British American Tobacco Group Research & Development

Method - Determination of semi-volatiles in mainstream cigarette smoke.

1 SCOPE OF APPLICATION
The method is applicable to quantitative determination of the yields of pyridine, styrene and quinoline in whole mainstream cigarette smoke, using gas chromatography-mass selective detection.

2 NORMATIVE REFERENCES
ISO 3308:2000 – Cigarettes - Routine analytical cigarette smoking machine – definitions and standard conditions
ISO 3402:1999 – Tobacco and tobacco products – atmospheres for conditioning and testing
ISO 4387:2000 – Cigarettes - Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine
ISO 8243:2006 – Cigarettes - Sampling

3 PRINCIPLE
Five conditioned cigarettes are smoked using a 20 port rotary Borgwaldt smoking machine. The mainstream smoke is collected on a 44mm Cambridge filter pad (CFP) with a XAD-4 sorbent tube behind the pad. After smoking, the CFP and the contents of the XAD-4 tube are combined and extracted using methanol. The samples are analysed by GC/MS, and quantified by Selective Ion Monitoring (SIM).

4 HEALTH & SAFETY
Read and understand the Material Safety Data Sheets for the chemicals used in this method. Read and understand the method risk assessment. Ensure that you understand the hazards and follow control measures relevant to the operation of this method. All preparation of standards and extraction of samples must be performed in a fume cupboard.

5 REAGENTS AND MATERIALS
All reagents are Analytical Grade or equivalent unless otherwise stated.

Pyridine – anhydrous 99.8%
Quinoline – 98%
Styrene – 99%
Pyridine-d5
Quinoline-d7
Styrene-d8
Methanol

6 APPARATUS
Borgwaldt-KC RM20CSR rotary 20 port smoking machine
Soap bubble manometer to measure puff volume
Analytical Balance capable of measuring to at least four decimal places
44mm Cambridge Filter Pads
500µL syringe
7 PRELIMINARY SAMPLE PREPARATION
Cigarettes should be conditioned according to normal procedures (ISO 3402:1999). Unless specifically requested, cigarettes are not subjected to any selection criteria other than the rejection of any obviously defective or damaged cigarettes. Butt marking is as specified in ISO 3308:2000 unless otherwise requested.

8 ANALYTICAL PROCEDURE – SOLUTION PREPARATION
All standards should be clearly and permanently labelled, including expiry date, and stored in a freezer.

8.1 Pyridine, Quinoline and Styrene stock standards
Prepare standard solutions of the analytes according to the table below. Weigh to the nearest mg, 100mg of each of the standard materials and transfer to separate volumetric flasks (class A). Make up to volume with methanol.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Weight (mg)</th>
<th>Final Volume (mL) in MeOH</th>
<th>Stock Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>100</td>
<td>10</td>
<td>10mg/mL</td>
</tr>
<tr>
<td>Quinoline</td>
<td>100</td>
<td>100</td>
<td>1mg/mL</td>
</tr>
<tr>
<td>Styrene</td>
<td>100</td>
<td>10</td>
<td>10mg/mL</td>
</tr>
</tbody>
</table>

This stock standard is stable for at least six months if stored at -20°C.

8.2 Mixed primary standard (pyridine and styrene 20µg/mL, Quinoline 2µg/mL)
Using a syringe measure 100µL of each individual stock (8.1) into a 50mL volumetric flask (class A) and make up to volume with methanol.
8.3 Deuterated internal standards (ISTD)

Pyridine, Quinoline and Styrene deuterated internal stock (pyridine and styrene 10mg/mL, quinoline 1mg/mL)

Prepare standard solutions of the analytes according to the table below. Weigh to the nearest mg, the required mass of each of the standard materials and transfer to separate volumetric flasks (class A). Make up to volume with methanol.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Weight (mg)</th>
<th>Final Volume (mL)</th>
<th>Stock Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine-d&lt;sub&gt;5&lt;/sub&gt;</td>
<td>100</td>
<td>10</td>
<td>10mg/mL</td>
</tr>
<tr>
<td>Quinoline-d&lt;sub&gt;7&lt;/sub&gt;</td>
<td>25</td>
<td>25</td>
<td>1mg/mL</td>
</tr>
<tr>
<td>Styrene-d&lt;sub&gt;8&lt;/sub&gt;</td>
<td>100</td>
<td>10</td>
<td>10mg/mL</td>
</tr>
</tbody>
</table>

