

## CORESTA RECOMMENDED METHOD N° 58

### **DETERMINATION OF BENZO[a]PYRENE IN CIGARETTE MAINSTREAM SMOKE – GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD**

*(February 2004)*

#### **0. INTRODUCTION**

Between 1999 and 2003, a Task Force composed of CORESTA members has studied the existing methodologies for the determination of Benzo[a]pyrene (B[a]P) in the mainstream smoke of cigarettes. Several methods have been proposed for this determination, which mainly are based on two types of analytical methodologies: HPLC with fluorescence detection and GC-MS. In both cases, it is necessary to purify the smoke condensate extract before performing the chromatography in order to obtain a correct separation of the B[a]P peak.

The Task Force decided in the first instance to develop a method using HPLC with fluorescence detection. However, after several collaborative experiments it appeared that achieving a significant reduction of the initially observed variability would be technically very difficult. The Task Force then decided to investigate a GC-MS method as an alternative and was able to demonstrate through collaborative experiments, that a lower variability can be obtained with this methodology.

This Recommended Method, produced through collaborative experiments involving many laboratories in many countries, provides an optimised procedure for the determination of Benzo[a]pyrene in cigarette mainstream smoke, and constitutes the accepted reference procedure by the Task Force. The repeatability and reproducibility of this method have been assessed according to ISO recommendations and are included.

#### **1. FIELD OF APPLICATION**

This recommended method is applicable to the determination of Benzo[a]pyrene (B[a]P) in the total particulate matter of cigarette mainstream smoke. The described method is specified using ISO 3308:2000 smoking parameters, but is technically compatible with other smoking regimes.

#### **2. REFERENCES**

*ISO 3308:2000*

*Routine analytical smoking machine – Definition and standard conditions*

*ISO 4387:2000*

*Cigarettes – Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine*

*ISO 3402:1999  
Tobacco and tobacco products – Atmosphere for conditioning and testing*

*ISO 8243:1991  
Cigarettes - Sampling*

*CORESTA Recommended Method N°21 (August 1991)  
Atmosphere for conditioning and testing tobacco and tobacco products.*

*CORESTA Recommended Method N°22 (August 1991)  
Routine analytical cigarette-smoking machine specifications, definitions and standard conditions.*

*CORESTA Recommended method N°23 (August 1991)  
Determination of total and nicotine-free dry particulate matter using routine analytical cigarette-smoking machine --Determination of total particulate matter and preparation for water and nicotine measurements.*

*CORESTA Recommended Method N°24 (August 1991)  
Cigarettes - Sampling*

### **3. PRINCIPLE**

Sampling of the test cigarettes. Conditioning of the test cigarettes. Smoking of the test cigarettes according to the smoking procedure specified in ISO 4387:2000. Extraction of the total particulate matter, collected on the glass-fibre filter pad, with methanol. Dilution of the methanol extract with water. Elution of the water/methanol solution through a CH SPE cartridge, followed by the elution of B[a]P with cyclohexane. Analytical determination of B[a]P by gas chromatography – mass spectrometry (GC/MS) using single ion monitoring (SIM) detection mode.

### **4. APPARATUS**

Normal laboratory apparatus and equipment and in particular the following items:

#### **4.1. Routine analytical cigarette-smoking machine**

Complying with the requirements of ISO 3308:2000 and equipped for smoking according to ISO 4387:2000.

#### **4.2. Gas chromatography – Mass spectrometry system**

Equipped with its computerised control and data acquisition and processing system. This system must be able to pilot the mass spectrometer in order to obtain chromatographic data under Single Ion Monitoring (SIM) detection mode. The gas chromatograph must be configured to perform splitless injections on a capillary column. It is recommended to equip the gas chromatograph with an autosampler for sample injection.

#### 4.3. Column

Fused silica capillary column with a methylphenyl(5 %)polysiloxane stationary phase. For example: a 30 m, 0,25 mm internal diameter, with a 0,25 µm film thickness column is suitable for this analysis.

