Cooperation Centre for Scientific Research Relative to Tobacco

Smoke Analysis Sub-Group

CORESTA Recommended Method No. 75

DETERMINATION OF TOBACCO SPECIFIC NITROSAMINES IN MAINSTREAM SMOKE BY LC-MS/MS

September 2022

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Title:

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July 2014	Version 2
August 2019	Version 3 - Reference added to ISO 20778; r&R data and sample descriptions added
September 2022	Version 4 - Instructions and r&R for cigar analysis added

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0. INTRODUCTION

Between 1999 and 2005, the CORESTA Special Analytes Task Force studied the existing methodologies for the determination of Tobacco Specific Nitrosamines (TSNAs) in the mainstream smoke of cigarettes. Two types of analytical methodologies had been mainly proposed for this determination: GC-TEA (gas chromatography with a thermal energy analyser) and LC-MS/MS (liquid chromatography - tandem mass spectrometry). The Task Force decided in the first instance to develop a method using GC-TEA, because this methodology was the most widely used in laboratories at that time.

However by 2009, it was ascertained that most laboratories applied an LC-MS/MS technique to measure yields of TSNAs. The Sub-Group (changed from Task Force) then investigated an LC-MS/MS method to complement the GC-TEA technique already available as CORESTA Recommended Method (CRM) N° 63. Several such methods have been described in the literature and are referenced herein. A joint experiment was carried out in which 14 laboratories participated, using their in-house LC-MS/MS methodologies. The reproducibility data was better for LC-MS/MS than for GC-TEA and methodology was very similar across laboratories. In general, cigarette mainstream smoke was collected on a Cambridge filter (CF) pad, an internal standard solution was added and, after extraction, an aliquot was separated and quantitatively analysed by LC-MS/MS and a general methodology was agreed, incorporating key learnings from the joint experiment.

This Recommended Method was produced through a final collaborative experiment involving 20 laboratories from 12 countries that was conducted in 2011. The repeatability and reproducibility values were calculated from cigarette smoke generated under ISO 3308 and Health Canada Intense smoking conditions. The method includes some notes to inform other laboratories that might wish to adopt it about some of the main features that need to be well controlled to provide data as robust and consistent as the repeatability and reproducibility data provided. Statistical evaluations were made according to ISO recommendations and are included.

At that time, when the collaborative study was conducted, the protocol stipulated the use of Health Canada Official Method (T-115) for Intense smoking conditions as there was not an ISO standard that defined Intense smoking conditions. ISO 20778, Routine analytical cigarette-smoking machine — Definitions and standard conditions with an intense smoking regime was published in 2018 and is equivalent to Health Canada Intense conditions and will be referenced hereafter.

In 2019, the Smoke Analysis Sub-Group conducted a collaborative study to extend the scope of the CRM to include cigars. The study was conducted using four reference cigars and one CORESTA Monitor (CM) test piece cigarette smoked under the cigar smoking regime (CRM No. 64). Statistical analysis was carried out following the ISO standard 5725 to generate initial repeatability (r) and reproducibility (R) values which are included.

1. FIELD OF APPLICATION

This method is applicable to the quantification of four tobacco specific nitrosamines (TSNAs) in the total particulate matter of mainstream cigarette and cigar smoke by using reversed phase high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS). The quantified TSNAs are: N-Nitrosonornicotine (NNN), N-Nitrosoanatabine (NAT), N-Nitrosoanabasine (NAB) and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK).

The use of these machine smoking parameters reflects their inclusion in the reporting requirements of various national regulations rather than an endorsement of their appropriateness by CORESTA.

2. NORMATIVE REFERENCES

2.1 *ISO 3308:2000/Amd 1:2009*
Routine analytical cigarette-smoking machine – Definitions and standard conditions

2.2 *ISO 8243:2006*
Cigarettes – Sampling

2.3 *ISO 3402:1999*
Tobacco and tobacco products – Atmosphere for conditioning and testing

2.4 *ISO 4387:2000/Amd 1:2008*
Cigarettes – Determination of Total and Nicotine-free Dry Particulate Matter Using a Routine Analytical Smoking Machine

2.5 *ISO 20778*
Routine analytical cigarette-smoking machine — Definitions and standard conditions with an intense smoking regime

2.6 CORESTA Recommended Methods No. 46, 64 and 65 for smoke collection parameters for cigar smoking

3. METHOD SUMMARY

3.1 After conditioning, cigarettes or cigars are smoked on a standard smoking machine. Mainstream smoke is trapped on a glass fiber filter pad.

