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PMI Research & Development

2017

Analysis of TSNA in Platform 1 aerosol

PRODUCT TESTING LABORATORY AND GOVERNANCE

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1. ANALYSIS OF TSNA IN PLATFORM 1 AEROSOL

1.1. Abstract

The aerosol samples are generated with a linear smoking machine and collected on a Cambridge filter pad. Target compounds are then extracted with a 100 mM ammonium acetate solution containing the internal standards.

The extracts are analyzed by High Performance Liquid Chromatography (HPLC) using a tandem mass spectrometry with an Heat Electrospray Ionization (HESI) interface in positive mode. The HPLC system is equipped with a column Supelco Discovery HC-C18 column, 50 x 2.1 mm ID x 3.0 µm.

Results are expressed as ng/item for P1.

1.2. Applicability

The method described is used for the determination of four Tobacco Specific Nitrosamines (TSNA) in platform 1 (P1) aerosols generated under International Organization for Standardization (ISO) and Health Canada (HC) conditions.

1.3. Reagents

- N-Nitrosonornicotine (NNN)
- 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl)-1-Butanone (NNK)
- N-Nitrosoanabasine (NAB)
- N-Nitrosoanatabine (NAT)
- 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl-D_4)-Butanone (D_4-NNN)
- N-Nitrosonornicotine-2,4,5,6-D_4 (D_4-NNN)
- N-Nitrosoanabasine (D_4-NAB)
- N-Nitrosoanatabine (D_4-NAT)
- Acetic acid
- Ammonium acetate
- Acetonitrile, LC-MS purity grade
- Methanol, LC-MS purity grade
- Deionized water

1.4. Aerosol generation

P1 items are conditioned in opened pack for at least 48 hours at target conditions of 22 ± 1°C and relative humidity of 60 ± 3% before to be used for aerosol generation.

Cambridge filters are conditioned for at least 12 hours at target conditions of 22 ± 1°C and relative humidity of 60 ± 3% before to be used for aerosol generation.

The aerosol samples are generated on a linear smoking machine under ISO or HC smoking regimens and collected on a Cambridge filter pad. The collection conditions for the different smoking regimes are summarized in Table **1**.

Four replicates per sample are prepared. With each series of analytical batch, two blank Cambridge filter pads are processed and analyzed to ensure that no contamination occurred throughout the sample preparation process.

Table 1: Aerosol Collection Condition

Regimen	Accumulation number	Puff number	Regimen Condition [puff volume/Puff duration/Puff Interval] [ml/s/s]
ISO	5	6	35/2/60
HC	5	12	55/2/30

1.5. Internal standard solutions

Individual 40 µg/mL D_4-NNN, 40 µg/mL D_4-NNK, 40 µg/mL D_4-NAT and 10 µg/mL D_4-NAB solutions are prepared by dissolution of the deuterated compounds in acetonitrile.

The intermediate ISTD solution is then prepared by dilution of the individual internal standard stock solutions in acetonitrile.

1.6. Extraction solution

The extraction solution consists in a 100 mM ammonium acetate solution with internal standards (by addition of the intermediate ISTD solution). This solution is used for sample extraction.

1.7. Samples preparation

Directly after aerosol generation, the Cambridge Filter Pad (CFP) is placed in a Filtrona tube and 10 mL of extration solution are added; The Filtrona tube is shaked at 250 rpm for 60 ± 30 minutes. Approximately 5 mL of the aerosol extract are then filtrated into a glass vial with a 0.45 µm filter using a disposable syringe.

1.8. Calibration solutions preparation

1.8.1. TSNA stock solutions

Individual 40 µg/mL NNN, 40 µg/mL NNK, 40 µg/mL NAT and 10 µg/mL NAB solutions are prepared by dissolution of the compounds in acetonitrile.

1.8.2. Intermediate TSNA standard solution

Intermediate TSNA standard solution is prepared by dilution of the individual TSNA stock solutions in a 30% acetonitrile / 70% water solution.

1.8.3. TSNA calibration standards

Six standard (STD) solutions are prepared by dilution of the intermediate TSNA standard solution in 10 mL of 100 mM ammonium acetate solution, 1 mL of intermediate ISTD solution and variable volumes of deionized water. The ranges of concentrations cover the ranges relevant for analysis and is provided in Table 2.

