

British American Tobacco Group Research & Development

Method - Determination of aromatic amines in mainstream cigarette smoke

1 SCOPE OF APPLICATION

The method is applicable to quantitative determination of the yields of 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, and 4-aminobiphenyl (henceforth collectively termed "Aromatic Amines") in mainstream cigarette smoke, using gas chromatography with mass selective detection.

2 NORMATIVE REFERENCES

- ISO 3308:2000 – 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

Twenty conditioned cigarettes are smoked using a 20 port rotary Borgwaldt smoking machine. The mainstream smoke is collected onto a 92mm Cambridge filter pad. After smoking the Cambridge filter pad is extracted in a 5% HCl solution. Two SPE steps using different retention mechanisms are used to clean the HCl extract and to isolate the aromatic amines. The aromatic amines are then eluted with toluene, derivatised with heptafluorobutyric anhydride and analysed by GC/MS using 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.

- 1-Aminonaphthalene 10ng/μL in toluene
- 2-Aminonaphthalene 10ng/μL in toluene
- 3-Aminobiphenyl 10ng/μL in toluene
- 4-Aminobiphenyl 10ng/μL in toluene
- 2-Aminonaphthalene-d₇ 0.1mg/mL in toluene
- 4-Aminobiphenyl-d₉ 0.1mg/mL in toluene
- Hydrochloric acid (37%)
- Ammonium hydroxide (29%)
- Methanol – HPLC grade
- Toluene
- Pyridine – HPLC grade
- Heptafluorobutyric anhydride

Deionised water

6 APPARATUS

Borgwaldt RM200 20 port rotary smoking machine

Soap bubble manometer to measure puff volume

Analytical balance capable of measuring to at least four decimal places

92mm Cambridge filter pads

50mL Volumetric flasks (class A) with stoppers

20mL Volumetric flasks (class A) with stoppers

250mL Schott bottles with lids

10mL Measuring cylinder

100mL Measuring cylinders

1L Measuring cylinder

Calibrated variable volumetric multipipette capable of dispensing up to 2mL volume

Air-displacement pipette capable of dispensing 4 μ L

A range of calibrated positive-displacement pipettes capable of dispensing from 10 μ L to 1000 μ L

12mL Plastic (styrene) disposable test tubes

Wrist action shaker

2mL amber GC vials with crimp top caps (PTFE coated)

2mL clear vials

Vial crimper

50mL dispenser (accuracy \pm 5mL)

6mL 150mg MCX (cation exchange) SPE cartridges

6mL 200mg HLB (hydrophobic-lipophilic balance) SPE cartridges

Heating block capable of holding 2 L GC vials and incubating to 80°C

Manifold vacuum block capable of holding SPE cartridges

40mL SPE Polypropylene filter tubes with plastic connectors

5.5cm diameter filter paper

SPE elution racks capable of holding 12mL tubes and 2mL glass vials

pH 11 indicator sticks

Agilent GC/MS with autosampler

J&W DB5 MS Chromatography column (30m x 0.25mm i.d x 0.25 μ m)

1m Deactivated fused silica column

7 PRELIMINARY SAMPLE PREPARATION

Cigarettes should be conditioned according to normal procedures (ISO 3402:1999). Unless specifically requested, cigarettes are not subject to any selection criteria other than the rejection of any obviously defective or damaged cigarettes. Butt marking is as specified in ISO 4387:2000 unless otherwise requested.

8 ANALYTICAL PROCEDURE – SOLUTION PREPARATION

8.1 Extracting solutions

8.1.1 5% Hydrochloric acid solution

135mL (\pm 5mL) concentrated Hydrochloric acid (37%) is carefully added to 865mL (\pm 5mL) de-ionised water using a 1L measuring cylinder and stored in a container with dispenser attached. Prepare at least weekly or with each batch of samples.

8.1.2 1% Hydrochloric acid solution

20mL (\pm 1mL) of the 5% hydrochloric acid solution (8.1.1) is added to 80mL (\pm 1mL) de-ionised water using a 100mL measuring cylinder and stored in a Schott bottle. Prepare at least weekly or with each batch of samples.

8.1.3 5% Ammonium Hydroxide solution in methanol

17mL (\pm 1mL) ammonium hydroxide (29%) solution is added to 83mL (\pm 1mL) methanol using a 100mL measuring cylinder and stored in a Schott bottle. Prepare at least weekly and refrigerate when not in use.

8.1.4 pH11 Ammonium Hydroxide solution

75mL (\pm 5mL) ammonium hydroxide (29%) solution is added to 425mL (\pm 5mL) de-ionised water using a 500mL measuring cylinder. Check the alkalinity (pH 11 ± 1) using an appropriate pH stick and remake if necessary before storing in a Schott bottle. Prepare at least weekly.

8.1.5 30/70 Methanol/water solution

30mL (\pm 1mL) methanol is added to 70mL (\pm 1mL) de-ionised water using a 100mL measuring cylinder and stored in a Schott bottle. Prepare at least weekly.

8.2 Standard Preparation

The following standards are stable for 12 months and should be refrigerated when not in use.

