British American Tobacco Group Research & Development

Method - Determination of tobacco-specific nitrosamines in mainstream smoke

1 SCOPE OF APPLICATION

The method is applicable to the quantitative determination of Tobacco Specific Nitrosamines (TSNAs) in mainstream smoke of cigarettes by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The TSNAs determined are N-nitrosoanabasine (NAB), N-nitrosoanatabine (NAT), N-nitrosonornicotine ketone (NNK) and N-nitrosonornicotine (NNN).

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 cigarettes are smoked on to a 44mm glass fiber filter pad. After the last cigarette is smoked, the filter pad is added to a centrifuge tube containing 20mL of methanol and 100µL of a mix containing four different internal standards. These internal standards are the deuterated equivalents of the four TSNAs that are quantified. The centrifuge tube is then shaken on an orbital shaker for 30 minutes at 200rpm. From the suspension, about 1mL is transferred to an autosampler vial and analysed by LC-MS/MS. The system is calibrated with a set of internally standardised TSNA standards.

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.

5.1 Reagents

Acetonitrile: HPLC grade (Rathburn Chemicals Ltd., Wakerburn, UK)

Ammonium acetate: ReagentPlus™, 99.99+% (Sigma-Aldrich Inc., St. Louis, MO, USA)

Methanol: HPLC grade (Rathburn Chemicals Ltd., Wakerburn, UK)

TSNA standards stock solution (Kinesis, Cambs., UK)

TNSNA-d4 standards stock solution (Kinesis, Cambs., UK)

TSNA QC stock solution (Kinesis, Cambs., UK)

Water: de-ionized water at 18.2 MΩ (Elga Process Water, High Wycombe, UK)

6 APPARATUS

Cerulean SM450 linear 20 port smoking machine

API 5000 instrument (Applied Biosystems, Applera UK, Warrington, UK), consisting of the following elements:

- Enhanced high performance triple quadrupole mass spectrometer system with a mass range of m/z 5 to 1250
- Turbo V Source
- Turbo Ion Spray Probe
- Analyst Software Version 1.4.1

Agilent 1100 series LC system, (Agilent Technologies, Inc.), consisting of the following elements:

- Agilent 1100 Series Binary Pump
- Agilent 1100 TC Autosampler
- Agilent 1100 Micro Vac Degasser
- Agilent 1100 TC Column Compartment
- Agilent 1100 Series Control Module

- LC column: Luna 3µ C18(2) 100A; 100x2.00 mm (Phenomenex, Macclesfield, UK)
- Guard column cartridge: SecurityGuard Guard Cartridge Kit (Phenomenex, Macclesfield, UK)
- Guard column: C18 4.0 mm x 2.0 ID mm (Phenomenex, Macclesfield, UK)

Pipettes: 100µL, 250µL and 1000µL micropipettes (Gilson Inc, Middleton, WI, USA)

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 4387:2000 unless otherwise requested.

8 ANALYTICAL PROCEDURE –SOLUTION PREPARATION

Solution concentrations shown below are target levels, but will vary depending on the exact concentration of stock solutions as supplied.

8.1 Stock Standard Solution

A certified stock standard solution is obtained from Kinesis, with concentrations of 10, 20, 20, 20 µg/mL of NAB, NAT, NNK, NNN respectively. This is diluted 20-fold with methanol to give an intermediate stock standard solution ('STD_STOCK_1') with concentrations of 0.5, 1.0, 1.0, 1.0 µg/mL of NAB, NAT, NNK, NNN respectively.

8.2 Stock QC Solution

QC samples are prepared by dilution of a certified stock QC solution, which is also obtained from Kinesis. The concentrations in the stock QC solution are 100, 400, 400, 400 µg/mL of NAB, NAT, NNK, NNN respectively. This is successively diluted 100-fold and 200-fold with methanol to give a

QC standard solution ('STD_QC') containing 5, 20, 20, 20 ng/mL of NAB, NAT, NNK, NNN respectively.