#### 4.4. Rotary evaporator or equivalent equipment

#### 4.5. Vacuum sample preparation unit

#### 4.6. CH Solid Phase Extraction cartridges

Phase : Cyclohexyl bonded silica. A 6ml, 1g packing cartridge is suitable.

#### 4.7. Gas tight syringes

25 µl, 100 µl, 250 µl and 1000 µl

#### 4.8. General laboratory equipment

For the preparation of samples, standards and reagents. All glassware shall be cleaned before use to avoid any contamination.

### 5. REAGENTS

All reagents shall be of analytical grade quality.

#### 5.1. Methanol

#### 5.2. Distilled water

#### 5.3. Cyclohexane

#### 5.4. Toluene

#### 5.5. Benzo[a]pyrene

#### 5.6. Benzo[a]pyrene-d12

**Note : Benzo[a]pyrene and benzo[a]pyrene-d12 are suspected carcinogens. Appropriate safety precautions shall be taken when manipulating these compounds or any solution containing these compounds.**

### 6. STANDARDS

#### 6.1. Primary B[a]P stock solution

Dissolve approximately 10 mg B[a]P, weighted exactly to 0,01 mg, in 10 ml of toluene. This solution shall be stored at – 20 °C.

#### 6.2. Secondary B[a]P stock solution

Dilute 1 ml of the primary B[a]P stock solution to 100 ml with methanol. This solution shall be stored at – 20 °C.

### **6.3. B[a]P-d12 stock solution**

Dissolve approximately 10 mg B[a]P-d12, weighted exactly to 0,01 mg, in 10 ml of toluene. This solution shall be stored at – 20 °C.

### **6.4. B[a]P-d12 spiking solution**

Using a gas syringe, transfer 100 µl of the B[a]P-d12 stock solution into a 100 ml volumetric flask and complete to the mark with methanol. This solution has a concentration of approximately 1 µg/ml.

### **6.5. Working standard solutions**

Prepare 6 working standard solutions that cover the concentration range of interest. For example, transfer 20 µl of the B[a]P-d12 stock solution (6.3) and 10 to 2000 µl of the secondary B[a]P stock solution (6.2) into 100 ml volumetric flasks and complete to the mark with cyclohexane. These solutions have a concentration of approximately 0.2 µg/ml of B[a]P-d12 and concentrations from 1 to 200 ng/ml of B[a]P.

### **6.6. Storage**

The above standard solutions are stable for up to six months if stored at –20 °C.

## **7. SAMPLE PREPARATION**

### **7.1. Sampling**

Sampling is done in accordance with ISO 8243:1991.

### **7.2. Smoking**

Cigarettes are smoked according to ISO 4387:2000. Typically 10 cigarettes should be smoked onto a 44 mm Cambridge filter pad, and 20 cigarettes onto a 92 Cambridge filter pad. Cambridge filter pad of 44 mm diameter are capable of retaining up to 150 mg of total particulate matter (TPM) and pads of 92 mm diameter up to 600 mg. If this mass is exceeded, the number of cigarettes shall be reduced. For low tar products, a greater number of cigarettes may be smoked to achieve a nominal TPM of 10 mg for a 44 mm pad and 20 mg for a 92 mm pad.

### **7.3. Filter pads extraction**

**7.3.1.** Remove the filter pad from its holder, fold the filter two times (with the condensate inside) and wipe the inside of the holder with the pad.

**7.3.2.** Transfer the filter pad into an Erlenmeyer flask (100 ml for a 44 mm pad; 200 ml for a 92 mm pad).

**7.3.3.** For a 44 mm pad, add 20 ml of methanol into the flask, then add 200 µl of the B[a]P-d12 spiking solution (6.4) with a suitable syringe. For a 92 mm pad, add 50 ml of methanol into the flask, then add 400 µl of the B[a]P-d12 spiking solution (6.4) with a suitable syringe.

**7.3.4.** Shake the flask vigorously until the filter pad has disintegrated and filter the solution through a glass suction filter or using paper filtration.