8.2.1 Internal standard Solution

Pipette 2.5mL 2-aminonaphthalene-d₇ stock solution (0.1mg/mL) and 0.5mL 4-aminobiphenyl-d₉ Stock solution (0.1mg/mL) into a 50mL volumetric flask (class A) and make up to volume with toluene.

Concentration of the Internal Standard Solution will be:

5ng/ μ L 2-Aminonaphthalene-d₇

1ng/ μ L 4-Aminobiphenyl-d₉

8.2.2 Calibration standards preparation

Using positive displacement pipettes add the amounts of purchased aromatic amines stock solutions (10ng/ μ L) into volumetric flasks as set out in Table 1 and make up to volume with toluene.

Table 1 – Calibration Stock Solution Preparation

Calibration Standard	1-Aminonaphthalene μ L	2-Aminonaphthalene μ L	3-Aminobiphenyl μ L	4-Aminobiphenyl μ L	Volumetric Flask Size mL
1	50	50	10	10	50
2	250	250	50	50	50
3	500	500	100	100	50
4	400	400	80	80	20
5	600	600	120	120	20
6	800	800	160	160	20
7	1000	1000	200	200	20

To prepare the calibration standard vials, add 100µL of internal standard solution (8.2.1) to 900µL of each calibration stock solution standard in separate autosampler vials. This will achieve the amounts for each level of the working calibration standard as listed in Table 2.

Table 2 – Calibration Vials Summary

Calibration Standard	1-Aminonaphthalene (ng)	2-Aminonaphthalene (ng)	3-Aminobiphenyl (ng)	4-Aminobiphenyl (ng)
1	9	9	1.8	1.8
2	45	45	9	9
3	90	90	18	18
4	180	180	36	36
5	270	270	54	54
6	360	360	72	72
7	450	450	90	90

8.2.3 Add 4µL of the derivatisation agent heptafluorobutyric anhydride, and 10µL of pyridine to each vial. Incubate the vials for 30 minutes at 80°C ± 5°C using a suitable heating block.

8.3 QC Preparation

8.3.1 Prepare the QC standard as described in Table 3.

Table 3 –QC Stock solution preparation

	Amount of QC Standard (10ng/µL) added (µL)	Volume (mL)	Concentration ng/mL
1-Aminonaphthalene	500	20	250
2-Aminonaphthalene	500		250
3-Aminobiphenyl	100		50
4-Aminobiphenyl	100		50

8.3.2 Add 4µL of the derivatisation agent Heptafluorobutyric anhydride, and 10µL of pyridine to each vial. Incubate the vials for 30 minutes at 80°C (± 5°C) using a suitable heating block.

9 ANALYTICAL PROCEDURE – SAMPLE PREPARATION

9.1 Sample Collection

Cigarettes are smoked on a Borgwaldt rotary 20 port smoking machine. Typically the RM200 is used, but if the cigarette style is not suitable, the RM20 CSR may be used. Warm-up the smoking machine for 20 minutes before smoking.

Check the linear airflow is 200 mm/s (±30 mm/s), the system has no leaks and puff volume is 35mL (± 0.3mL) (for ISO smoking).

20 cigarettes are loaded and smoked. Record the number of lit puffs and the weight of Total Particulate Matter (TPM).

9.2 Sample Extraction

After smoking, cut the pad into quarters and add 100mL (\pm 10mL) of 5% HCl to the pad in a 250mL Schott bottle using a 50mL dispenser.

Add 100 μ L (\pm 2 μ L) of deuterated internal standard solution to the acidified sample using a positive displacement pipette.

Place on a wrist-action shaker for 30 minutes at 180rpm (\pm 10rpm).

9.3 Sample Clean Up

9.3.1

Apply all of the HCl extract to an unconditioned Waters Oasis MCX cartridge. Use a slight vacuum to initiate the drip-through of the acidified sample.

9.3.2

Rinse the MCX cartridge with 2mL 1% HCl using the variable volume autopipette. Rinse with 2mL methanol. Discard the acidified waste.

9.3.3

Elute the compounds retained on the MCX with 2mL of 5% ammonium hydroxide in methanol into the plastic test tubes. Use the vacuum to remove any residual eluting solvent from the cartridge.

9.3.4

Using a 10 mL measuring cylinder dilute the eluant with 10mL (\pm 2mL) of pH 11 ammonium hydroxide solution to modify the sample for the reverse-phase HLB cartridge.

9.3.5

Condition the HLB cartridge with 3mL of methanol followed by 2mL of pH 11 ammonium hydroxide solution using an autopipette.

9.3.6

Apply the diluted eluant to the HLB cartridge to retain the aromatic amines.

9.3.7

Rinse the cartridge with 2mL of pH 11 ammonium hydroxide and then rinse with 2mL of 30/70 methanol/water to remove some of the more polar and less hydrophobic compounds. Dry the cartridge using the vacuum manifold for at least 15 minutes to ensure that no water remains in the final extract. Discard the waste solvent from the manifold.

9.3.8

Elute the Aromatic Amines from the HLB cartridge into the clear 2mL vials using 1.5mL of toluene. Transfer the toluene eluant to an autosampler vial using glass pasteur pipettes. Should the final extract contain traces of water ensure this is not transferred to the autosampler vial. Add 4 μ L of the derivatization reagent, Heptafluorobutyric Anhydride.