8.4 Mixed deuterated internal standard spiking solution (pyridine-d<sub>5</sub> and styrene-d<sub>8</sub> 200µg/mL, quinoline-d<sub>7</sub> 20µg/mL)

Pipette 2.0mL of each internal standard stock solution (8.3) into a 100mL volumetric flask (class A) and make to volume with methanol.

8.5 Calibration standards

Dilute the mixed primary standard (8.2) as follows and add the internal standard (8.4) then make up to volume with methanol.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrated Standard</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

The calibration standards are stable for three months if stores at -20°C.

8.6 Quality control standard stock solution (pyridine and styrene 10mg/mL, quinoline 1mg/mL)

Weigh to the nearest 0.1mg 100mg of pyridine and styrene and 10mg quinoline in a volumetric flask and make up to volume with methanol.

This stock standard is stable for at least six months if stored at -20°C.

8.7 Quality control standard (pyridine and styrene 4µg/mL, quinoline 0.4µg/mL)

Pipette 400µL of the QC stock solution (8.6), plus 1mL of the ISTD (8.4) into a 100mL volumetric flask (class A), make up to volume with methanol.

This QC standard is stable for three months if stored at -20°C.
9 ANALYTICAL PROCEDURE – SAMPLE PREPARATION

9.1 Sample Collection
Cigarettes are smoked on a Borgwaldt Rotary 20 port smoking machine. Typically the RM20 CSR is used. Warm-up the smoking machine for 20 minutes before smoking.

Check the linear airflow is 200 mm/s (± 30mm/s). Directly behind the Cambridge filter pad insert the XAD-4 tube. Check that the system has no leaks and puff volume is 35mL (± 0.3mL) (for ISO smoking).

5 cigarettes are loaded and the Cambridge filter pad is spiked with 200µL of internal standard spiking solution (8.4) and smoked. Record the number of lit puffs.

9.2 Sample Extraction
Add 20mL (± 1mL) of methanol to each conical flask via dispenser (check delivery with a volumetric flask) and seal flask and shake at 180rpm for 20 minutes.

Transfer an aliquot of approximately 2mL of extract to a crimp top GC vial.

10 ANALYTICAL PROCEDURE – INSTRUMENTAL ANALYSIS

10.1 Instrument Set Up Parameters
Analysis is performed on an Agilent 6890 Gas Chromatograph (GC) fitted with an autosampler and 5973 Mass Selective Detector (MSD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column type</td>
<td>J&amp;W DB-WAX 30m x 0.25mmID x 0.25µm film, or equivalent, with retention gap (approximately 1m)</td>
</tr>
<tr>
<td>Injection type and temperature</td>
<td>Splitless/ 250°C</td>
</tr>
<tr>
<td>Column temperature programme</td>
<td>40°C (2 minutes)/10°C per minute to 150°C/20°C per minute to 240°C Hold for 20 minutes.</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium (1.0 mL/minute)</td>
</tr>
<tr>
<td>Transfer line Temperature</td>
<td>240°C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>1µL</td>
</tr>
<tr>
<td>Solvent Delay</td>
<td>5.50 minutes</td>
</tr>
<tr>
<td>MS Source temperature</td>
<td>230°C</td>
</tr>
<tr>
<td>MS Quadrupole temperature</td>
<td>150°C</td>
</tr>
<tr>
<td>MS Mode</td>
<td>SIM</td>
</tr>
<tr>
<td>Ion Dwell time</td>
<td>50 ms</td>
</tr>
</tbody>
</table>

