

CONFIDENTIAL

UK SMOKE CONSTITUENTS STUDY

ANNEX A Part 4 Method: Determination of Benzo[a]pyrene yields in mainstream cigarette smoke by gas chromatography - mass spectrometry

COMMISSIONED BY :

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*Setting standards
in analytical science*

1 Scope of the method

- 1.1 This method is applicable to the quantitative determination of benzo[a]pyrene yields in mainstream smoke of cigarettes of a range of deliveries.

2 Principle of the method

- 2.1 Five conditioned cigarettes are smoked using a 20 channel linear smoking machine. The mainstream smoke is collected onto a 44 mm glass fibre filter pad. The pad is extracted with cyclohexane and an aliquot of the solution concentrated to ca 1mL. The extract is quantitatively transferred to a silica cartridge. The cartridge eluted with hexane and the extract solution concentrated to approximately 1 mL under nitrogen. The solution is analysed using a gas chromatograph – mass spectrometer. The instrument is calibrated with a set of benzo[a]pyrene standards. An internal standard is used to compensate for variations in injection volume and a surrogate standard to compensate for losses in the extraction procedure.

3 Apparatus

- 3.1 20 Channel linear Filtrona smoking machine
- 3.2 44 mm glass fibre filter pad
- 3.3 150 mL conical flasks and glass stoppers
- 3.4 Orbital mechanical shaker
- 3.5 Volumetric flasks
- 3.6 2 mL capacity amber GC vials and appropriate caps
- 3.7 60 mL screw top amber vials (or equivalent) – used for storing standards.
- 3.8 Reacti vap block with nitrogen supply
- 3.9 Positive displacement automatic pipette
- 3.10 500 mg/10 mL silica cartridge
- 3.11 10 mL graduated test tubes
- 3.12 Rotary evaporator
- 3.13 50 mL round bottom flasks (for 3.12), stoppers & cork rings

4 Reagents

Reagents are Analytical Grade or equivalent unless otherwise stated.

- 4.1 Elgastat UHP water (or equivalent)
- 4.2 Cyclohexane
- 4.3 Hexane
- 4.4 Anhydrous sodium sulphate

5 Standards

- 5.1 Benzo[a]pyrene (10 mg per vial).
- 5.2 Benzo[a]pyrene (10 mg per vial) – different manufacturer.
- 5.3 Stock syringe spike – p-terphenyl-d₁₄.
- 5.4 Stock surrogate standard – benzo[a]pyrene-d₁₂ (nominal 10 µg mL⁻¹) in cyclohexane.
- 5.5 The unopened standards should be stored in a refrigerator and have a manufacturers expiry date.

6 Preparation of stock and working standard solutions

Benzo[a]pyrene is carcinogenic. Handling of concentrated solutions (>10 mg mL⁻¹) can only be carried out by trained personnel in designated areas.

- 6.1 **Stock Benzo[a]pyrene (BaP) standard (100 mg mL⁻¹)** – Open the vial and weigh accurately, approximately 0.0100 g of the Benzo[a]pyrene (5.1) into a 100 mL volumetric flask. Add ca 25 mL of hexane (4.3) and swirl to dissolve the material. Make up to the mark with hexane, stopper and shake to mix. Transfer to two appropriately labelled vials (3.7). The standard is stable for 6 months, stored in a refrigerator. Use one of the vials to prepare the standards and keep the other as a reference.
- 6.2 **Working BaP standard (1000 ng mL⁻¹)** - Using an automatic pipette (3.9), add 500 µL (2 × 250 µL) of the 100 µg mL⁻¹ BaP standard (6.1) into a 50 mL volumetric flask containing approximately 25 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 3 months, stored in a refrigerator.
- 6.3 **GC Calibration standard (nominal 25ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 250 µl of the 1000ng mL⁻¹ working BaP standard (6.2) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.
- 6.4 **GC Calibration standard (nominal 15 ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 150 µl of the 1000ng mL⁻¹ working BaP standard (6.2) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.
- 6.5 **GC Calibration standard (nominal 10 ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 100 µl of the 1000 ng mL⁻¹ working BaP standard (6.2) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.
- 6.6 **GC Calibration standard (nominal 5 ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 50 µl of the 1000 ng mL⁻¹ working BaP standard (6.2) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.

