

CONFIDENTIAL

UK SMOKE CONSTITUENTS STUDY

ANNEX

Part 7: Method

Determination of SVC yields in mainstream cigarette smoke

Commissioned by:

Tobacco Manufacturers Association

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1 Scope of the method

- 1.1 This method is applicable to the determination of pyridine, quinoline and styrene yields (SVC) in mainstream tobacco smoke using Gas Chromatography/Mass Spectrometry.

2 Principle of the method

- 2.1 For each sample, five conditioned cigarettes are smoked on a Borgwaldt rotary smoking machine. The mainstream smoke is passed through a Cambridge filter pad and the vapour phase passed through a solid sorbent tube (XAD-4). After smoking, both the pad and contents of the sorbent tube are transferred to a flask. Triethylamine is added to stabilise the extract. Methanol is added and the resulting solution shaken to extract the semi-volatile compounds. An aliquot of the The solution is analysed by gas chromatography/mass spectrometry. For low tar brands the amount of extraction solvent is reduced. For high SVC yield brands an aliquot of the extract solution is diluted before analysis. An internal standard is used to compensate for variations in injection volume and a surrogate standard to compensate for losses in the extraction procedure.
- 2.2 The instrument is calibrated with a set of standards containing the analytes under investigation. A blank is analysed with each set of samples.

3 Apparatus

- 3.1 Borgwaldt rotary smoking machine and ancillary equipment
- 3.2 XAD-4 tubes 940/80mg solid sorbent beds)
- 3.3 44 mm Cambridge filters pads
- 3.4 Cambridge filter trap consisting of pad (3.3) plus holder
- 3.5 125 mL conical flasks and stoppers
- 3.6 Orbital shaker
- 3.7 60 mL screw top amber vials (or equivalent) – used for storing standards.
- 3.8 2 mL GC amber vials and caps
- 3.9 Positive displacement pipette (0 – 250 μ L)
- 3.10 Glass pipette

4 Standards and Reagents

Reagents are AR grade (or equivalent) - unless otherwise stated.

- 4.1 Methanol
- 4.2 Triethylamine
- 4.3 2-picoline-d₇
- 4.4 Pyridine (density = 0.978*)
- 4.5 Pyridine-d₅, 99% purity – supplied in 1 mL ampoules
- 4.6 Quinoline (density = 1.093*)

* Aldrich

- 4.7 Quinoline-d₇ - supplied in 1 mL ampoules
- 4.8 Styrene (containing inhibitor, density = 0.909*)
- 4.9 Styrene-d₈ (contains inhibitor), 98% purity - supplied in 1 mL ampoules
- 4.10 Water (Elgastat – UHP) or equivalent

5 Preparation of standard and reagent solutions

- 5.1 **Extraction solvent.** Using a pipette (3.9), add 250 µL of triethylamine (4.2) to a Winchester (2.5 L) of methanol. Label the bottle and store in a fire safe. The solution is stable for three months.
- 5.2 **Stock syringe spike - (200 µg mL⁻¹):** Open the vial and weigh accurately, approximately 0.0100 g of the 2-picoline-d₇ (4.3) into a 100 mL volumetric flask. Add ca 25 mL of extraction solvent (5.1) and swirl to dissolve the material. Make up to the mark with methanol, stopper and shake to mix. Transfer to two appropriately labelled bottles (3.7). The standard is stable for 3 months, stored in a refrigerator. Use one of the vials to prepare the standards and keep the other as a reference.
- 5.3 **Working syringe spike - (20 µg mL⁻¹):** Using a pipette, add 5 mL of the stock syringe spike (5.2) to a 50 mL volumetric flask containing approximately 25 mL of extraction solvent (5.1). Make up to the mark with methanol, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The solution is stable for 2 months, stored in a refrigerator.
- 5.4 **Stock standard solutions 1** (nominal concentration 1000 µg mL⁻¹ pyridine and styrene, 500 µg mL⁻¹ quinoline): Prepare three stock solutions, one for each analyte. For each analyte, weigh a 100 mL stoppered volumetric flask half full of extraction solvent (5.1). Using a pipette, transfer 0.1 mL of pyridine (4.4), 0.1 mL of styrene (4.8) and 0.05 mL of quinoline (4.6) to the appropriate flask, stopper and reweigh. Make up to the mark with extraction solvent, stopper and shake to mix. Expiry date 3 months. Store in a glass bottle (3.7)
- 5.5 **Mixed stock standard solution 2** (nominal concentration 50 µg mL⁻¹ pyridine, styrene & 5 µg mL⁻¹ quinoline): Pipette 5 mL aliquot of the pyridine and styrene stock solutions (5.4) and 1 mL of the quinoline stock solution into a 100 mL volumetric flask. Make up to the mark with extraction solvent (5.1). Stopper and shake to mix. Expiry date 1 month. Store in a glass bottle (5.5)
- 5.6 Using pipettes (3.10 & 3.9), pipette the following volumes of the mixed stock standard 2 (5.5) into a series of 100 mL volumetric flasks and dilute to volume with extraction solvent (5.1). Stopper and shake to mix. Prepare weekly.