3.2 After addition of an internal standard, the total particulate matter collected on the glass fiber filter pad is extracted into 20 mL of 100 mM ammonium acetate solution using a shaker for 60 minutes.

3.3 The extract is syringe filtered through a 0,45 µm PTFE column directly into an auto sampler vial.

The samples are subjected to reversed phase high performance liquid chromatography (HPLC) and quantified via tandem mass spectrometry (MS/MS).

4. APPARATUS AND EQUIPMENT

Usual laboratory apparatus and equipment and in particular the following items:

- Equipment needed to perform conditioning of test articles.
- Equipment needed to perform marking for butt length of test articles.
- Equipment needed to perform smoking of test articles complying with ISO 3308, ISO 20778, or CRM No. 64, as applicable.

The necessary general laboratory equipment for the preparation of samples, standards, and reagents (examples):

- Analytical balance, capable of measuring to at least four decimal places
- Erlenmeyer flask: 100 mL
- Centrifuge tube: 50 mL
- Dispenser (20 mL for extracting solutions)
- Gas-tight syringes: 250 µL
- Volumetric pipettes: 0.5 mL, 1 mL, 2 mL, 4 mL, 5 mL, 10 mL, 20 mL and 50 mL
- Automated volumetric pipette
- Volumetric flasks: 10 mL, 100 mL, 200 mL, 500 mL and 2000 mL
- Shaker

High performance liquid chromatograph coupled to tandem mass spectrometer (LC-MS/MS) consisting of:

- Binary pump
- Autosampler
- Tandem mass spectrometer
- Data collection system
- LC Column: Waters XBridge BEH C18® , 2,5 µm, 2,1 mm × 50 mm or equivalent[1]

5. REAGENTS AND SUPPLIES

Note: All reagents shall be, at the least, recognized as analytical reagent grade in quality.

- N-Nitrosonornicotine (NNN) (min. 98 %)
- N-Nitrosoanatabine (NAT) (min. 98 %)
- N-Nitrosoanabasine (NAB) (min. 96 %)
- 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) (min. 97 %)
- N-Nitrosonornicotine-2,4,5,6-d4 (NNN-d4) (min. 98 %)
- N-Nitrosoanatabine-2,4,5,6-d4 (NAT-d4) (min. 98 %)
- N-Nitrosoanabasine-2,4,5,6-d4 (NAB-d4) (min. 98 %)
- 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone-2,4,5,6-d4 (NNK-d4) (min. 98 %)
- Ammonium acetate (min. 97 %)
- Acetonitrile – HPLC Grade

[1] Waters XBridge BEH is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

- Methanol – HPLC Grade
- Acetic Acid (min. 99,7 %)
- De-ionized water > 18,2 MΩ
- Syringe Filter – 0,45 µm PTFE or equivalent
- Disposable syringes – 5 mL
- Autosampler vials (amber), caps and Teflon faced septa

Warning notice: The solvents and chemicals to be used for this method are classified as toxic, highly toxic, harmful, carcinogenic, mutagenic, sensitising, teratogenic, irritant, corrosive, easily flammable and dangerous for the environment. The instructions specified in the individual material safety data sheets concerning safe handling, storage and waste disposal as well as protective equipment must be followed.

6. PREPARATION OF GLASSWARE

Glassware should be cleaned and dried in such a manner to ensure that contamination does not occur.

Note: It is important that all possible sources of contamination which may interfere with the analytical process are removed from the work area.

7. PREPARATION OF SOLUTIONS

7.1 Extraction Solution (100 mM ammonium acetate solution)

- Weigh 15,4 g ± 0,05 g of ammonium acetate. Put into a 2000 mL volumetric flask and dilute to the mark with de-ionized water.

7.2 HPLC Mobile Phase A (0,1 % acetic acid solution in water)

- Add 1 mL of acetic acid into a 1000 mL volumetric flask and dilute to the mark with deionized water.

7.3 HPLC Mobile Phase B (0,1 % acetic acid solution in methanol)

- Add 1 mL of acetic acid into a 1000 mL volumetric flask and dilute to the mark with methanol.

Note: Extraction solution and mobile phases are stable for up to three months at room temperature.