- 6.7 **GC Calibration standard (nominal 2.5 ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 25 µl of the 1000 ng mL⁻¹ working BaP standard (6.2) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.
- 6.8 **GC Calibration standard (nominal 1 ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 10 µl of the 1000 ng mL⁻¹ working BaP standard (6.2) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.
- 6.9 **Working surrogate standard (nominal 200 ng mL⁻¹ BaP-d₁₂):** Using an automatic pipette (3.9), add 1000 µL (4 x 250 µL) of the 10 µg mL⁻¹ standard (5.3) into a 50 mL volumetric flask containing approximately 25 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 3 months, stored in a refrigerator.
- 6.10 **Stock syringe spike - (100 mg mL⁻¹):** Open the vial and weigh accurately, approximately 0.0100 g of the p-terphenyl-d₁₄ (5.2) into a 100 mL volumetric flask. Add ca 25 mL of hexane (4.3) and swirl to dissolve the material. Make up to the mark with hexane, stopper and shake to mix. Transfer to two appropriately labelled vials (3.7). The standard is stable for 6 months, stored in a refrigerator. Use one of the vials to prepare the standards and keep the other as a reference.
- 6.11 **Working syringe spike - (1000 ng mL⁻¹):** Using an automatic pipette (3.9), add 500 µL (2 x 250 µL) of the 100 µg mL⁻¹ stock syringe spike (6.10) to a 50 mL volumetric flask containing approximately 25 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The solution is stable for 3 months, stored in a refrigerator.
- 6.12 **Recovery standard:** Using automatic pipettes (3.9), add 250 µL of the working surrogate standard solution (6.9), 750 µL of hexane (4.3) and 100 µL of the working syringe spike (6.11) to the vial. Crimp the vial immediately.
- 6.13 **Quality Control stock standard (1000 ng mL⁻¹ BaP):** Prepare a standard, ideally from a different source (5.2), containing ca 1000 ng mL⁻¹ of benzo[a]pyrene (see 6.1 & 6.2).
- 6.14 **QC standard (nominal 20 ng mL⁻¹ BaP):** - Using an automatic pipette (3.9), add 200 µl of the 1000 ng mL⁻¹ working BaP standard (6.13) into a 10 mL volumetric flask containing approximately 5 mL of hexane (4.3). Make up to the mark with hexane, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The standard is stable for 1 month, stored in a refrigerator.

7 Method

- 7.1 Cigarettes are conditioned¹ at a temperature of 22 ± 1°C and 60 ± 2 % relative humidity for a minimum of 48 hours but not exceeding 10 days.
- 7.2 Butt marking is to ISO butt length specifications². Filtered cigarettes are smoked to a measured butt length equal to either the tipping paper + 3 mm or filter length + 8 mm whichever is longer. The minimum butt length is 23 mm and this is also used for non filter brands. All smoking is conducted in an environment of temperature 22 ± 2°C and 60 ± 5 % relative humidity¹.