Standard No.	Volume of mixed stock solution (5.5) added /mL	Nominal concentration of analyte (Pyr & Styr., Quinoline) / $\mu\text{g mL}^{-1}$	Nominal concentration of analyte (Pyr & Styr., Quinoline) in cigarette smoke / $\mu\text{g cig}^{-1}$
1	5	2.5, 0.25	15, 1.5
2	4	2.0, 0.20	12, 1.2
3	3	1.5, 0.15	9, 0.9
4	2	1.0, 0.10	6, 0.6
5	1	0.5, 0.05	3, 0.3
6	0.5	0.25, 0.025	1.5, 0.15
7	0.25	0.125, 0.0125	0.75, 0.075
8	0.125	0.0625, 0.00625	0.375, 0.0375

- 5.7 Recovery standard stock solution pyridine d_5 (nominal concentration $10\,000\ \mu\text{g mL}^{-1}$ pyridine). Weigh the ampoule containing the pyridine d_5 (4.5). Break open and quantitatively transfer to a 100 mL volumetric flask half full of extraction solution (5.1). Make up to the mark with extraction solution, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The solution is stable for 3 months, stored in a refrigerator. Allow the ampoule to dry and re-weigh to determine the amount of recovery standard added.
- 5.8 Recovery standard stock solution quinoline d_7 (nominal concentration $10\,000\ \mu\text{g mL}^{-1}$ quinoline). Using quinoline- d_7 (4.7) prepare a stock solution using the procedure above (5.7).
- 5.9 Recovery standard stock solution styrene d_8 (nominal concentration $10\,000\ \mu\text{g mL}^{-1}$ styrene). Using styrene- d_8 (4.9) prepare a stock solution using the procedure above (5.7).
- 5.10 Recovery working stock solution pyridine (nominal concentration $200\ \mu\text{g mL}^{-1}$). Pipette 1 mL of the stock pyridine d_5 solution (5.7) into a 50 mL volumetric flask half full of extraction solution (5.1). Make up to the mark with extraction solution, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The solution is stable for 3 months, stored in a refrigerator.
- 5.11 Recovery working stock solution quinoline (nominal concentration $100\ \mu\text{g mL}^{-1}$). Pipette 0.5 mL of the stock quinoline d_7 solution (5.8) into a 50 mL volumetric flask half full of extraction solution (5.1). Make up to the mark with extraction solution, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The solution is stable for 3 months, stored in a refrigerator.
- 5.12 Recovery working stock solution styrene (nominal concentration $200\ \mu\text{g mL}^{-1}$). Pipette 1 mL of the stock styrene d_8 (5.8) into a 50 mL volumetric flask half full of extraction solution (5.1). Make up to the mark with extraction solution, stopper and shake to mix. Transfer to an appropriately labelled vial (3.7). The solution is stable for 3 months, stored in a refrigerator.
- 5.13 Mixed working recovery standard solution (nominal concentration $20\ \mu\text{g mL}^{-1}$ styrene and pyridine, $10\ \mu\text{g mL}^{-1}$ quinoline). Using a pipette, transfer 1 mL of each working recovery standard (5.10, 5.11 & 5.12) to a 50 mL volumetric flask. Make up to the mark with extraction solvent (5.1), 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 Method

