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Cigarettes — Determination of tobacco specific nitrosamines in mainstream cigarette smoke with an intense smoking regime — Method using LC-MS/MS

Cigarettes — Dosage des nitrosamines spécifiques du tabac dans le courant principal de la fumée de cigarette avec un régime de fumage intense — Méthode par CL-SM/SM

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 126, *Tobacco and tobacco products*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

In 2009 the CORESTA (www.coresta.org) Special Analytes Sub-Group focused on the development of a method for the determination of Tobacco Specific Nitrosamines (TSNAs) in mainstream cigarette smoke. The Sub-Group investigated a liquid chromatography- tandem mass spectrometry (LC-MS/MS) method to complement the gas chromatography with a thermal energy analyser (GC-TEA) technique already available as CORESTA Recommended Method N° 63. Several LC-MS/MS methods have been described in the literature and are referenced herein[3][4]. 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 summary, mainstream cigarette smoke was collected on a glass fibre filter pad, an internal standard solution was added and after extraction, an aliquot was separated and quantitatively analysed by LC-MS/MS. A general methodology was agreed, incorporating key learnings from the joint experiment.

This document was produced through a CORESTA collaborative experiment conducted in 2011, involving 20 laboratories from 12 countries[5][6]. Cigarettes were smoked with the intense smoking regime specified in Health Canada Official Method T-115 (equivalent to ISO 20778) and statistical evaluations were made according to the recommendations provided in ISO 5725[1].

No machine smoking regime can represent all human smoking behaviour.

— It is recommended that cigarettes also be tested under conditions of a different intensity of machine smoking than those specified in this document;

— Machine smoking testing is useful to characterize cigarette emissions for design and regulatory purposes, but communication of machine measurements to smokers can result in misunderstandings about differences in exposure and risk across brands;

— Smoke emission data from machine measurements may be used as inputs for product hazard assessment, but they are not intended to be nor are they valid as measures of human exposure or risks. Communicating differences between products in machine measurements as differences in exposure or risk is a misuse of testing using International Standards.

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1 Scope

This document specifies a method for the quantification of four tobacco specific nitrosamines (TSNAs) in the total particulate matter of cigarette mainstream smoke with the intense smoking regime specified in ISO 20778 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-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3402, *Tobacco and tobacco products — Atmosphere for conditioning and testing*

ISO 8243, *Cigarettes — Sampling*

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

ISO 20779, *Cigarettes — Generation and collection of total particulate matter using a routine analytical smoking machine with an intense smoking regime*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
tobacco specific nitrosamines
TSNAs
four nitrosamines found predominantly in tobacco: N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

[SOURCE: ISO 22303:2008, 3.1]

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4 Principle

Cigarettes are smoked on a routine analytical cigarette smoking machine according to ISO 20778. The mainstream smoke is trapped on a glass-fibre filter pad. After addition of an internal standard, the total particulate matter collected on the glass-fibre filter pad is extracted with 100 mM ammonium acetate solution using a shaker.

The extract is syringe filtered through a 0,45 µm PTFE syringe filter directly into an autosampler vial.

The samples are quantitated by LC-MS/MS.

5 Apparatus

In addition to the list provided below, usual laboratory apparatus and equipment are needed for preparation of samples and standards. All glassware shall be cleaned before use to avoid any contamination.

5.1 Analytical balance, capable of measuring to at least four decimal places.

5.2 Extraction container, of approximately 50 ml.

5.3 Dispenser, of capacity 20 ml.

5.4 Gas-tight syringes, of capacity 250 µl.

5.5 Mechanical volumetric pipette.

5.6 Shaker.

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

5.7.1 Binary pump.

5.7.2 Autosampler.

5.7.3 Tandem mass spectrometer.

5.7.4 Data collection system.

5.7.5 LC column: Waters XBridge BEH C18®[1)], 2,5 µm, 2,1 mm × 50 mm or equivalent.

6 Reagents

Use only reagents of recognized analytical reagent grade.