- 7.3 ISO conditions³ for smoking cigarettes apply. The smoking machine puffing parameters are $35 \pm 0.2 \text{ cm}^3$ puff volume with 2.0 ± 0.02 second puff duration once every 60.0 ± 0.5 seconds.
- 7.4 A minimum of five determinations are performed for each brand. The smoking of the cigarette brands are randomised so that samples from the same brand are smoked on different days on different channels.
- 7.5 Five cigarettes are smoked and the mainstream smoke is collected onto a glass fibre filter pad (3.2).
- 7.6 Using tweezers, remove each pad and place into labelled 150 mL flasks (3.3). Wipe the inside surfaces with further portions of the filter material and put in the flask.
- 7.7 For each set of samples, prepare a blank sample using a blank filter pad and/or pad where an unlit cigarette has been “smoked”.
- 7.8 Using an automatic pipette (3.9) add 250 μL of the working surrogate standard solution (6.9) to the pad in each flask.
- 7.9 Add 50 mL of cyclohexane (4.2) using a glass pipette.
- 7.10 Place the flasks on the shaker (3.4) and shake at moderate speed for 20 minutes.
- 7.11 Using a measuring cylinder, add 10 mL of water (4.1) to each flask and shake at moderate speed for 15 minutes.
- 7.12 Using an automatic pipette, transfer 20 mL of each solution (cyclohexane layer) to labelled round bottom flasks (3.13). Stopper the flasks. NB For samples containing significant amounts of tar (e.g $>10 \text{ mg cig}^{-1}$ NFDPM), a smaller aliquot (10 mL) is taken. Similarly for low tar samples (e.g $< 5 \text{ mg cig}^{-1}$), use a larger aliquot (30 mL).
- 7.13 For each solution, concentrate to ca $1/5^{\text{th}}$ volume using the rotary evaporator (3.12).
- 7.14 Transfer each solution to labelled graduated test tubes (3.11).
- 7.15 Rinse each flask with ca 1mL of cyclohexane and transfer to the appropriate graduated test tube.
- 7.16 Concentrate to ca 1 mL by blowing down under nitrogen on a Reacti-vap block (3.8) heated to ca $50 \pm 5 \text{ }^\circ\text{C}$. Do not allow to go dry.
- 7.17 Prepare a set of cartridges (3.10) by conditioning each with one bed volume of hexane (ca 10 mL). Do not allow the cartridges to dry out.
- 7.18 Quantitatively transfer each solution (7.16) to the appropriate cartridge. Do not allow the cartridge to dry out.
- 7.19 Elute the BaP with 10 mL of hexane (4.3) into labelled graduated test tubes (3.11)
- 7.20 Concentrate to ca 1 mL by blowing down under nitrogen on a Reacti-vap block.
- 7.21 Quantitatively transfer to a GC vial, add 100 μL of the working syringe spike (6.11) and immediately crimp the vial. Analyse within 48 hours.
- 7.22 With each batch of samples, analyse a set of standards, recovery standard, blank sample, solvent blank and QC standard.
- 7.23 Standards are treated in the same way as samples – pipette 1 mL into a vial, add 100 μL of the working syringe spike (6.11) and immediately crimp the vial. Analyse within 48 hours.

7.24 A typical running order would be: Standards (high to low), solvent blank (4.3), 5 samples, QC standard, blank sample, 5 samples, QC standard, blank sample, 5 samples, QC standard, blank sample, 5 samples, QC standard, solvent blank