- 6.1 Cigarettes are conditionedⁱ at a temperature of $22 \pm 1^\circ\text{C}$ and $60 \pm 2\%$ relative humidity for a minimum of 72 hours but not exceeding 10 days.
- 6.2 Butt marking is to ISO butt length specificationsⁱⁱ. 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 $22 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ relative humidityⁱ.
- 6.3 As far as practicable, ISO conditionsⁱⁱⁱ for smoking cigarettes apply. The smoking machine puffing parameters are $35 \pm 0.2\text{ cm}^3$ puff volume with 2.0 ± 0.05 second puff duration once every 60.0 ± 0.5 seconds. The smoking machine may have to be adjusted slightly to achieve a 35 cm^3 puff volume.
- 6.4 A minimum of five determinations are performed for each brand. The smoking of the cigarette brands are randomised as far as practicable so that samples from the same brand are smoked on different days.
- 6.5 Assemble a Cambridge filter trap (3.4), and XAD-4 trap (see picture) for each channel
- 6.6 Five cigarettes are smoked using ISO specifications. Insert an unlit cigarette in the next "smoking" port – this is so that a clearing puff is taken after a set of puffs from the 5 lit cigarettes. At the end of the smoking run five clearing puffs are taken. NB Weigh the Cambridge trap (3.4) before and after smoking so that the tpm (total particulate matter) can be determined.
- 6.7 Transfer the pad from the trap (6.5) to a labelled flask (3.5). Wipe the inside of the holder with a quarter piece of pad and also add to the flask. Stopper the flask.
- 6.8 Using a pipette (3.9), add $200\ \mu\text{L}$ of the mixed recovery standard solution (5.13) to the pad.
- 6.9 Remove the XAD-4 tube. Transfer the contents of the tube (including glass wool) to the flask.
- 6.10 Using a glass pipette, transfer 30 mL of extraction solvent (5.1) to each flask and stopper. For low tar brands ($\text{NFDPM} < 2.5\text{ mg cig}^{-1}$) use 15 mL of solvent.
- 6.11 For each set of samples, prepare a blank sample as well (unsmoked 1R4F or blank matrix)
- 6.12 Place the set of flasks on a shaker (3.6) and shake for 20 minutes.
- 6.13 Using a pipette (3.10), transfer 1 mL of the solution (6.12) to a labelled GC vial (3.8). If appropriate for high yield brands, transfer 0.5 mL of the sample plus 0.5 mL of the extraction solvent (5.1) to the vial to keep the solution concentration within the calibration range.
- 6.14 Prepare a recovery standard sample by using a pipette (3.9) to add $200\ \mu\text{L}$ of the mixed recovery solution (5.13) to a vial followed by $4 \times 200\ \mu\text{L}$ extraction solvent (5.1).
- 6.15 Add $100\ \mu\text{L}$ of the working syringe spike (5.3) to each vial and seal.
- 6.16 With each batch of samples, analyse within 48 hours a set of standards, recovery standard (6.14), blank sample and QC solution. Standards and blanks are treated in the same way as samples (6.13 - 6.14).

7 GC/MS conditions

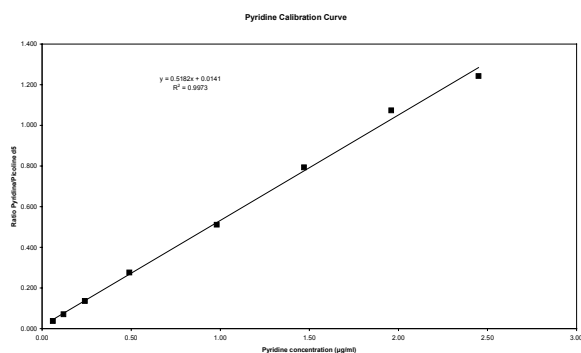
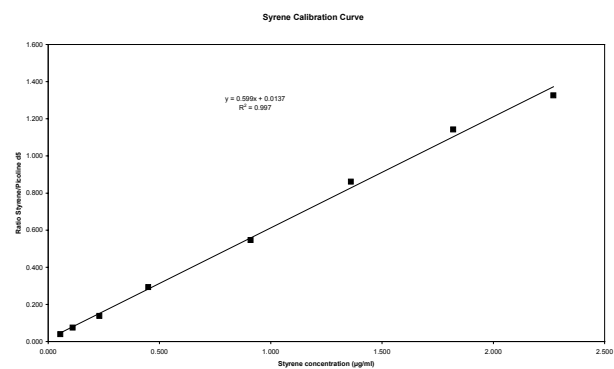
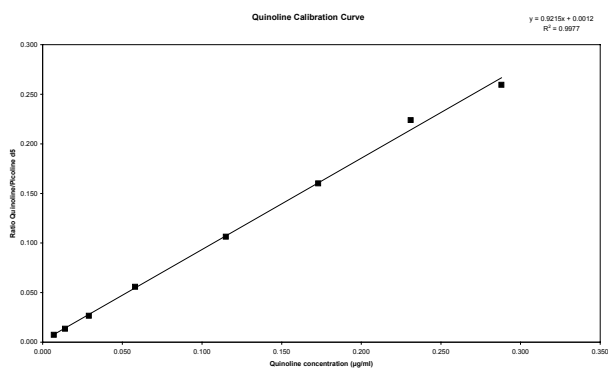
7.1 The following conditions were used