6.1 N-Nitrosonornicotine, (NNN) CAS-No: 80508-23-2, $w \geq 98$ % (mass fraction).

6.2 N-Nitrosoanatabine, (NAT) CAS-No: 71267-22-6, $w \geq 98$ % (mass fraction).

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 by ISO of this product.

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6.3 N-Nitrosoanabasine, (NAB, CAS-No: 1133-64-8), $w \geq 98$ % (mass fraction).

6.4 4-(N-Methylnitrosamino)-1-(3-pyridyl)-1-butanone, (NNK) CAS-No: 64091-91-4), $w \geq 98$ %.

6.5 Deuterated (N-Nitrosonornicotine), (NNN-d4) CAS-No: 66148-19-4, w ≥ 98 %, isotopic purity $w \geq 99$ %.

6.6 Deuterated (N-Nitrosoanatabine), (NAT-d4) CAS-No: 1020719-69-0, $w \geq 98$ %, isotopic purity $w \geq 99$ %.

6.7 Deuterated (N-Nitrosoanabasine), (NAB-d4) CAS-No: 1020719-68-9, $w \geq 98$ %, isotopic purity $w \geq 99$ %.

6.8 Deuterated 4-(N-Methylnitrosamino)-1-(3-pyridyl)-1-butanone, (NNK-d4) CAS-No: 764661-24-7, $w \geq 98$ %, isotopic purity $w \geq 99$ %.

6.9 Ammonium acetate, $w \geq 97$ % (mass fraction).

6.10 Acetonitrile, HPLC grade.

6.11 Methanol, HPLC grade.

6.12 Acetic acid, $w \geq 99{,}7$ %.

6.13 De-ionized water, ≥ 18,2 MΩ·cm.

6.14 Syringe filter, 0,45 µm polytetrafluoroethylene (PTFE) or equivalent.

6.15 Disposable syringes, 5 ml.

6.16 Autosampler vials (amber), caps and PTFE faced septa.

7 Preparation

7.1 Preparation of glassware

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

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

7.2 Preparation of solutions

7.2.1 Extraction solution, 100 mM ammonium acetate solution.

Weigh 15,40 g ± 0,05 g of ammonium acetate. Put into a 2 000 ml volumetric flask and dilute to the mark with de-ionized water.

7.2.2 HPLC mobile phase A, 0,1 % (volume fraction) acetic acid solution in water.

Add 1 ml of acetic acid into a 1 000 ml volumetric flask and dilute to the mark with de-ionized water.

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7.2.3 HPLC Mobile Phase B, 0,1 % (volume fraction) acetic acid solution in methanol.

Add 1 ml of acetic acid into a 1 000 ml volumetric flask and dilute to the mark with methanol.

NOTE Extraction solution and mobile phases have been shown to be stable for up to three months when stored at room temperature.

7.3 Preparation of standards

7.3.1 General

For the preparation of standard solutions volumetric pipettes should be used.

7.3.2 Preparation of internal standard solutions

7.3.2.1 Primary internal standard solution

Weigh, to the nearest 0,1 mg, 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 1 000 µg/ml.

7.3.2.2 Combined secondary internal standard 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.

7.3.2.3 Working internal standard 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.

7.3.3 Preparation of calibration standard solutions

7.3.3.1 Primary single TSNA solutions

Weigh, to the nearest 0,1 mg, 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 1 000 µg/ml.

7.3.3.2 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.

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7.3.3.3 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.

7.3.3.4 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 (see Table 2).

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.

Table 1 — Preparation of working standard solutions for calibration

Solutions	S0 ml	S1 ml	S2 ml	S3 ml	S4 ml	S5 ml	S6 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

Concentrations	S0 ng/ml	S1 ng/ml	S2 ng/ml	S3 ng/ml	S4 ng/ml	S5 ng/ml	S6 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	4	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

7.3.3.5 Storage

The above standard solutions have been shown to be stable for up to six months if refrigerated below 5 °C. Stability of all standard solutions shall be assessed by each laboratory.