**7.3.5.** Wash the filter remainder with approximately 15 ml of methanol for a 44 mm pad or 25 ml of methanol for a 92 mm filter pad respectively . Add this washing solution to the filter extract and complete to a volume which is : at least 40 ml for a 44 mm filter pad, or at least 80 ml for a

92 mm pad, with methanol. For convenience bigger final volumes can be used, but without unnecessarily diluting the solution.

- 7.3.6.** Transfer an aliquot of the obtained solution into a separatory funnel. The volume of this aliquot shall not exceed 40 ml which is convenient for this procedure. However a smaller aliquot can be used in order to shorten the elution time during the clean-up step (see below 7.4.2).
- 7.3.7.** Add distilled water into the funnel in order to obtain a solution containing 60 % of water and 40 % of methanol, and mix. For example, if an aliquot of 40 ml is used in 7.3.6, add 60 ml of distilled water.

#### **7.4. Sample clean-up**

- 7.4.1.** The CH SPE cartridge is pre-conditioned before use by passing through it 10 ml of methanol and 10 ml of a mixture of water and methanol (60 : 40 w/w).
- 7.4.2.** In the vacuum sample preparation unit, let the extraction solution pass through the CH SPE cartridge under vacuum at a flow rate of approximately 2 ml/min (1 drop per second). Rinse the funnel with 10 ml of a mixture of water and methanol (60 : 40 v/v). Dry the cartridge with a stream of air for at least 30 minutes.
- 7.4.3.** Elute the cartridge with 15 ml of cyclohexane.
- 7.4.4.** Reduce the volume of the cyclohexane solution to about 0.5 ml. Then add cyclohexane in order to obtain a volume of 1 ml in a volumetric flask.

**Note :** In spite of the drying procedure described in 7.4.2, the cyclohexane solution obtained in 7.4.3 may still contain a significant amount of water and a two phase solution can be obtained after the volume reduction prescribed in 7.4.4. In this case, the cyclohexane phase shall be separated from the water phase before adjusting the final volume to 1 ml. Alternatively the cyclohexane solution in 7.4.3 may be dried on a water adsorbent before volume reduction.

- 7.4.5.** Transfer the obtained solution into a sample vial with a screwcap and Teflon faced septum.

## **8. DETERMINATION**

### **8.1. GC/MS operating conditions**

Set up and operate the GC/MS system in accordance with the manufacturer's instructions.

The following condition are suitable for this analysis:

Injector temperature:	290 °C
Mode:	constant flow
Initial flow:	0.9 ml/min
Injection:	1µl splitless
Column temperature:	80 °C for 3 min 5 °C/min to 290 °C hold at 290°C for 20 min
Transfer line temp:	270°C
MS Source:	230°C
Ion traces:	B[a]P: m/z 252 (quantification) and 250 (confirmation) B[a]P-d12: m/z 264 (quantification) and 260 (confirmation)

These chromatographic conditions shall be adapted in order to obtain a correct resolution of the B[a]P and B[a]P-d12 peaks. A typical chromatogram is given in Annex 1.

## 8.2. Calibration

Inject successively each working standard solution (6.5) in the GC/MS system. Record the area of the B[a]P and the B[a]P-d12 peaks. A calibration curve for B[a]P is generated by calculating a linear equation regression of the area ratios of B[a]P to B[a]P-d12 peaks as a function of the B[a]P concentrations. The intercept of this regression line should be close to zero.

Inject one working standard solution (6.5) after 10 sample analysis and if the measured concentration for this solution is different by more than 15 % of the nominal value then repeat the calibration procedure.

## 8.3. Determination of B[a]P

Inject the sample, calculate the area ratio of B[a]P to B[a]P-d12 peaks and obtain the concentration of B[a]P in the solution by comparing this ratio with the B[a]P calibration line.

**Note :** During a normal analysis sequence, it has been observed by several laboratories that the absolute value of the B[a]P-d12 peak area may show significant variations. The reasons for this observed variability of the GC-MS response have not been investigated thoroughly. However, this phenomenon has no effect on the final result because the internal standard procedure used in this method compensates for these variations.