Injection Type	Splitless		
Column Type	ZB-Wax		
Column Length	30 m		
Column ID	0.25 mm		
Film Thickness	0.25 μm		
Carrier Gas	Helium 1.0 mL min ⁻¹ Constant Flow		
Injector Temperature	250°C		
Transfer Line Temperature	240°C		
Oven Program	40°C for 2min - rising at 10°C/min to 150°C - rising at 20°C/min to 240°C – 240°C for 20min		
Injection Volume	1 μl		
Initial Valve	Off		
Relay Event	0.5 min On		
SIM Program		Time/min	Ions Monitored
Group 1	Pyridine	7.0	84, 56, 79, 52
Group 2	Picoline	8.1	100, 72
Group 3	Styrene	8.6	104, 78, 112, 84
Group 4	Quinoline	15	129, 102, 136, 108

7.2 A typical running order would be: Standards (high to low), solvent blank (5.1), 10 samples, QC std, blank sample, 10 samples, blank sample, QC std, Standards (high to low).

8 Validation of method

8.1 Typical calibration curves are shown below



8.2 The following data was obtained for 1R4F and 1R5F using the method

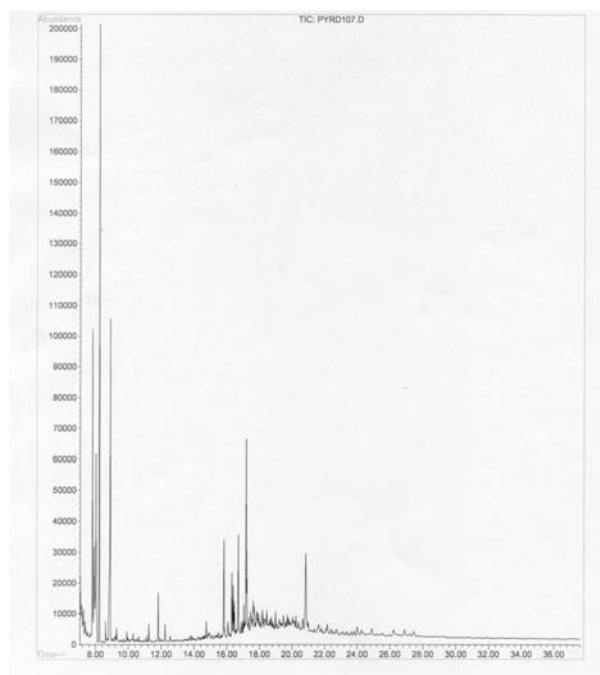
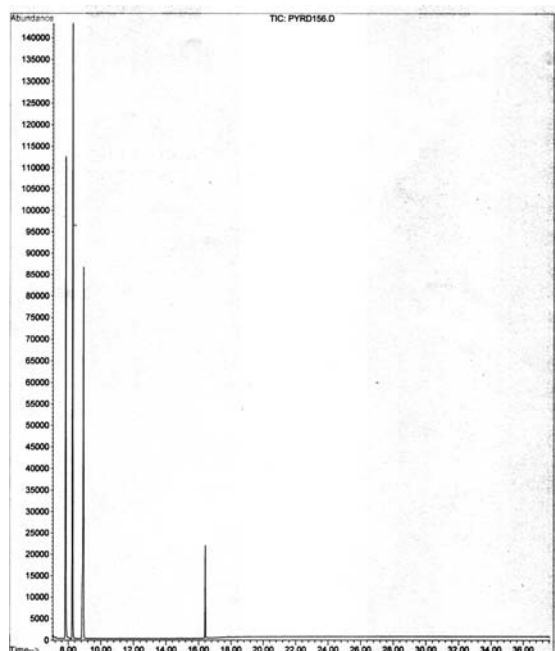
Brand	No of determinations	Mean Pyridine yield $\mu\text{g cig}^{-1}$	Mean Quinoline yield $\mu\text{g cig}^{-1}$	Mean Styrene yield $\mu\text{g cig}^{-1}$
1R4F	5 (same day)	4.63 ± 0.24	0.22 ± 0.01	6.52 ± 0.22
1R4F	5 (results from study)	4.14 ± 0.24	0.22 ± 0.04	5.63 ± 0.26
1R5F	5 (same day)	0.73 ± 0.11	0.057 ± 0.005	1.45 ± 0.15
1R5F	5 (results from study)	1.70 ± 0.20	0.08 ± 0.02	2.93 ± 0.23