8.3 Internal Standards

Pyridine-ring-tetradeuterated equivalents of the four different TSNAs (NAB-d_4, NAT-d_4, NNK-d_4 and NNN-d_4) are used as internal standards. They are purchased as a solution containing 40, 80, 80, 80 µg/mL NAB-d_4, NAT-d_4, NNK-d_4 and NNN-d_4 respectively. This is diluted 40-fold with methanol to give an intermediate internal standard stock solution ('IS_2') with concentrations of 1.0, 2.0, 2.0 and 2.0 µg/mL NAB-d_4, NAT-d_4, NNK-d_4 and NNN-d_4 respectively. A further 20-fold dilution with methanol gives a working internal standard solution ('IS_0.1') with concentrations of 50, 100, 100 and 100 ng/mL NAB-d_4, NAT-d_4, NNK-d_4 and NNN-d_4 respectively.

8.4 Calibration Standard Solutions

8.4.1 Preparation of calibration standards

Calibration standard solutions are prepared by serial dilution with methanol, starting from the intermediate stock standard solution ('STD_STOCK_1') to give concentrations as shown:

Calibration Standard	NAB ng/mL	NAT ng/mL	NNK ng/mL	NNN ng/mL
8	50	100	100	100
7	25	50	50	50
6	5	10	10	10
5	2.5	5	5	5
4	0.5	1	1	1
3	0.05	0.1	0.1	0.1
2	0.025	0.05	0.05	0.05
1	0.005	0.01	0.01	0.01

Note: The entire calibration range shown may not be required, depending on the type of samples being tested.

8.4.2 Addition of internal standards

The internal standard solution IS_0.1 (100µL) is added to each calibration standard (900µL) in autosampler vials prior to the analytical run. The internally standardised calibration standards are of the concentrations shown below

Calibration Standard (internally standardised)	NAB ng/mL	NAT ng/mL	NNK ng/mL	NNN ng/mL
8	45	90	90	90
7	22.5	45	45	45
6	4.5	9	9	9
5	2.25	4.5	4.5	4.5
4	0.45	0.9	0.9	0.9
3	0.045	0.09	0.09	0.09
2	0.0225	0.045	0.045	0.045

1	0.0045	0.009	0.009	0.009

The standards each contain 5, 10, 10 and 10 ng/mL of NAB-d_4, NAT-d_4, NNK-d_4 and NNN-d_4 respectively.

8.5 QC Standard Solution

The internally-standardised QC standard solution is obtained by adding 100µL of the internal standard solution IS_0.1 to 900µL of the STD_QC solution in autosampler vials to give the concentrations shown below. It also contains the four deuterated TSNAs at a concentration of 5ng/mL (NAB-d_4) and 10ng/mL (NAT-d_4, NNK-d_4 and NNN-d_4).

	[NAB] (ng/mL)	[NAT] (ng/mL)	[NNK] (ng/mL)	[NNN] (ng/mL)
QC standard (internally standardised)	4.5	18	18	18

All stock solutions may be kept in the freezer (at -20°C) for a maximum of one year, the working standard solutions can be kept in the freezer for a maximum of six months and the internally standardised calibration standards can be kept in a refrigerator (4°C) or freezer for up to one week. However, an internally standardised QC sample should be prepared for every analytical run.

8.6 HPLC mobile phases

The mobile phases should be prepared at least weekly.

8.6.1 100 mM ammonium acetate solution:

In a 250mL bottle, weigh out 1.93 (± 0.01g) of ammonium acetate (1.93g of ammonium acetate = 1.93g / (77.08 g/mol) = 0.025 mol). Add 250mL of water and shake to ensure all ammonium acetate dissolves.

8.6.2 Mobile phase A:

In a 1000mL LC bottle, add 50mL of 100mM ammonium acetate solution. Fill the bottle to 1000mL with water. This will result in a solution of 5mM ammonium acetate in water.

8.6.3 Mobile phase B:

In a 1000mL LC bottle, add 50mL of 100mM ammonium acetate solution. Fill the bottle to 1000mL with acetonitrile. This will result in a solution of 5mM ammonium acetate in 95% acetonitrile and 5% water (vol. %).

9 ANALYTICAL PROCEDURE – SAMPLE PREPARATION

A 20-port linear smoking machine is used to smoke the test cigarettes. Each smoke port should be equipped with a pad holder containing a 44-mm Cambridge filter pad. Weigh each holder/pad assembly before smoking commences. Five cigarettes are normally smoked per port. A reference or monitor cigarette is included in every smoke run.

9.1 Sample Collection

Samples are smoked according to ISO 3308:2000 unless otherwise specified.

