
**Tobacco and tobacco products —
Determination of tobacco-specific
nitrosamines in tobacco products —
Method using LC-MS/MS**

*Tabac et produits du tabac — Dosage des nitrosamines spécifiques du
tabac dans les produits du tabac — Méthode par CL-SM/SM*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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*.

This second edition cancels and replaces the first edition (ISO 21766:2018), of which it constitutes a minor revision.

The main changes compared to the previous edition are as follows:

- the title and CAS number for NNK-d4 (see [5.12](#)) have been updated;
- the nomenclature of the deuterated nitrosamines in [5.6](#) to [5.13](#) have been harmonized.

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.

Introduction

The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Smokeless Tobacco Sub-Group studied various widely-used procedures for the determination of tobacco specific nitrosamines (TSNAs) in smokeless tobacco products. A study was conducted in 2009 that evaluated several different methodologies. This study included both liquid chromatography tandem mass spectrometry methods (LC-MS/MS) and gas chromatography combined with nitrogen chemiluminescence detection methods. The results generated with a supplied LC-MS/MS method proved to be the most consistent and was used as the basis for CORESTA Recommended Method N° 72^[2]. Nine laboratories provided data using this method. This study included nine commercial smokeless tobacco products covering eight different product styles. CORESTA Recommended Method N° 72 was updated in 2016 to include repeatability and reproducibility for the four CORESTA reference products.

CORESTA Recommended Method N° 72 was used as the basis for this document. However, the scope of this document was broadened to include ground tobacco, cigarette fillers and cigar fillers in addition to smokeless tobacco products. The respective values for repeatability (r) and reproducibility (R) for ground tobacco, cigarette fillers and cigar fillers have been determined through an international collaborative study that was conducted in 2017 and involved 18 laboratories.

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1 Scope

This document specifies a method for the quantification of four tobacco specific nitrosamines (TSNAs) in tobacco and the following tobacco products: cigarettes, cigars and smokeless tobacco products using reversed phase high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS). The TSNAs determined with this method are: N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

2 Normative references

There are no normative references in this document.

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-(N-Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

[SOURCE: ISO 22303:2008, 3.1]

4 Principle

Deuterium-labelled (d₄) internal standards are added to the tobacco sample and subsequently extracted with an aqueous buffer. The sample extracts are filtered and then analysed by reversed phase high performance liquid chromatography (HPLC) and quantified by tandem mass spectrometry (MS/MS). The amounts of TSNAs in the tobacco products are reported as ng/g, as is wet mass.

5 Reagents

Use only reagents of recognized analytical grade during the analysis. Solvents shall be of HPLC-grade or better.

5.1 Water, deionized, resistivity $\geq 18,2 \text{ M}\Omega \cdot \text{cm}$ at 25 °C.

5.2 Acetonitrile, HPLC grade or better.

5.3 Methanol, HPLC grade or better.

5.4 Ammonium acetate, $w \geq 98 \%$ (mass fraction).

5.5 Acetic acid, $w \geq 98 \%$ (mass fraction).

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

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

5.8 4-(N-Methylnitrosamino)-1-(3-pyridyl)-1-butanone, (NNK, CAS-No: 64091-91-4), $w \geq 98 \%$ (mass fraction).

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

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

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

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

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

6 Apparatus

Usual laboratory apparatus and supplies, and in particular the following items. All glassware shall be cleaned before use to avoid any contamination.

6.1 High performance liquid chromatograph tandem mass spectrometer (LC-MS/MS) with electrospray ion source (ESI), consisting of the following.

6.1.1 Binary pump.

6.1.2 Autosampler.

6.1.3 Column oven.

6.1.4 Data collection system.

6.2 HPLC column: reversed-phase C18¹⁾, 2,5 μm particle size, 2,1 mm $\times$ 50 mm, or equivalent.

6.3 Orbital shaker, wrist action shaker, or similar.

1) Waters XTerra® MS C18, 2,5 μm , 2,1 $\times$ 50 mm has been shown to be a suitable column. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent columns may be used if they can be shown to lead to the same results, i.e. that the analytes and internal standards are sufficiently resolved from interferences.