8 GC-MS Operation

8.1 The operating conditions for the instrument are listed below:

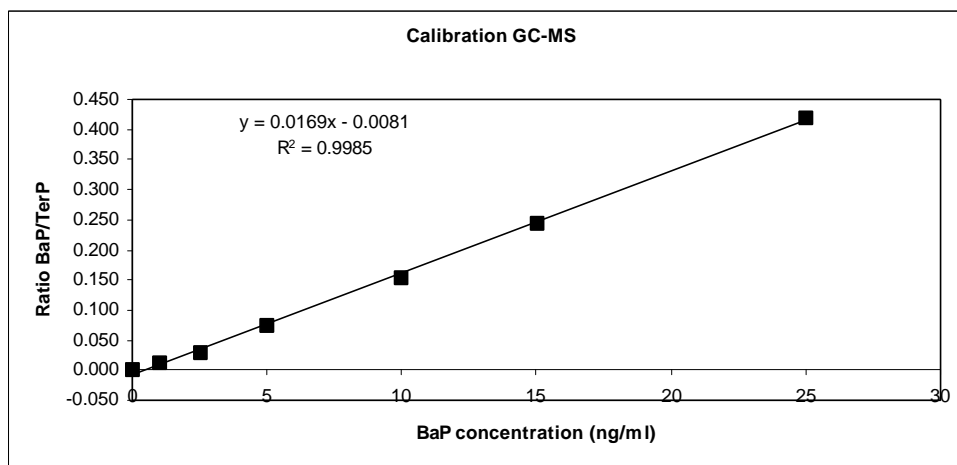
Parameter	Setting
Instrument	Trace GC
Injector type	Split/Splitless
Wash solvent	Hexane
Column length	30m
Column ID	0.25mm
Phase	DB-17HT
Film Thickness	0.15µm
Carrier Gas	Helium
Carrier Flow	0.8ml/min
Injector Temperature	270°C
GC Interface Temperature	280°C
Source temperature	180°C
Oven Program	60°C for 1min, then rising at 20°C/min to 140°C, hold for 1 min, then rising at 5°C/min to 300°C, hold for 1min
Total Run Time	43.00min
Detector	MSD
Injection Volume	2ul
MSD Delay Time	18min
Emission Current	355µA
Mode:	Selected ion monitoring (SIM)
<i>SIM programme:</i>	
<i>p</i> -Terphenyl d ₁₄	23.70min – 24.50min, <i>M/Z</i> 244.21
Benzo [a] pyrene	35.5min – 36.00min <i>M/Z</i> 252.10
Benzo [a] pyrene d ₁₂	35.5min – 36.00min <i>M/Z</i> 264.20

8.2 A QC chart is kept of the concentration determined each day by analysis of the QC standard (6.13). The purpose being to check that the daily calibration of the instrument is satisfactory and that no significant change in the calibration occurs within or between runs. Appropriate limits will be determined and analytical results accepted only if the QC std concentration is within the set limits.

9 Validation

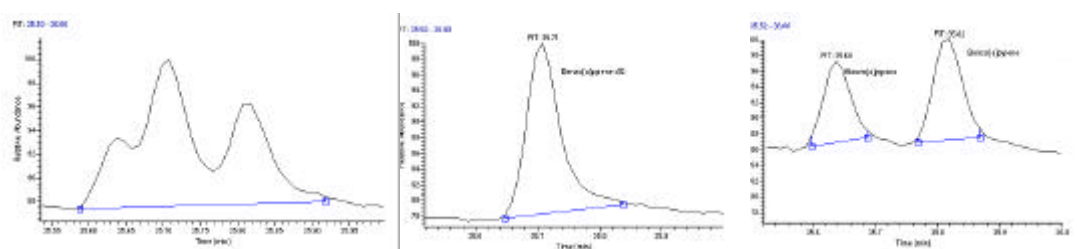
9.1 *Calibration:* – Figure 1 shows an example of a calibration curve

Figure 1 Calibration curve for benzo[a]pyrene.



9.2 *Chromatography:* Figure 2 shows the chromatograms obtained of the analysis of mainstream cigarette smoke (1R4F) for benzo[a]pyrene.

Figure 2 Chromatogram of mainstream cigarette smoke (1R4F) for benzo[a]pyrene



Total ion chromatograph (TIC) of all components in 1R4F in time range 35.50min to 36.00min

Selected ion monitoring (SIM) of benzo[a]pyrene d₁₂ at M/Z 264.20

Selected ion monitoring (SIM) of benzo[a]pyrene and benzo[e]pyrene at M/Z 252.10

9.3 *Recovery:* – The recovery standard was used to determine recovery. The recovery varies depending on the volume of cyclohexane taken, e.g. if the initial sample extract volume is 50 mL and a 20 mL aliquot was put through the clean up stage then 40% should be achieved. In practice for a 20 mL aliquot, recoveries were in the range 28.3% to 50.10%.