Crimp the vial, shake, and place in a pre-heated heating block for 30 minutes at 80°C (\pm 5°C). The sample is ready for analysis on a GC/MS.

10 ANALYTICAL PROCEDURE – INSTRUMENTAL ANALYSIS

10.1 Instrument Set Up Parameters

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

Column Type	J&W DB5 MS 30m x 0.25mm i.d x 0.25µm
Injection type and temperature	Splitless/ 280°C
Column temperature programme	Initial 80°C hold for 2 minutes Ramp 30°C/min to 150°C 16°C/min to 210°C hold for 2 minutes 30°C/min to 280°C hold for 2 minutes Total run time is 14.42 minutes
Carrier gas	Helium
Transfer line temperature	280°C
Injection Volume	1µL
Column Flow	1mL/min (constant flow)
Solvent Delay	5 minutes
MS Source temperature	230°C
MS Quadrupole temperature	150°C
MS Mode	SIM
Ion Dwell time	

The following ions are used as target ions:

	Target
1-Aminonaphthalene	339 (100%)
2-Aminonaphthalene	339 (100%)
3-Aminobiphenyl	365 (100%)
4-Aminobiphenyl	365 (100%)
2-Aminonaphthalene-d ₇	346 (100%)
4-Aminobiphenyl-d ₉	374 (100%)

Standards and samples are quantified on the target ions stated above.

10.2 System Suitability Criteria

10.2.1 MS Tuning

Tune the MS weekly, or if the system has been vented. Check the following criteria are met on the tune report:

Air and water peaks <10%

EM Volts 1000 – 3000

Ion ratios of m/z 69:219:502 are approximately 10:4:1

Peak width approximately 0.6 ($\pm 10\%$)

10.2.2 Peak check

System suitability is based on 1-aminonaphthalene. If this analyte passes the system suitability criteria it is assumed that the other aromatic amines will also pass. For the QC:

Peak height must be 500,000m or above

USP tailing not greater than 2.0 and not less than 0.9

10.2.3 Calibration linearity

The R^2 value of the calibration graph must be >0.995

10.3 Run Order

Start run with two conditioning vials (the first of blank toluene and the second of a previously analysed smoked sample)

Standards in ascending order (1-7)

QC standard

Reference cigarette sample 1

Smoked samples (1 x 5 replicates)

Reference cigarette sample 2

Smoked samples (1 x 5 replicates)

QC standard

If further samples are on the sequence at least one QC standard should be run for every 12 sample vials. A QC standard should be run to complete the sequence.

11 CALCULATIONS

Using the instrument software, plot a calibration graph of calibration standards concentration against peak area ratio, without forcing the line through zero.

Peak area ratio = 2-aminonaphthalene peak area/2-aminonaphthalene- d_7 peak area

Check the plots, coefficient of determination (R^2) and intercept before accepting the calibrations. Calculate the concentration of the aromatic amines in the sample solutions.

The results obtained from the GCMS are in ng. To convert to ng/cigarette divide the result in ng by the number of cigarettes smoked for each sample (usually 20).

12 PRECISION AND REPORTING LIMITS

Five replicate smokings and analyses are performed to determine the precision of the the analysis. Longer term precision is monitored through the maintenance of control charts.

The lower reporting limit is defined by the concentration of the lowest calibration standard and equates to 0.45ng/cigarette for 1- and 2-aminonaphthalene and 0.09ng/cigarette for 3- and 4-aminobiphenyl.

13 QUALITY ASSURANCE AND CONTROL

Control charts of the QC standard and the reference cigarette are maintained to allow inspection of the method performance.

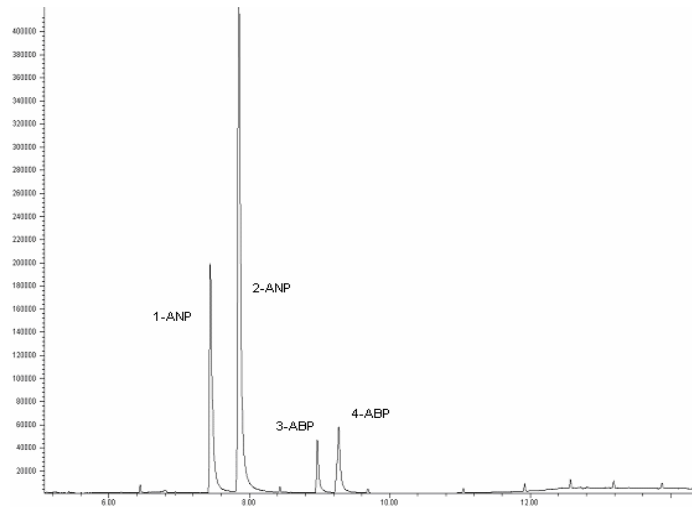
14 SPECIAL CASES

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

QC Standard : 225 ng/mL 1-ANP and 2-ANP, 45 ng/mL 3-ABP and 4-ABP



Kentucky Reference Cigarette (2R4F)