6.4 Autosampler vials (amber).**6.5 Disposable syringes**, of appropriate size for filtering samples.**6.6 Syringe filter**, of diameter 25 mm and pore size 0,45 µm, made of polytetrafluoroethylene (PTFE) or equivalent.

NOTE Various filter materials were evaluated during the collaborative study and PTFE had the highest recovery from those verified.

Other filter materials may also be suitable, however, they should be evaluated before routine use.

6.7 Extraction containers, glass, of capacity 50 ml to 100 ml.**6.8 Amber volumetric flasks, class A**, in a range of sizes.**6.9 Glass volumetric pipettes, class A, and/or positive-displacement pipettes**, in a range of sizes.**6.10 Analytical balance**, capable of measuring to at least four decimal places (gram).**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 may interfere with the analytical process are removed from the work area.

Standard solutions and sample extracts shall be protected from light.

7.2 Preparation of solutions**7.2.1 Extraction solution, 100 mM ammonium acetate in water**

Weigh 15,4 g $\pm$ 0,05 g of ammonium acetate. Quantitatively transfer into a 2 000 ml volumetric flask and dilute to the mark with deionized water.

7.2.2 HPLC Mobile Phase A: Water, resistivity $\geq$ 18,2 M Ω ·cm at 25 °C**7.2.3 HPLC Mobile Phase B: 0,1 % acetic acid in methanol**

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

Stability studies should be performed by the laboratory to determine the shelf life of these solutions.

7.3 Preparation of standards**7.3.1 General**

All standard solutions shall be prepared in amber, or light protected glassware and stored at about -20 °C, except the calibration standards which shall be stored in a refrigerator. Produce a series of calibration standards to cover the range of expected results to be found in the test samples, as in [7.3.3.4](#). Stability studies should be performed by the laboratory to determine the shelf life of these solutions.

7.3.2 Preparation of internal standard solutions

7.3.2.1 Stock solution

Weigh, to the nearest 0,1 mg, approximately 10 mg each of NNN-d4, NAT-d4, NAB-d4 and NNK-d4. Quantitatively transfer 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 4,00 ml of each of the four internal standard stock solutions into a 100 ml volumetric flask and dilute to volume with acetonitrile. Mix well. The concentration is approximately 40 µg/ml of NNN-d4, NNK-d4, NAT-d4 and NAB-d4.

7.3.2.3 Internal standard spiking solution

Transfer 5,00 ml of mixed internal standard solution into a 100 ml volumetric flask and dilute to volume with acetonitrile. Mix well. The concentration is approximately 2 000 ng/ml of NNN-d4, NNK-d4, NAT-d4 and NAB-d4.

7.3.3 Preparation of calibration standard solutions

7.3.3.1 Stock solution

Weigh, to the nearest 0,1 mg, approximately 10 mg each of NNN, NAT, NAB and NNK. Quantitatively transfer 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 TSNA standard solution (I)

Transfer 4,00 ml of each of the three TSNA stock solutions NNN, NNK, NAT and 1,00 ml of the TSNA stock solution NAB into a 100 ml volumetric flask and dilute to volume with acetonitrile. Mix well. The concentration will be approximately 40 µg/ml of NNN, NNK, NAT and 10 µg/ml of NAB.

7.3.3.3 Mixed TSNA standard solution (II)

Transfer 2,50 ml of the mixed TSNA standard solution (I) into a 250 ml volumetric flask and dilute to volume with acetonitrile/deionized water (30 %/70 %). Mix well. The concentration of NNN, NNK and NAT will be approximately 400 ng/ml, and the concentration of NAB will be approximately 100 ng/ml.

7.3.3.4 TSNA calibration standards

Prepare seven working standard solutions that cover the concentration range of interest. [Table 1](#) provides an example of calibration standard preparation.