9.2 Sample Extraction

After the last cigarette has been smoked, remove the pad holders and weigh to obtain the weight of total particulate matter trapped on the filter pad. Remove the filter pad from the filter pad holder and place it in a 50mL centrifuge tube. Wipe the filter holder twice with quarter sections of a filter pad and add the quarter filter pads to the centrifuge tube. Add 20mL of methanol and 100µL of the internal standard solution IS_2, tightly close the centrifuge tube, and shake it for 30 minutes at 200rpm on an orbital shaker.

9.3 Sample Clean Up

No further clean-up is required. Take about 1mL of liquid from the centrifuge tube and transfer it to an autosampler vial. The vial is then crimp-sealed and submitted for analysis.

10 ANALYTICAL PROCEDURE – INSTRUMENTAL ANALYSIS

10.1 Instrument Set-up Parameters

10.1.1 HPLC Set-up

An Agilent 1100 LC system is used for chromatographic separation. The system is fitted with a Luna C18(2) column and a C18 guard column, which are both heated to 40°C. A gradient of two mobile phases is used. Mobile phase A is 5 mM ammonium acetate in water and mobile phase B is 5mM ammonium acetate in 95% acetonitrile and 5% water (vol. %). Flow rate is set at 0.2 mL/minute. Injection volume is 5µL of sample or calibration standard. The gradient programme is as shown:

Time (min)	mobile phase A(%)	mobile phase B(%)
0	95	5
4	30	70
4.1	95	5
10	95	5

10.1.2 MS/MS Set-up Parameters

An Applied Biosystems API 5000 mass spectrometer is used for the MS/MS analysis. The instrument is operated in positive ESI mode. Curtain gas (CUR) is set at 25psi, gas 1 (GS1) is set at 50psi, gas 2 (GS2) is set at 50psi, source temperature (TEM) is set at 450°C, interface heater (IHE) is on, collision gas (CAD) is set at 6psi, the electrospray voltage (IS) is set at 5500, declustering potential (DP) is set at 60V, entrance potential (EP) is set at 20V and the dwell time for each MRM transition is set at 50ms. For each compound, one MRM transition is recorded:

compound	parent ion	daughter ion	CE*	CXP*
	(m/z)	(m/z)	(eV)	(V)
NAB	192	162	18	12
NAT	190	79	45	12
NNK	208	122	19	9
NNN	178	148	16	9
NAB-d4	196	166	18	12
NAT-d4	194	83	45	12
NNK-d4	212	126	18	15
NNN-d4	182	152	16	10

*CE = collision energy; CXP = collision cell exit potential

10.2 System Suitability Criteria

When the system is ready, run one standard two or three times (*e.g. calibration standard 5*) to check chromatography and sensitivity, comparing with previous runs. The chromatography should be stable after two injections. The last injection of the calibration standard 5 is used to check the system suitability. The first eluting compound (NNN) and the last eluting compound (NAB) are used for this test.

10.2.1 Chromatographic performance

To evaluate the chromatography, the integrated peaks representing NAB and NNN are examined for retention time and peak width. The peak width at 50% peak height of the NAB peak should be less than or equal to 0.10 minutes and the peak width at 50% peak height of the NNN peak should be less than or equal to 0.12 minutes. The retention time of NAB should be 8.8 – 9.0 minutes and the retention time of NNN should be 8.0 – 8.2 minutes. However retention times may vary slightly from column to column.

10.2.2 Peak shape

A number of the compounds have relatively stable rotamer forms. These rotamers are visible as shoulders in the MRM chromatograms. Because they represent exactly the same compound, this shoulder should be included in the total area of the peak.

10.2.3 Sensitivity check

To evaluate the sensitivity of the mass spectrometer, the peaks in the MRM traces representing NAB (m/z192→m/z162) and NNN (m/z178→m/z148) are integrated. The peak areas of NAB and NNN should be more than or equal to 1.00E+05. If any of these values is below the set limit, corrective action should be taken (*e.g.* clean the curtain plate of the mass spectrometer).

10.3 Run Order

Blank (methanol)

Calibration standards 1-12 (in order of increasing concentration)

Blank (methanol), QC sample, blank (methanol)

Test samples

Blank (methanol), QC sample, blank (methanol) – after every 15 test samples, and at end of run

11 CALCULATIONS

The concentrations are calculated from the standard calibration curve. Peaks are automatically integrated using the Applied Biosystems Analyst software. Where necessary, the peak integration should be examined and corrected manually.

