) in accordance with the practices of the individual laboratory.

**20.4** Make a new calibration curve after every 20 injections of test sample (20 sample extracts).

## **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 nanograms per cigarette

### **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 a known amount of standard (after smoking) onto a filter pad with cigarette smoke and extracting the pad by the same method used for extracting samples. Unspiked pads are also analysed. The recovery is calculated from the following equation and is shown in Table 3:

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



Table 3. Mean and recovery of B[a]P spiked onto the matrix

Spiked amount (ng/cig)	Mean (ng/cig)	Recovery (%)
2.91	2.93	100.58
6.79	6.82	100.39
9.70	10.08	103.92

### 21.3 Analytical specificity

GC–MS provides analytical specificity. The retention time and molecular mass to charge ratio ( $m/z$ ) are used to verify the specificity of the results for an unknown sample.

### 21.4 Linearity

The B[a]P calibration curves established are linear over the standard concentration range of 2–60 ng/mL.

### 21.5 Possible interference

No known components have both similar retention times and  $m/z$  as B[a]P or B[a]P-d12.

## 22 REPEATABILITY AND REPRODUCIBILITY LIMITS

An international collaborative study [2.9] conducted in 2012, involving testing of three reference cigarettes (1R5F, 3R4F and CM6) and two commercial brands by eight laboratories, gave the precision limits for the method indicated in Table 4.

The difference between two single results found for matched cigarette 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 in the normal, correct application of the method.

Single results for matched cigarette 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 4.

**Table 4. Precision limits for the determination of B[a]P (ng/cigarette) in mainstream cigarette smoke from reference cigarettes**

Reference cigarette	ISO smoking regimen				Intense smoking regimen			
	$n$	$m_{cig}$	$r_{limit}$	$R_{limit}$	$n$	$m_{cig}$	$r_{limit}$	$R_{limit}$
1R5F	8	1.46	0.47	0.99	8	6.47	1.52	2.91
3R4F	8	5.99	1.03	2.09	8	14.51	2.52	3.15
CM6	7	13.70	1.43	5.83	7	25.86	2.81	8.45

Where 1R5F, 3R4F and CM6 are three reference cigarettes analysed in this study,  $n$  is the number of laboratories that participated,  $m_{cig}$  is the mean value of B[a]P per cigarette,  $r_{limit}$  is the repeatability limit of B[a]P, and  $R_{limit}$  is the reproducibility limit of B[a]P.

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 (five cigarettes smoked per pad) from linear smoking machines and one Cambridge filter pad (20 cigarettes smoked per pad) from rotary smoking machines. Under intense smoking conditions, one test result was defined as the average of seven individual replicates of the mean yield of two sets of three Cambridge filter pads (three cigarettes smoked per pad) from linear smoking machines and two sets of one Cambridge filter pad (10 cigarettes smoked per pad) from rotary smoking machines. For more information, see reference [2.9].

## 23 TEST REPORT

The following information shall be included in the test report:

- A reference to this method, i.e. WHO TobLabNet SOP 05
- Date of receipt of the sample
- The results and their units



**ANNEX 1. Typical chromatograms obtained in the determination of benzo[a]pyrene in mainstream cigarette smoke**

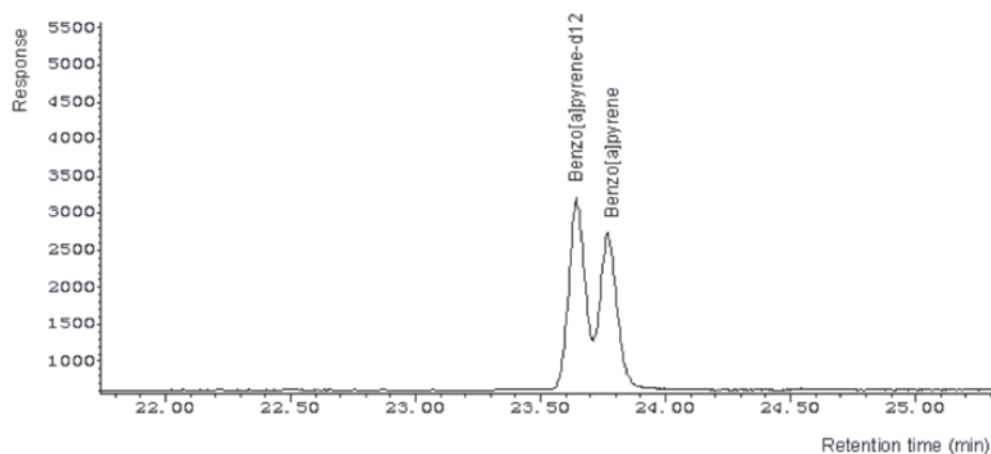


Figure 1. Representative chromatogram of a standard solution

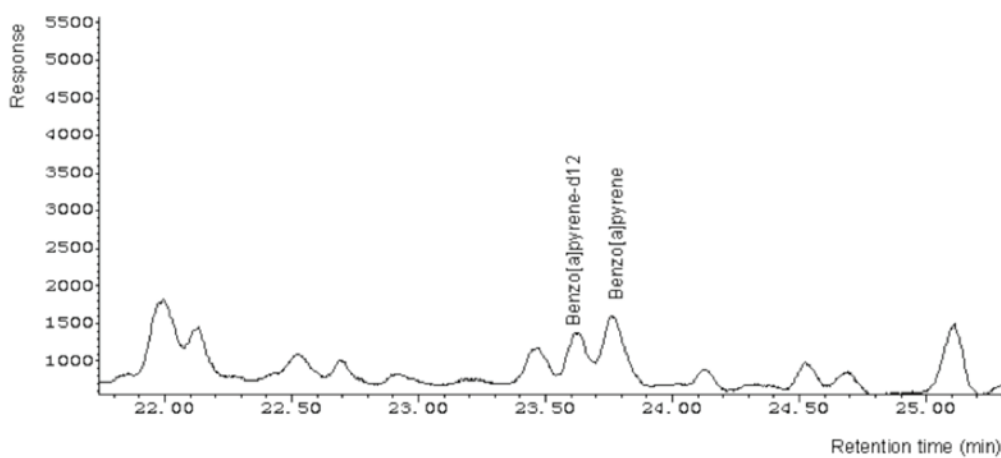


Figure 2. Representative chromatogram of a sample solution





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 benzo[a]pyrene in mainstream cigarette smoke under International Organization for Standardization (ISO) and intense smoking conditions.