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8 Sampling

Carry out sampling in accordance with ISO 8243.

9 Tobacco product preparation

Condition the cigarettes in accordance with ISO 3402.

10 Sample generation — Smoking of cigarettes

10.1 General

Cigarettes are smoked in accordance with ISO 20778 and ISO 20779.

10.2 Linear smoking

Typically, 3 cigarettes are smoked per 44 mm diameter glass fibre filter pad constituting one replicate.

10.3 Rotary smoking

Typically, 5 cigarettes are smoked per 92 mm diameter glass fibre filter pad constituting one replicate.

Glass fibre filter pads of 44 mm diameter are capable of retaining up to 150 mg of TPM and pads of 92 mm diameter are capable of retaining 600 mg of TPM. If, during smoking, this mass is exceeded, the number of cigarettes should be reduced and a calculation made to allow for the reduced number of cigarettes smoked.

11 Sample analysis

11.1 Preparation of sample

11.1.1 General

Remove the filter pad and place it into an extraction container. Wipe the inside of the holder with back side (clean) of the glass fibre filter pad and add the filter pad to the extraction container.

11.1.2 Extraction for linear smoking (44 mm pad)

After adding 200 µl of the working internal standard solution (7.3.2.3) to the glass fibre filter pad, add 20 ml of 100 mM ammonium acetate extraction solution (7.2.1) to each extraction container containing a filter pad and cap.

11.1.3 Extraction for rotary smoking (92 mm pad)

After adding 400 µl of the working internal standard solution (7.3.2.3) to the glass fibre filter pad, add 40 ml of 100 mM ammonium acetate extraction solution (7.2.1) to each extraction container containing a filter pad and cap.

The extraction volume can be adjusted in each laboratory.

NOTE It is acceptable to extract the glass fibre filter pads with 100 mM ammonium acetate extraction solution containing the internal standards instead of spiking internal standard solution directly to the filter pads.

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11.1.4 Final sample preparation

Perform extractions by using a shaker and agitate for 60 min at 210 r/min. The shaking should be sufficiently vigorous to ensure adequate extraction.

Filter the pad extract directly into vials through the 0,45 µm PTFE syringe filter.

NOTE The above sample extracts have been shown to be stable for up to six days if refrigerated below 5 °C.

11.2 Analysis of samples by LC-MS/MS

11.2.1 General

An adjustment to the chromatographic conditions can be required depending on the instrument configuration and the column chosen for separation.

11.2.2 HPLC set-up parameters (Example)

— Column temperature: 65 °C

— Autosampler tray temperature: 5 °C

— Injection volume: 5 µl

— Flow rate: 250 µl/min

11.2.3 Mobile phase (Example)

— A: 0,1 % acetic acid in water (volume fraction)

— B: 0,1 % acetic acid in methanol (volume fraction)

11.2.4 Mobile phase: Gradient (Example)

See Table 3 for an example of a gradient programme.

Table 3 — Gradient programme

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

11.2.5 MS/MS Set-up parameters (Example)

The following conditions are suitable for the analysis.

The instrument is operated in electrospray ionization (ESI), positive mode.

— Gas 1 (Nebulizer gas): N_2, 350 kPa

— Gas 2 (Drying/Evaporation gas): N_2, 400 kPa

— Temperature heater gas 2: 700 °C

— Curtain gas (CUR): N_2, 275 kPa

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— Collision activated dissociation (CAD): N_2, 20 kPa

— Ion spray voltage (IS): 4 500 V

Other suitable instruments may be used as well. Based on the instrument make and model, the optimal MS/MS parameters can be different and may be used.

Inject 5 µl of each sample onto the HPLC column and analyse as per the chromatographic conditions listed above.