## 8.4. Calculation

The amount of B[a]P per cigarette is calculated as follows:

$$M = \frac{C * V * V_e}{n * V_c}$$

Where :

- M is the mass of B[a]P in cigarette smoke expressed in ng/cig
- C is the concentration of B[a]P in the sample solution expressed in ng/ml.
- V is the volume of the sample solution expressed in ml (V = 1ml)
- n is the number of cigarettes smoked
- Ve is the volume of the extraction solution (7.3.5)
- Vc is the volume of the aliquot of the extraction solution used during the clean-up (7.3.6).

## 9 REPEATABILITY AND REPRODUCIBILITY

A major international study involving 13 laboratories and 7 cigarette samples including the 2R4F (a reference cigarette produced by the University of Kentucky) and covering a wide range of blends and constructions was conducted in 2003 and the following values for repeatability (r) and reproducibility (R) were obtained for this method.

The difference between two single results found on matched cigarette samples by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability value (r) on average not more than once in 20 cases in the normal and correct operation of this method.

Single results on 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.

Data analysis for the 7 cigarette samples gave the estimates as summarised in the following table :

Cigarette sample	B[a]P (ng/cigarette)		
	Mean	r	R
2R4F	7,28	1,27	2,52
A	1,81	0,49	1,01
B	5,27	1,06	2,52
C	6,54	1,11	2,21
D	7,76	1,47	2,88
E	8,71	1,39	2,72
F	14,07	2,26	5,94

## 10 REPORT

The test report shall indicate the method used and the results obtained. It shall also mention any operating conditions not specified in this recommended method, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details required for complete identification of the sample. Where appropriate, record the information in 10.1 to 10.4.

### 10.1. Characteristic data about the cigarette

Cigarette identification. In the case of a commercial cigarette this may include:

- Name of manufacturer, country of manufacture;
- Product name;
- Packet number (of that product sampled that day);
- Marks on any tax stamp;
- Printed mainstream smoke yields (if any);
- Length of cigarette;
- Length of filter;
- Length of overwrap.
- Diameter

### 10.2. Sampling

- Type of sampling procedure.
- Number of cigarettes in laboratory sample.
- Date and location of purchase.
- Place of purchase or sampling;
- Kind of sampling point;
- Sampling point (e.g. address of retail outlet or machine number);