The following ions should be used as target and qualifier ions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Qualifier1</th>
<th>Qualifier 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>79.0(100%)</td>
<td>51.0(25-35%)</td>
<td>53.0(5-15%)</td>
</tr>
<tr>
<td>Pyridine-d₅</td>
<td>84.0(100%)</td>
<td>56.0(45-58%)</td>
<td>54.0(15-25%)</td>
</tr>
<tr>
<td>Styrene</td>
<td>104.0(100%)</td>
<td>103.0(40-50%)</td>
<td>78.0(30-40%)</td>
</tr>
<tr>
<td>Styrene-d₈</td>
<td>112.0(100%)</td>
<td>84.0(35-45%)</td>
<td>86.0(0-10%)</td>
</tr>
<tr>
<td>Quinoline</td>
<td>129.0(100%)</td>
<td>128.0(16-20%)</td>
<td>102.0(20-28%)</td>
</tr>
<tr>
<td>Quinoline-d₇</td>
<td>136.0(100%)</td>
<td>108.0(20-30%)</td>
<td>109.0(1.5-5%)</td>
</tr>
</tbody>
</table>
These ion ratios should be used to confirm that the peaks in the standards are the correct compounds.

The ion ratios of the sample peaks should be within 20% of the standard ion ratios in that run. There may be exceptions when the qualifier ion ratios fall outside these limits, it may be that there are other compounds present in the sample that have the same ion. The compound can be reported as present if the retention time and one of the qualifier ions matches the corresponding standard peak.

10.2 System Suitability Criteria

10.2.1 MS Tuning
Tune MS weekly, or if system has been vented. Check the following criteria are met on the tune report;
air and water peaks <10%
EM volts 1000 – 3000
Ion ratio of 502:219:69 is 10:4:1 respectively.
Peak width approx. 0.6 (+ 0.1)

10.2.2 Peak shape check
Open a chromatogram of calibration standard 3, and use the instrument software to assess the pyridine-d$_5$ peak shape. The value for USP tailing, as defined in the instrument software should be <3.5. If the result is >3.5 the problem should be investigated before further analysis.

10.2.3 Ion ratio check
Check ion ratios are within the limits shown in section 10.1.

10.2.4 Calibration linearity
The R$^2$ value of the calibration graphs must be >0.99.

10.3 Run Order
Two conditioning samples
Calibration Standards in ascending order
10 samples
QC standard
10 samples
QC standard
Continue until all samples are analysed
Calibration Standards in ascending order

11 CALCULATIONS
Using the instrument software, plot a calibration graph of calibration standards concentration against peak area ratio, without forcing the line through zero.
For example:
Peak area ratio = (Pyridine peak area)/(Pyridine-d$_5$ peak area)
Check the plots, coefficient of determination (R$^2$) and intercept before accepting the calibrations. Calculate the concentration of pyridine, styrene and quinoline in the sample solutions.
The results obtained from the GCMS are in µg/mL. To convert results to µg/cigarette, use the following equation:
Yield per cig (µg/cigarette) = \frac{\text{concentration in extract (µg/mL) x V}}{N}

N = Number of cigarettes smoked (normally 5).
V = Extract volume (normally 20mL).

12 PRECISION AND REPORTING LIMITS
Five replicate smokings and analyses are performed to determine the precision of the analysis. Longer-term precision is monitored through the maintenance of control charts.

The method detection limits are defined as ten times the standard deviation of the lowest calibration standard analysed ten times. The practical reporting limits are defined by the concentration of the lowest calibration standard and are as follows:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detection Limit (µg/cigarette)</th>
<th>Lower Reporting Limit (µg/cigarette)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>0.15</td>
<td>0.8</td>
</tr>
<tr>
<td>Styrene</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Quinoline</td>
<td>0.02</td>
<td>0.8</td>
</tr>
</tbody>
</table>

13 QUALITY ASSURANCE AND CONTROL
Control charts of the QC standard and the reference cigarette are maintained to allow inspection of the method performance.

14 SPECIAL CASES
Under more intense smoking regimes, the number of cigarettes per smoking run may need to be reduced in order to avoid smoke breakthrough on the Cambridge filter pad.

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APPENDIX A SAMPLE CHROMATOGRAMS

Standard 1 - extracted ion chromatogram of pyridine

Ky2R4F - extracted ion chromatogram of styrene