8. PREPARATION OF STANDARDS

8.1 Preparation of Internal Standard Solutions

8.1.1 Primary Solution

- Weigh approximately 10 mg each of NNN-d4, NAT-d4, NAB-d4 and NNK-d4.
- Put into individual 10 mL volumetric flasks and dilute each flask to the mark with acetonitrile and mix well.
- The concentration in each solution is approximately 1000 µg/mL.

8.1.2 Combined Secondary Solution

- Transfer 5 mL of each primary solution of NNN-d4, NAT-d4 and NNK-d4 and 1 mL of NAB-d4 into a 100 mL volumetric flask. Dilute to the mark with acetonitrile and mix well.
- The concentration in this solution is approximately 50 µg/mL of NNN-d4, NAT-d4 and NNK-d4 and 10 µg/mL of NAB-d4.

8.1.3 Working Solution

- Transfer 50 mL of the combined secondary solution into a 500 mL volumetric flask. Dilute to the mark with acetonitrile and mix well.
- The concentration in this solution is approximately 5 µg/mL of NNN-d4, NAT-d4 and NNK-d4 and 1 µg/mL of NAB-d4.

8.2 Preparation of Calibration standard solutions

8.2.1 Primary Single TSNA Solutions

- Weigh approximately 10 mg each of NNN, NAT, NAB and NNK.
- Put into individual 10 mL volumetric flasks and dilute each flask to the mark with acetonitrile and mix well.
- The concentration in each solution is approximately 1000 µg/mL.

8.2.2 Mixed TSNAs Stock Solution (I)

- Transfer 4 mL of the primary single TSNA solutions of NNN, NAT and NNK and 1 mL of the primary single TSNA solution of NAB into a 100 mL volumetric flask. Dilute to the mark with acetonitrile and mix well.
- The concentration in this solution is approximately 40 µg/mL of NNN, NAT and NNK and 10 µg/mL of NAB.

8.2.3 Mixed TSNAs Stock Solution (II)

- Transfer 2 mL of the mixed TSNAs stock solution (I) into a 200 mL volumetric flask. Dilute to the mark with acetonitrile and de-ionized water mixed solution (30:70 volume fraction) and mix well.
- The concentration in this solution is approximately 400 ng/mL of NNN, NAT and NNK and 100 ng/mL of NAB.

8.2.4 Working Standard Solutions

- Prepare 7 working standard solutions that cover the concentration range of interest.
- Add selected volumes of solutions listed in Table 1 in a 100 mL volumetric flask and dilute to the mark with de-ionized water.
- These solutions have concentrations of approximately 50 ng/mL of NNN-d4, NAT-d4 and NNK-d4, 10 ng/mL of NAB-d4, from 0 ng/mL to 80 ng/mL of NNN, NAT and NNK and from 0 ng/mL to 20 ng/mL of NAB (Table 2).

Note: Each laboratory should establish the most suitable calibration range depending on the equipment used and the type of samples to be analysed. The standard preparation procedure is given as an example and is applicable for the range of the products in a collaborative study.

Note: Opportunity to order customized mixed TSNA standard stock solutions from the supplier.

Table 1 – Preparation of Working Standard Solutions for Calibration

	Working standard solutions for calibration						
	S0	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Solutions	**mL**	**mL**	**mL**	**mL**	**mL**	**mL**	**mL**
Internal standard solution	1	1	1	1	1	1	1
Mixed TSNAs stock solution (II)	0	0,5	1	2	5	10	20
Ammonium acetate (100 mM)	10	10	10	10	10	10	10
Acetonitrile	10	10	10	10	8	7	4
Final volume	100	100	100	100	100	100	100

Table 2 – Concentration of Each Calibration Standard

	S0	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Concentrations	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**
NNN	0	2	4	8	20	40	80
NAT	0	2	4	8	20	40	80
NAB	0	0,5	1	2	5	10	20
NNK	0	2	4	8	20	40	80
NNN-d4	50	50	50	50	50	50	50
NAT-d4	50	50	50	50	50	50	50
NAB-d4	10	10	10	10	10	10	10
NNK-d4	50	50	50	50	50	50	50

8.2.5 Storage

- The above standard solutions are stable for up to six months if refrigerated below 5 °C.

9. SAMPLING

Sampling is done in accordance with ISO 8243:2006.