To compensate for any suppression effect, the peak area of each compound is divided by the peak area of its corresponding internal standard. This ratio is used as a measure of response at the corresponding concentration.

All calibration plots should be linear with a correlation coefficient (r) of at least 0.975 (or $r^2 \geq 0.95$). A weighting factor of 1/x is used. The slopes and intercepts of the calibration curves are used to calculate the concentrations of the individual compounds in the extracts.

The Analyst software will produce concentrations in ng/mL. The amount of each TSNA (X_{TSNA}) in the mainstream smoke (ng per cigarette) is calculated as:

$$X_{TSNA}(ng/cig) = \frac{[TSNA](ng/mL) \times 20(mL)}{n(cig)}$$

in which n is the number (normally 5) of cigarettes smoked per port (cig = cigarette).

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 reporting limit for the method as normally operated is defined as the concentration of the second-lowest standard, and equates to 0.09ng/cigarette for NAB, and to 0.18ng/cigarette for NAT, NNK and NNN.

13 QUALITY ASSURANCE AND CONTROL

Cambridge filter pads containing smoke particulate matter were spiked with the TSNAs at three levels. Typical recoveries were in the range 90 – 103%.

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

14 SPECIAL CASES

When very low delivery cigarettes are tested the number of cigarettes smoked per port may be increased to obtain the required sensitivity. When smoking cigarettes at intense regimes, the number of cigarettes smoked per port may need to be reduced to avoid overloading the Cambridge filter pad.

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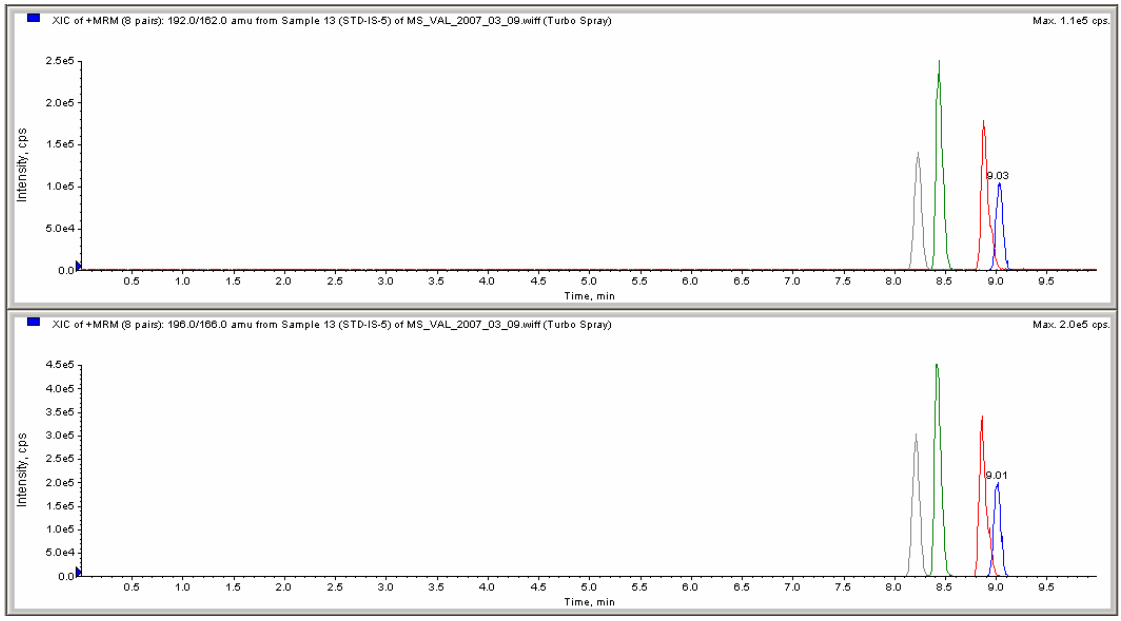
APPENDIX A SAMPLE CHROMATOGRAMS

1) Calibration Standard 5 ng/ml.

Key: upper chromatogram = TSNA: NAB (blue), NAT (red), NNK (green) and NNN (grey)
lower chromatogram = internal standards: NAB-d_4 (blue), NAT-d_4 (red), NNK-d_4 (green) and NNN-d_4 (grey)



2) Reference Cigarette 2R4F (key: as above)