The retention time in the chromatogram can be different depending on the choice of column.

NOTE 1 Depending on the chromatographic system used, peak splitting and peak fronting can be observed in particular for the early eluting compounds (e.g. NNN, NNN-d4).

NOTE 2 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 (volume fraction)] improved sensitivity.

The mass spectrometric parameters are given in Table 4.

Table 4 — Mass spectrometric parameters

Compounds	Precursor ion	Quantifier	Qualifier	DP[a]	CE[b]	CXP[c]	Dwell time
	m/z	*m/z*	*m/z*	V	V	V	ms
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

[a] DP: Declustering potential.

[b] CE: Collision energy.

[c] CXP : Collision cell exit potential.

11.3 Calculation

11.3.1 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.

When laboratories either have problems obtaining 4 internal standards or have checked/validated that using 2 internal standards gives comparable data then the method may 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 cigarettes containing such blends.

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11.3.2 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.

NOTE 1 Depending on manufacturer and model of MS-MS a dilution of the sample extract and the concentration of the calibration could be required to ensure operating the instrument within the manufacturer advised ranges (Detector saturation area).

11.3.3 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 Annex A, Figures A.1 and A.2.

TSNA concentrations are reported in ng/ml by the chromatography software.

11.3.4 Determination of mainstream smoke TSNA deliveries

The mainstream smoke TSNA deliveries, M, expressed in nanograms per cigarette, are given by Formula (1):

$$M = \frac{C \times V}{N} \tag{1}$$

where

C is the concentration of the analyte reported in ng/ml by the chromatography software;

V is the volume of the extraction solution used, in millilitres;

N is the number of cigarettes smoked.

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 of cigarette, in nanograms per cigarette, should be rounded to the nearest 0,1 ng.

12 Repeatability and reproducibility

A major international collaborative study was conducted in 2011 involving 20 laboratories and 10 cigarette samples including the reference cigarettes KR 1R5F and KR 3R4F and the CORESTA Monitor 6[5],[6]. The cigarettes were smoked with the intense smoking regime specified in Health Canada Official Method T-115 (equivalent to ISO 20778). The samples covered a wide range of blends and constructions. The samples are identified in Table 5. The values for repeatability limit, r, and reproducibility limit, R, obtained from this study are provided in Table 6. The statistical evaluation was performed according to ISO 5725-2.

The difference between two single results found on matched cigarette samples by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit, r, on average not more than once in 20 cases in the normal and correct operation of this method.

Single results on matched cigarette samples reported by two laboratories will differ by more than the reproducibility limit, R, on average not more than once in 20 cases in the normal and correct operation of the method.

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Table 5 — Sample identification

Sample ID	Intense NFDPM[a] yield (mg/cig)	Product/ Blend type
Sample 1	25,2	Dark air-cured
Sample 2	23,6	American blended
Sample 3	23,2	American blended
Sample 4	18,4	Virginia blended
Sample 5	12,5	Virginia blended
Sample 6	22,9	Virginia blended
Sample 7	15,7	Charcoal filtered
CM6	29,1	CORESTA Monitor 6 Test Piece
KR 1R5F	17,6	Kentucky Reference 1R5F
KR 3R4F	25,9	Kentucky Reference 3R4F

[a] NFDPM: nicotine-free dry particulate matter

Table 6 — Results overview

NNN (ng/cigarette)						
Sample	**No. of laboratories**	**Mean**	s_r	s_R	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	**No. of laboratories**	**Mean**	s_r	s_R	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

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Table 6 *(continued)*

NAB (ng/cigarette)						
Sample	**No. of laboratories**	**Mean**	s_r	s_R	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	**No. of laboratories**	**Mean**	s_r	s_R	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 Test report

The test report shall state the yield of TSNAs in nanograms per cigarette, the method used, and shall include all conditions not specified in this document or regarded as optional. It shall also give all details necessary for the identification of the cigarettes smoked.

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