10. TOBACCO PRODUCT PREPARATION

Conditioning of the cigarettes is done in accordance with ISO 3402:1999. Conditioning of cigar samples is done in accordance with CRM No. 46.

11. SAMPLE GENERATION – SMOKING OF CIGARETTES

Test articles are smoked according to ISO 3308, ISO 20778, or CRM No. 64, as applicable, and with the following modifications:

- Check the puff volume(s) of the smoking machine and adjust accordingly.
- Cigarettes or cigars typically smoked per trap are as noted below.

Regime	ISO 3308		ISO 20778		CRM 64	
Pad Size (mm)	44	92	44	92	44 or 55	92
Cigarettes/trap	5	20	2-3	10		
Cigars/trap					1-3	1-3

Note: The number of cigarettes or cigars smoked is adjusted as needed to avoid total particulate matter (TPM) breakthrough.

12. SAMPLE ANALYSIS

12.1 Sample preparation

Remove the filter pad and place it into an Erlenmeyer flask. Wipe the inside of the holder with two quarter sections of a pad, and add the quarter pads to the flask.

Note: Solvent volumes may need to be adjusted to ensure analytes are within the calibration range but IS ratios should be maintained.

12.1.1 Extraction for 44 mm pad or 55 mm pad

- After adding 200 µL of internal standard solution to the pad, add 20 mL of 100 mM ammonium acetate solution to each Erlenmeyer flask containing a pad from the analytical run and cap.

12.1.2 Extraction for 92 mm pad

- After adding 400 µL of internal standard solution to the pad, add 40 mL of 100 mM ammonium acetate solution to each Erlenmeyer flask containing a pad from the analytical run and cap.

Note: The extraction volume can be adjusted in each laboratory.

Note: It is acceptable to extract the filter pads with 100 mM ammonium acetate solutions containing the internal standards instead of spiking internal standard solution directly to the filter pads.

12.1.3 Final Sample Preparation

- Perform extractions by using a shaker and agitate for 60 minutes at 210 rpm.
- Filter the pad extract directly into vials through a syringe filter (0,45 µm PTFE).

Note: The above sample extracts are stable for up to six days if refrigerated below 5 °C.

12.2 Reversed Phase High Performance Liquid Chromatography

Note: The choice / adjustment to the chromatographic conditions may be required depending on the different instrument configuration and columns applied for separation.

12.2.1 HPLC Set-up Parameters (Example)

- Column Temperature: 65 °C
- Autosampler Tray Temperature: 5 °C
- Injection Volume: 5 µL
- Flow Rate: 250 µL/min

12.2.2 Mobile Phase (Example)

- A: 0,1 % Acetic acid in water
- B: 0,1 % Acetic acid in methanol

12.2.3 Mobile Phase: Gradient (Example)

Table 3 – Gradient Program

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	98	2
4	2	98
7	2	98
8	98	2
20	98	2

12.2.4 MS/MS Set-up Parameters (Example)

Note: The following conditions are suitable for the analysis:

- The instrument is operated in electrospray ionization (ESI) mode.
- Gas 1 (GS1): N_2, 50 psi (345 kPa)
- Gas 2 (GS2): N_2, 60 psi (414 kPa)
- Turbo Ion Spray Temperature: 700 °C
- Interface Temperature: off
- Curtain Gas (CUR): N_2, 40 psi (276 kPa)
- Collision Gas (CAD): N_2, 3 psi (21 kPa)
- Ion Spray Voltage (IS): 4500 V
- Inject 5 µL of each sample onto the HPLC column and analyze as per the chromatographic conditions listed above.

Note: The retention time in the chromatogram may be different depending on the choice of column.

Note: According to the described chromatographic system, peak splitting and peak fronting might be observed in particular for the early eluting compounds (e.g. NNN, NNN-d4).

Note: Some laboratories reported that NNN-d4 peak could not be found. A reduction of the acetic acid in the mobile phases did not improve the sensitivity. Another mobile phase (A: 2 mM ammonium acetate / B: Methanol and 0,01 % formic acid) improved sensitivity.