Table 2: TSNA calibration standards typical concentrations

Compound	STD1 (ng/mL)	STD2 (ng/mL)	STD3 (ng/mL)	STD4 (ng/mL)	STD5 (ng/mL)	STD6 (ng/mL)
NNN	1	4	8	20	40	80
NAT	1	4	8	20	40	80
NAB	0.25	1	2	5	10	20
NNK	1	4	8	20	40	80
D_4-NNN	20	20	20	20	20	20
D_4-NNK	20	20	20	20	20	20
D_4-NAT	20	20	20	20	20	20
D_4-NAB	5	5	5	5	5	5

1.9. Instrumental Conditions

The samples are analyzed by High Performance Liquid Chromatography (HPLC) coupled to a tandem mass spectrometry following tables below:

Table 3: Chromatographic Conditions for Determination of TSNA

Column	Supelco Discovery HC-C18 column, 50 x 2.1 mm ID x 3.0 µm or equivalent
Guard-column	Waters Symmetry C-18 column, 3.5 µm or equivalent
In-Line Filter	Filter end fitting 0.5 µm PEEK Upchurch Scientific or equivalent
Mobile Phase A1	MilliQ Water
Mobile Phase B1	0.1% acetic acid in MeOH
Flow rate	220 µL /min
Flow mode	Gradient
Column oven temperature	60°C
Injection volume	10 µL
Loop volume	10 µL
Syringe speed	8 µL/s
Run time	12 min
Sample tray temperature	20°C
Flush volume	1000 µL
Flushing solution	Methanol

Table 4 Mobile phase gradient

Time (min)	A (%)	B(%)
0	97	3
0.05	97	3
3.0	10	90
4.0	10	90
5.0	1	99
6.0	97	3
12.0	97	3

Table 5 Mass spectrometer settings

Parameter	Description
Ionization source	HESI
Ionization polarity	Positive
Scan type	SRM
Acquisition time	10.0 min
Capillary voltage	3.5 kV
Capillary temperature	350 °C
Vaporizer temperature	270 °C

1.10. Testing procedure

The following typical analytical sequence is used for the determination of four TSNA:

- 3 conditioning injections (STD level 1 or 5)
- Calibration curve (STD 1 to 6)
- Blanks
- Samples
- After every 5 samples, inject a quality check (STD level 5)
- Wash injection (acetonitrile)

1.11. Verification of results

1.11.1. Calibration curve

A calibration curve is used to quantify the unknown samples using the response ratio of analyte to the internal standard. The peak area ratio is applied to generate the curve. The linear or quadratic regression is calculated automatically by LC Quan software. Specific information about regressions are provided in Table 6:

Table 6: Parameters used for calibration curves

Compound	Internal standard	Regression type	Weighting factor	R^2
NNN	D_4-NNN	linear	1/x	≥ 0.995
NNK	D_4-NNK	linear	1/x	≥ 0.995
NAT	D_4-NAT	linear	1/x	≥ 0.995
NAB	D_4-NAB	linear	1/x	≥ 0.995

1.11.2. Quality Check

The validity of the calibration is continuously verified during the batch analysis by analysing the calibration control standard injections. Each control standard must be within ±10% of its theoretical value.