8.3 The following yields were obtained for:

		Pyridine $\mu\text{g cig}^{-1}$	Quinoline $\mu\text{g cig}^{-1}$	Styrene $\mu\text{g cig}^{-1}$	Comments
Brand A	5	8.09 ± 0.62	0.25 ± 0.01	9.79 ± 0.52	Pyr. = 5.5 – 12.1, Quin. = 0.35 – 1.42, Sty. = 2.4 – 23.8 [†]
Brand B	5	2.50 ± 0.35	0.14 ± 0.01	3.44 ± 0.34	Pyr. = 2.2 – 4.4, Quin. = 0.19 – 0.38, Sty. = 1.2 – 7.8 [†]
Brand C	2	10.7 ± 0.53	0.38 ± 0.01	6.88 ± 0.02	Plain cigarette (UK blend – high tar)
Brand D	2	18.2 ± 4.91	0.43 ± 0.01	9.56 ± 2.51	Filter cigarette (dark air cured tobacco)
Brand E	2	1.83 ± 0.06	0.12 ± 0.02	2.68 ± 0.27	Filter cigarette (menthol UK blend medium/low tar)
Brand F	2	9.44 ± 0.08	0.30 ± 0.004	9.79 ± 0.34	Filter cigarette (USA blend – high tar)
Brand G	2	0.71 ± 0.13	0.06 ± 0.002	1.22 ± 0.20	Filter cigarette (UK blend – low tar)

8.4 Chromatograms for standard solution 3 and 1R4F are shown below

The peak order in the standard is pyridine, internal standard, styrene and quinoline. 1R4F is the extract solution which includes the deuterated recovery standards and the internal standard.



[†] Results from inter comparison exercise. Most results at lower end of range.

8.5 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 environmental conditions • Establish correct butt mark lengths • Determine TPM
To show calibration is satisfactory	<ul style="list-style-type: none"> • Print graphs and “look at curve” - check for linearity • Check R² (>0.99) • Monitor intercept for significant change
To show analytical instrument is calibrated and operating satisfactorily	<ul style="list-style-type: none"> • Use analytical standards which are in date • Quality control solution – run with every batch of samples • Analyse a blank pad/matrix/sample with every batch of samples • Monitor internal standard peak areas • Monitor recoveries for each analyte – reject any which give abnormal high or low recoveries.
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 Results

9.1 Results should be reported as $\mu\text{g cig}^{-1}$

9.2 The intercept on the calibration curve changes between runs and effects the limit of detection. Therefore the lowest standard concentration is used to determine a limit of quantitation and reporting limit. With 5 cigarettes and 15 mL volume, the reporting limit is set at $0.2 \mu\text{g cig}^{-1}$ ($0.02 \mu\text{g cig}^{-1}$ quinoline).

9.3 Mean, standard deviation and relative standard deviation will be determined for each brand of cigarettes and Dixon’s outlier test will be performed (5% level).

10 Calculations

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

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

10.2 Using a spreadsheet package, plot calibration graphs of concentration versus peak area ratio. Calculate linear regression equations ($y = mx + c$) 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).

10.3 Using the spreadsheet and the sample peak area ratios, calculate the concentration of each SVC in the sample solutions.

10.4 The concentration for each sample is adjusted to allow for recovery:

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

10.5 The percentage recovery is calculated using peak area ratios of the surrogate in the samples compared to the recovery standard. NB One would expect similar recoveries for all analytes. Tag the result if recovery outside normal range for a set of samples.

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

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

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

10.7 Calculate each SVC yield per cigarette:

$$\text{SVC yield per cig.} (\mu\text{g cig}^{-1}) = \frac{\text{sample solution conc.} (\mu\text{g mL}^{-1}) \times \text{sample volume (mL)}}{\text{number of cigarettes (cig)}}$$

10.8 NB The sample solution concentration must be corrected for any dilution of the original solution (6.13).

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

ⁱⁱ 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

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