Table 4 – Mass Spectrometric Parameters

Compounds	Precursor ion (m/z)	Quantifier (m/z)	Qualifier (m/z)	DP* (V)	CE* (V)	CXP* (V)	Dwell time (m sec)
NNN	178	148	120	41	15	10	150
NAT	190	160	106	41	15	10	150
NAB	192	162	133	36	17	10	150
NNK	208	122	79	41	17	8	150
NNN-d4	182	152	124	41	15	8	150
NAT-d4	194	164	110	41	15	10	150
NAB-d4	196	166	137	36	17	10	150
NNK-d4	212	126	83	41	17	8	150

** DP: Declustering potential; CE: Collision energy; CXP: Collision cell exit potential*

12.3 Calculations

12.3.1 Calibration Curve

- A calibration curve is generated by calculating a linear regression of the area ratios of each TSNA to corresponding internal standard peak as a function of the concentration ratios of each TSNA to corresponding internal standard.

Note: When laboratories either have problems obtaining 4 internal standards or have checked / validated that using 2 internal standards gives comparable data, then the method can be run using NNN-d4 as substitute for the deuterated NAT / NAB standards. There is low NAT in some blends and it is recommended using 4 internal standards for test articles containing such blends.

12.3.2 Determination of the TSNAs Concentrations

- Inject the sample, calculate the area ratio of each TSNA to corresponding internal standard peak and obtain the concentration ratio by comparing the area ratio with the calibration curve.

12.3.3 Sample Quantification

- The amount of the various TSNA compounds in smoke samples is quantified by the internal standard method. Examples of chromatograms are shown in Figures 1 and 2.
- TSNA concentrations are reported in (ng/mL) by the chromatography software.
- Determination of Mainstream Smoke TSNA Deliveries, M in [ng/cig]

$$M = C * Ws * \frac{V}{N}$$

where

C: the ratio by weight obtained from the calibration curve

Ws: the amount in ng of the internal standard added to the sample

V: the volume of 100 mM ammonium acetate solution used for extraction

N: the number of cigarettes or cigars smoked

13. REPEATABILITY AND REPRODUCIBILITY

- The repeatability and reproducibility values for cigarettes were determined from an international study conducted in 2011 involving 20 laboratories and ten cigarette samples including the reference cigarettes KR 1R5F and KR 3R4F and the CORESTA Monitor CM6. The samples are identified in Table 5. The seven commercially available cigarettes and the references covered a wide range of blends and constructions.
- The repeatability and reproducibility values for cigars were determined from an international study conducted in 2019 involving eight laboratories and four cigar reference samples (1C1, 1C2, 1C3, and 1C4) and CORESTA Monitor CM8. The samples are identified in Table 6. Due to the small number of laboratories and the typically high relative variability of cigars, the r&R values should be viewed as preliminary.

Table 5 – Cigarette Sample Identification

Sample ID	ISO 3308 NFDPM Yield* (mg/cig)	ISO 20778 NFDPM Yield* (mg/cig)	Product/ Blend Type
Sample 1	9,5	25,2	Dark air-cured
Sample 2	8,0	23,6	American blended
Sample 3	6,4	23,2	American blended
Sample 4	3,6	18,4	Virginia blended
Sample 5	1,8	12,5	Virginia blended
Sample 6	9,7	22,9	Virginia blended
Sample 7	1,3	15,7	Charcoal filtered
CM6	14,2	29,1	CORESTA Monitor 6 Test Piece
KR 1R5F	1,7	17,6	Kentucky Reference 1R5F
KR 3R4F	8,2	25,9	Kentucky Reference 3R4F

[*] *NFDPM: nicotine-free dry particulate matter*

Table 6 – Cigar Sample Identification

Sample ID	Description	Diameter, mm	Nominal avg Mass, g
1C1	Large machine-made cigar	15.9	6.4
1C2	Machine-made filtered cigar	<12.1	1.4
1C3	Machine-mad Cigarillo	<12.1	2.7
1C4	Large machine-made cigar with natural wrapper	12.8	3.2
CM8	Monitor Cigarette	<12.1	0.96

- The statistical evaluation was performed according to ISO 5725-2:1994 “Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability (r) and reproducibility (R) of a standard measurement method.”

13.1 Determination of Outliers[2]

- Individual data points reported as non-numeric (i.e. below the LOQ) were removed prior to evaluation of numeric data for outliers.
- Raw data was checked for consistency both within labs and among labs using Mandel's k and Mandel's h statistics, respectively. These statistics are represented graphically as plots across all labs for each cigarette sample and analytical parameter (not shown).
- Actual removal of data prior to the estimation of the repeatability (r) and reproducibility (R) limits were made based on the Cochran's C and Grubbs' numerical outlier tests, with outliers (α = 1 %) being removed and stragglers (α = 5 %) retained.