1.12. Example Chromatograms

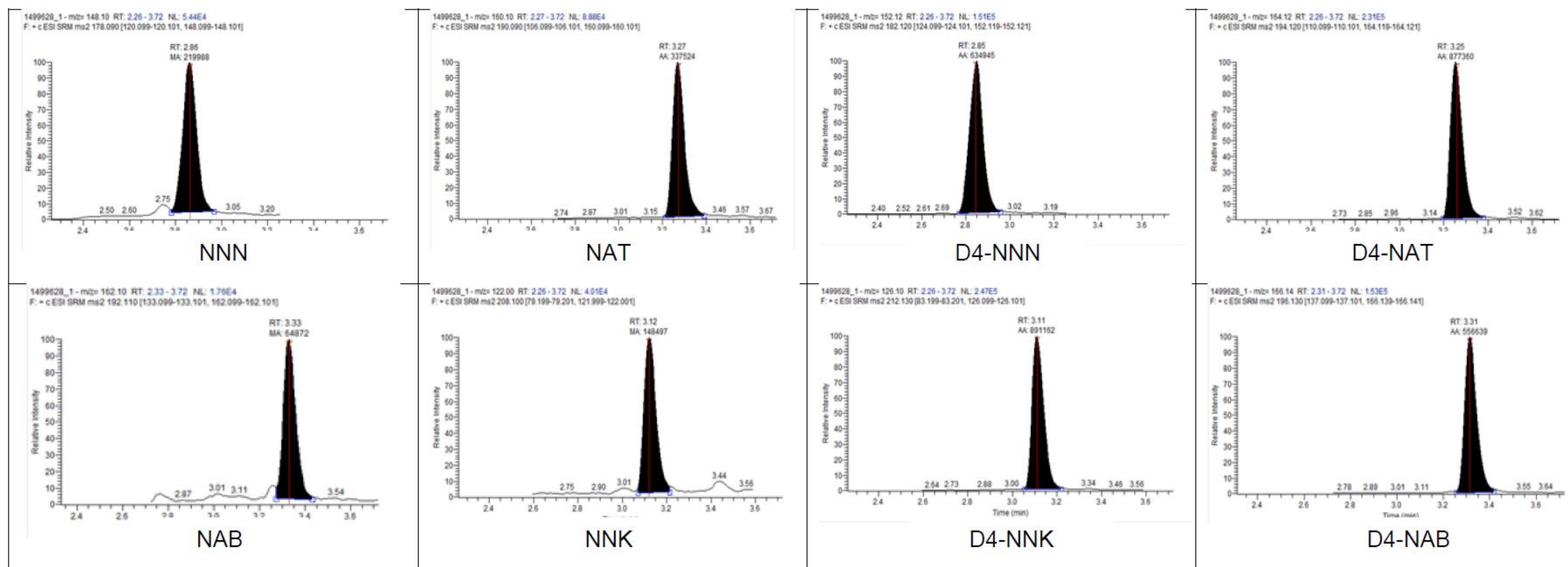

Figure 1: Example Chromatogram for P1 samples

1.13. Limit of Detection (LOD) / Lower Limit of Quantitation (LLOQ)

The LOD and LLOQ were both calculated in terms of the standard deviation of five different measurements of the lowest calibration standard for all other analytes produced under intermediate precision conditions (five different preparations from at least two different operators and analyzed on five different days).

$$LOD = 3 \times SD_{level1}$$

$$LLOQ = 10 \times SD_{level1}$$

Level 1 is the lowest calibration level.

Detailed results are provided in Table 7.

Table 7 LOD, LLOQ and ULOQ for P1 samples

	P1, ISO and HC regimens			
Compound	**LOD [ng/item]**	**LLOQ [ng/item]**	**ULOQ [ng/item]**	**STD1 [ng/item]**
NNN	0.114	0.380	160	2.00
NNK	0.141	0.469	160	2.00
NAT	0.079	0.264	160	2.00
NAB	0.087	0.289	40	0.500

ULOQ = upper limit of quantitation (highest calibration level)

1.14. Repeatability limit (r) and Intermediate precision limit (IP)

$$r = 2 \cdot \sqrt{2} \cdot s_r$$

$$IP = 2 \cdot \sqrt{2} \cdot s_{IP}$$

s_r is the standard deviation of repeatability.

s_{IP} is the standard deviation of intermedidate precision.

Repeatability limit and intermediate precision limit are determined during four different days using different smoking machines for the aerosol generation and standard solutions preparation. Different operators are involved in both aerosol generation and analysis.

IP and r values for P1 are depicted in Table 8.

Table 8: Repeatability r and Intermediate precision IP for P1 samples (ISO and HC Regimens)

	P1, ISO regimen			P1, HC regimen		
Compound	**Mean conc [ng/item]**	**r [ng/item]**	**IP [ng/item]**	**Mean conc [ng/item]**	**r [ng/item]**	**IP [ng/item]**
NNN	6.24	0.905	1.06	15.7	3.38	3.97
NNK	3.39	0.526	0.588	8.28	1.72	2.23
NAT	6.86	1.04	1.14	18.2	3.29	4.13
NAB	1.23	0.206	0.542	3.17	0.616	1.22

1.15. 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