9.4 *Accuracy and precision:* In terms of precision and accuracy the following results were achieved in this study:

Cigarette Brand	Benzo[a]pyrene yield/ ng per cigarette
1R4F	6.27 ± 0.51 (five determinations in a single smoking run)
1R5F	1.81 ± 0.14 (five determinations in a single smoking run)
1R4F	7.07 ± 0.91 (five determinations - different runs)
1R5F	1.80 ± 0.54 (four determinations - different runs)

9.5 Normal laboratory QC procedures should be in place as summarised in the table below:

Aim	How achieved
To show smoking is to ISO conditions	<ul style="list-style-type: none"> • Check puff volumes • Check puff profiles • Check CO yields (9.6) • Establish correct butt mark lengths
To show standards are stable	<ul style="list-style-type: none"> • Split one of the calibration standards between two 50 mL vials (3.7). Use one solution and keep the other to act as a check
To show calibration is satisfactory	<ul style="list-style-type: none"> • Print graph and “look at curve” - check for linearity • Check R² (>0.99), intercept close to the blank result.
To show analytical instrument is calibrated and operating satisfactorily	<ul style="list-style-type: none"> • Use certified standards which are in date • Quality control solution • Analysis of blank matrix
To show standard laboratory equipment is functioning satisfactorily	<ul style="list-style-type: none"> • Balance – check weight • Temperature check of refrigerators and freezers • Pipettes – volume check • Conditioning cabinet – temperature and relative humidity • Smoking environment – temperature and relative humidity • Air flows – within specification

9.6 As a check that cigarettes have been smoked in accordance with ISO standard conditions, carbon monoxide concentration will be measured and the yield compared with that normally achieved. Results for cigarettes which give significantly low or high carbon monoxide yields ($\pm 3 \times$ standard deviation) will be discarded.

10 Results

- 10.1 Results should be reported as ng cig⁻¹. A reporting limit of 1 ng cig⁻¹ was used in this study.
- 10.2 Mean, standard deviation and relative standard deviation are determined for each brand of cigarette and Dixon's outlier test was performed (5% level).

11 Calculation

- 11.1 Calculate the peak area ratio of the sample/standard to allow for variation in the injection volumes:

$$\text{Peak area ratio of standard/sample} = \frac{\text{peak area of sample/standard}}{\text{peak area of internal std for sample/standard}}$$

- 11.2 Using a spreadsheet package, plot a calibration graph of concentration versus peak area ratio. Calculate the linear regression equation ($y = mx + c$) without forcing the line through zero. Check the plots, coefficient of determination (r^2) and intercept before accepting the calibrations (see validation section for acceptance criteria).
- 11.3 Using the spreadsheet and the sample peak area ratios, calculate the concentration of BAP in the sample solutions.
- 11.4 The concentration for each sample is adjusted to allow for recovery:

$$\text{Corrected concentration of analyte in sample} = \frac{\text{concentration of analyte in sample} \times 100}{\text{percentage recovery (\%)}}$$

- 11.5 The percentage recovery is calculated using peak area ratios of the surrogate in the samples compared to the recovery standard. NB One would expect 40% recovery if a 20 mL aliquot has been taken from the original sample volume of 50 mL.

$$\text{Percentage recovery (\%)} = \frac{\text{peak area ratio of surrogate in sample} \times 100}{\text{peak area ratio of surrogate in recovery standard (6.11)}}$$

- 11.6 NB The peak area ratio of the surrogate in the samples and recovery standard is calculated as follows

$$\text{Peak area ratio of surrogate in sample} = \frac{\text{peak area of surrogate in sample}}{\text{peak area of internal std in sample}}$$

- 11.7 Calculate the concentration of BAP per cigarette:

$$\text{BAP yield per cig. (ng cig}^{-1}\text{)} = \frac{\text{sample solution conc. (ng mL}^{-1}\text{)} \times 1 \text{ (mL)} [6.12 \text{ volume exc. syringe spike}]}{\text{number of cigarettes (cig)}}$$

¹ ISO 3402: 2000 - Tobacco and tobacco products – atmosphere for conditioning and testing

² ISO :4387: 2000 - Methods for chemical analysis of tobacco and tobacco products – Part 14: Determination of total and nicotine- free dry particulate matter using a routine analytical smoking machine

³ ISO 3308:2000 – Routine analytical cigarette smoking machine – Part 1: Definitions and standard conditions