13.2 Determination of General Mean, Repeatability and Reproducibility Variance

- The general mean was determined as per ISO 5725-2 section 7.4.4 across the participating laboratories whose data remained following the removal of outliers.
- Repeatability variance (sr) was determined as per ISO 5725-2 section 7.4.5.1 and reproducibility variance (sR) was determined as per ISO 5725-2 section 7.4.5.5.
- Repeatability (r) and reproducibility (R) figures calculated for 95 % confidence level were also indicated.

13.3 Results

Calculated statistical data are indicated in the tables below.

13.3.1 Cigarettes - ISO 3308 Smoking Regime

	NNN [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	16	277	17	25	47	70
2	18	37,3	3,1	4,4	8,8	12,4
3	17	24,0	2,4	2,8	6,7	7,8
4	18	9,6	1,5	2,4	4,2	5,8
5	18	12,1	1,3	2,0	3,6	5,6
6	16	22,7	3,8	4,2	10,8	11,9
7	17	10,5	1,1	1,7	3,0	4,8
CM 6	18	20,0	1,8	2,9	5,1	8,1
KR 1R5F	19	44,4	3,1	5,9	8,6	16,7
KR 3R4F	18	115	6	12	18	34

[2] Due to the small number of reporting laboratories, outlier detection was not employed for the cigar results.

	NAT [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	16	145	10	26	27	74
2	16	40,5	2,9	8,0	8,2	22,5
3	16	27,9	2,4	5,7	6,7	16,2
4	15	11,0	1,2	2,2	3,4	6,2
5	16	12,8	1,2	2,5	3,5	7,2
6	16	27,9	3,0	5,7	8,5	16,1
7	16	14,4	1,2	2,4	3,5	6,8
CM 6	17	33,7	3,0	6,6	8,5	18,6
KR 1R5F	17	45,8	3,3	8,3	9,2	23,4
KR 3R4F	16	113	5	20	14	55

	NAB [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	17	20,0	1,8	3,2	5,2	9,0
2	14	5,3	0,5	1,0	1,4	2,7
3	14	3,7	0,5	0,7	1,5	2,1
4	13	1,5	0,2	0,3	0,6	0,9
5	11	1,8	0,2	0,3	0,5	0,9
6	13	3,6	0,5	1,3	1,3	3,5
7	14	1,8	0,2	0,3	0,6	0,9
CM 6	13	3,7	0,3	1,0	0,9	2,7
KR 1R5F	16	6,5	0,5	1,0	1,5	2,9
KR 3R4F	16	13,0	0,8	1,9	2,3	5,2

	NNK [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	18	133	12	14	33	41
2	17	24,5	2,8	3,6	7,8	10,2
3	18	17,9	1,9	3,2	5,3	9,0
4	14	3,6	0,6	1,1	1,7	3,0
5	13	3,3	0,6	0,7	1,8	2,1
6	15	7,2	0,9	2,0	2,7	5,7
7	15	7,4	0,7	1,1	1,9	3,1
CM 6	17	26,5	2,3	3,0	6,5	8,6
KR 1R5F	19	21,8	1,4	2,8	3,9	8,0
KR 3R4F	19	97,1	5,2	10,8	14,6	30,5

13.3.2 Cigarettes – ISO 20778 Intense Smoking Regime

	NNN [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	18	603	43	80	122	225
2	18	87,5	9,9	12,7	27,9	35,9
3	18	68,6	7,0	10,6	19,7	29,9
4	18	34,9	6,0	10,1	16,8	28,6
5	16	51,2	5,0	15,0	14,0	42,4
6	18	48,0	8,3	11,6	23,5	32,8
7	16	63,6	6,2	8,2	17,6	23,3
CM 6	18	37,9	5,1	7,5	14,4	21,3
KR 1R5F	18	237	17	26	49	72
KR 3R4F	19	297	26	31	73	88

	NAT [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	17	322	23	76	64	214
2	15	91,2	7,2	20,8	20,5	58,9
3	15	76,8	7,0	16,9	19,7	47,7
4	16	39,4	3,5	8,9	9,9	25,2
5	16	54,0	5,6	15,8	15,8	44,7
6	15	54,0	5,9	11,7	16,7	33,0
7	16	83,0	5,9	17,4	16,7	49,1
CM 6	17	64,8	6,1	14,2	17,1	40,3
KR 1R5F	16	230	13	49	37	138
KR 3R4F	17	279	22	47	63	132