### **10.3. Description of Test**

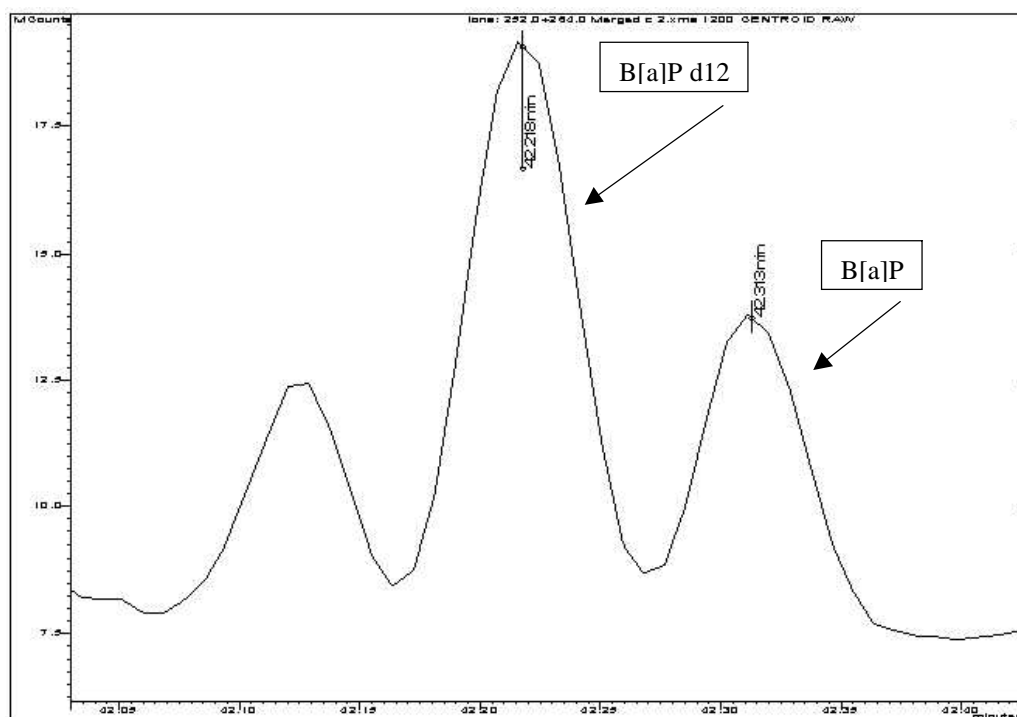
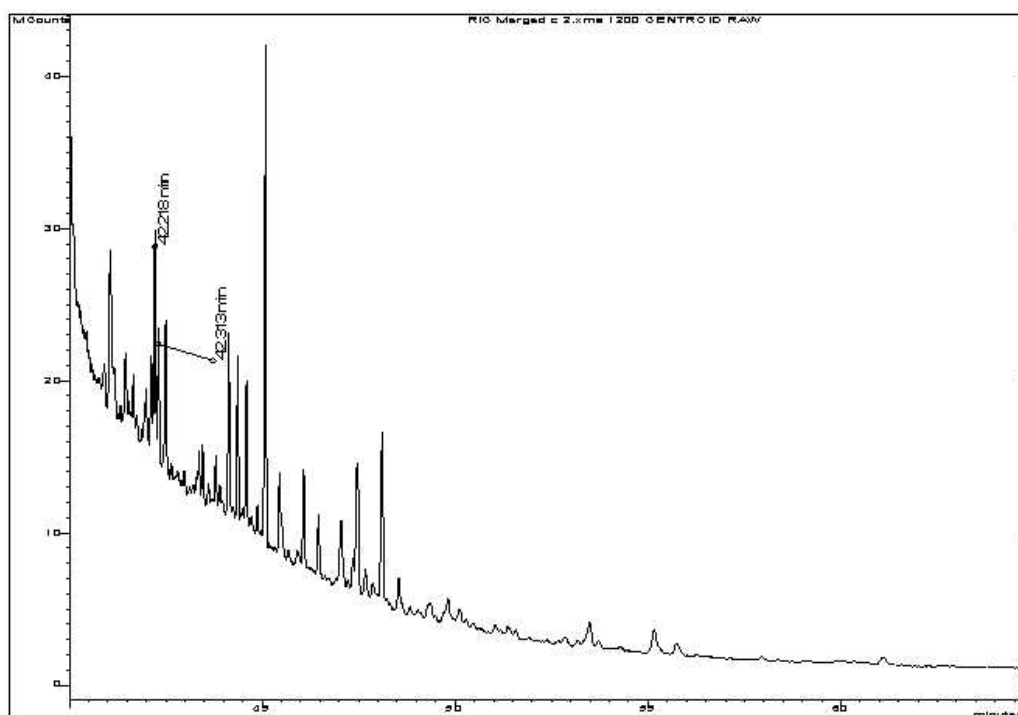
- Date of test.
- Type of smoking machine used.
- Type of smoke trap used.
- Number of cigarettes smoked into each smoke trap.
- Butt length.
- Room temperature (°C) during smoking operation.
- Relative humidity (%) during smoking operation.
- Atmospheric pressure (kPa) during smoking operation.

### **10.4. Test Results**

The expression of the laboratory data depends on the purpose for which the data are required, and the level of laboratory precision. Confidence limits shall be calculated and expressed on the basis of the laboratory data before any rounding has taken place.

- Amount of B[a]P in the mainstream smoke of cigarette in ng/cig to the nearest 0.1 ng

## ANNEX 1 : Example of a chromatogram of a cigarette smoke extract



Using the column described in 4.3 and the chromatographic conditions in 8.1, the retention times of the B[a]P and B[a]P-d12 peaks are between 40 and 45 minutes. The sum of ion traces 252 and 264 are displayed. The upper chromatogram shows the portion of chromatogram located between 40 and 65 min and the lower one is a zoom around the B[a]P and B[a]P-d12 peaks