	NAB [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	17	42,9	3,8	7,6	10,8	21,4
2	16	11,8	1,3	2,6	3,6	7,3
3	16	10,1	1,4	2,5	4,1	7,2
4	14	5,5	1,0	2,2	2,9	6,2
5	15	6,7	0,9	1,8	2,5	5,0
6	14	7,5	1,0	2,2	2,7	6,2
7	15	8,7	0,7	1,6	1,9	4,7
CM 6	15	7,5	0,8	2,3	2,2	6,4
KR 1R5F	16	27,8	1,9	4,3	5,3	12,2
KR 3R4F	17	31,2	2,7	4,7	7,7	13,2

	NNK [ng/cigarette]					
Sample	**# laboratories**	**Mean**	***sr***	***sR***	***r***	***R***
1	18	297	25	51	71	144
2	17	55,5	5,6	7,5	15,7	21,2
3	18	49,9	5,8	6,9	16,3	19,5
4	13	12,1	1,1	3,3	3,2	9,3
5	17	15,0	2,6	4,2	7,2	11,9
6	15	14,3	1,5	3,0	4,2	8,4
7	18	46,6	4,9	8,2	13,9	23,2
CM 6	19	50,8	5,8	9,7	16,5	27,4
KR 1R5F	19	121	7	19	20	52
KR 3R4F	19	252	20	33	58	92

13.3.3 Cigars – Cigar Smoking Regime

	Puff Count [/cigar]					
Sample	**N Labs**	**Mean**	**r**	**%r**	**R**	**%R**
1C1	6	87.6	18.2	20.80 %	74.6	85.10 %
1C2	8	20.5	2.2	11.00 %	7.4	36.30 %
1C3	7	35.4	5.4	15.10 %	9.8	27.60 %
1C4	6	41	7.1	17.40 %	12.2	29.70 %
CM8	7	12.1	1	8.40 %	2.3	19.00 %

	TPM [mg/cigar]					
Sample	**N Labs**	**Mean**	**r**	**%r**	**R**	**%R**
1C1	6	86.5	25.7	29.80 %	104.9	121.30 %
1C2	8	20.3	2.8	13.90 %	10.9	53.80 %
1C3	7	72.9	9.7	13.30 %	57.8	79.30 %
1C4	6	65.4	24.9	38.10 %	41.1	62.80 %
CM8	8	15.0	1.8	12.00 %	5.1	34.20 %

	NNN [ng/cigar]					
Sample	**N Labs**	**Mean**	**r**	**%r**	**R**	**%R**
1C1	4	580	150	25.90 %	361	62.30 %
1C2	7	241	53	22.00 %	201	83.40 %
1C3	7	992	288	29.00 %	772	77.80 %
1C4	6	3060	1503	49.10 %	1503	49.10 %
CM8	8	21.7	10.2	46.90 %	16.8	77.20 %

	NAT [ng/cigar]					
Sample	**N Labs**	**Mean**	**r**	**%r**	**R**	**%R**
1C1	4	417	90	21.60 %	275	66.00 %
1C2	7	148	31	21.10 %	159	107.00 %
1C3	6	359	84	23.30 %	157	43.80 %
1C4	6	1387	509	36.70 %	1002	72.30 %
CM8	8	45.2	13.9	30.70 %	36.2	80.20 %

	NAB [ng/cigar]					
Sample	**N Labs**	**Mean**	**r**	**%r**	**R**	**%R**
1C1	4	69.9	16.4	23.50 %	41.2	58.90 %
1C2	7	28.7	6.4	22.30 %	25.7	89.60 %
1C3	6	88	24.4	27.70 %	93.3	106.00 %
1C4	6	294	111	37.80 %	235	80.10 %
CM8	8	5.1	2	39.40 %	4.8	94.50 %
	NNK [ng/cigar]					
Sample	**N Labs**	**Mean**	**r**	**%r**	**R**	**%R**
1C1	4	303	116	38.50 %	276	91.20 %
1C2	7	206	41	20.00 %	171	83.10 %
1C3	7	432	145	33.60 %	249	57.70 %
1C4	6	2592	1527	58.90 %	1914	73.80 %
CM8	7	32	12.6	39.20 %	12.6	39.20%

14. RESULTS

14.1 Test results

- The expression of the laboratory data depends on the purpose for which the data are required, and the level of laboratory precision. Confidence limits should be calculated and expressed on the basis of the laboratory data before any rounding has taken place.
- TSNA yields in the mainstream smoke reported in ng/cig should be rounded to the nearest 0,1 ng.

15. REFERENCES

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[4] Wagner K.A., Finkel N.H., Fossett J.E., Gillman I.G., 2005: Development of a quantitative method for the analysis of tobacco-specific nitrosamines in mainstream cigarette smoke using isotope dilution liquid chromatography/electrospray ionization tandem mass spectrometry; Analytical Chemistry Volume 77, Issue 4, p. 1001-1006.

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[6] Xiong W., Hou H., Jiang X., Tang G., Hu Q., 2010: Simultaneous determination of four tobacco-specific N-nitrosamines in mainstream smoke for Chinese Virginia cigarettes by liquid chromatography-tandem mass spectrometry and validation under ISO and "Canadian intense" machine smoking regimes; Analytica Chimica Acta Volume 674, Issue 1, p. 71-78.

EXAMPLE CHROMATOGRAMS

Figure 1 – Chromatograms of a typical TSNAs calibration standard

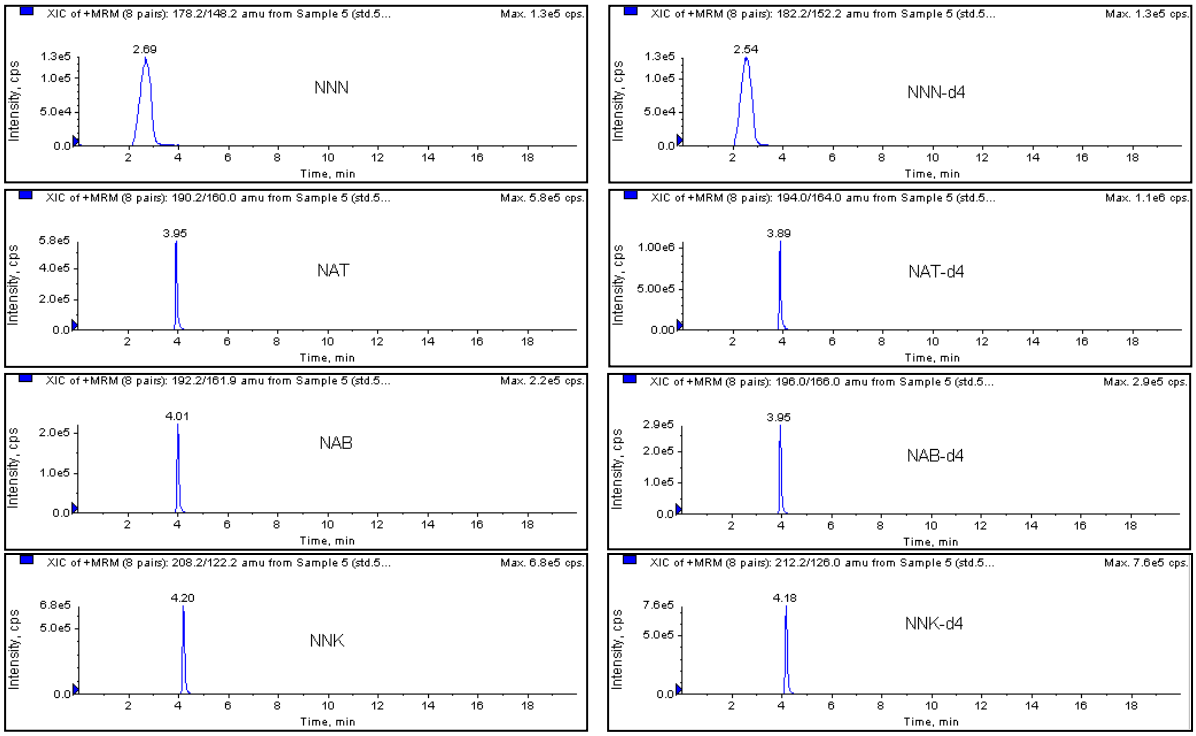

Figure 2 – Chromatograms of TSNAs in mainstream cigarette smoke extract (KR 3R4F)