The TSNA calibration standards are prepared in seven separate 100 ml volumetric flasks, each containing 10 ml of 100 mM ammonium acetate solution. Transfer 1,00 ml of the internal standard spiking solution (2 000 ng/ml) to each of the seven volumetric flasks. Next, add the appropriate volume of the mixed TSNA standard solution (II), given in [Table 1](#). Then add the volume of acetonitrile, given in [Table 1](#). Finally, dilute each of the seven flasks to volume with 100 mM ammonium acetate and mix well. Calculate the exact concentrations for each standard and record.

NOTE Stock solutions of the individual TSNA and deuterated internal standards in acetonitrile can be purchased at the required levels.

Table 1 — Preparation of working standard solutions for calibration

Calibration standard	Volume of mixed TSNA standard (II) (ml)	Volume of internal standard spiking solution 2 000 ng/ml (ml)	Volume of acetonitrile (ml)	Concentration of NNN (ng/ml)	Concentration of NNK (ng/ml)	Concentration of NAT (ng/ml)	Concentration of NAB (ng/ml)
Cal. 1	0,125	1,00	22	0,5	0,5	0,5	0,125
Cal. 2	0,250	1,00	22	1,0	1,0	1,0	0,250
Cal. 3	0,50	1,00	22	2,0	2,0	2,0	0,50
Cal. 4	1,00	1,00	22	4,0	4,0	4,0	1,00
Cal. 5	2,00	1,00	22	8,0	8,0	8,0	2,00
Cal. 6	5,00	1,00	21	20	20	20	5,00
Cal. 7	25,0	1,00	15	100	100	100	25,0

The linearity range shall be determined for the specific instrument utilized. Stability studies should be performed by the laboratory to determine the shelf life of the standard and internal standard solutions.

8 Sampling

8.1 General

Sampling is conducted such that the laboratory test sample is representative of the population to be tested.

8.2 Sample preparation

A test portion shall be prepared and analysed for each test sample.

Tobacco and tobacco products shall be ground unless the samples are homogeneous and have a particle size <4 mm. It is important that the grinding procedure does not generate excessive heat or cause sample degradation. For further information, see Reference [4].

Tobacco products supplied in the form of pouches should be analysed together with their pouch paper and shall be cut into two halves and added directly in the extraction flask.

At the time of analysis, samples should be allowed to fully equilibrate to room temperature for, typically, 2 h before weighing the sample. Samples removed from a freezer may require additional time to equilibrate.

Samples should be mixed prior to weighing to ensure sample homogeneity.

8.3 Sample extraction

- Using an analytical balance, weigh approximately 1 000 g (note the exact weight to thousandths of a gram) of sample into the 50 ml to 100 ml extraction container.
- Add 0,300 ml of the 2 000 ng/ml internal standard spiking solution (using a calibrated positive displacement pipette or equivalent).
- Add 30 ml of 100 mM ammonium acetate and cap the extraction container.
- Shake the sample(s) for 40 min $\pm$ 5 min at a rate that will ensure sufficient extraction.
- Filter each sample using a 25 mm, 0,45 μ m PTFE syringe filter directly into amber autosampler vials and cap each vial.

The samples may be extracted in a centrifuge tube and centrifuged after shaking.

- The extract is ready for injection into the LC-MS/MS system.

NOTE Sample clean-up using solid phase extraction (SPE) prior to injection into the LC-MS/MS has been shown to reduce contamination of the ion source and reduce the need for routine instrument maintenance. See [Annex A](#) for a suggested sample clean-up procedure using SPE.

9 Sample analysis

9.1 General

Moisture content may be determined on the laboratory test sample to present the final results on a dry-weight basis.

Set up and operate the LC-MS/MS system in accordance with the manufacturer's instructions. Equilibrate the system prior to use.

9.2 Suggested HPLC parameters

The following are recommended conditions for the HPLC system.

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

- Column temperature: 60 °C
- Injection volume: 10 µl
- Flow rate: 0,22 ml/min
- Mobile phase A: Water
- Mobile phase B: 0,1 % acetic acid in methanol

See [Table 2](#).

Table 2 — HPLC gradient

Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)	Gradient type
0	0,22	100	0	Initial
3,0	0,22	10	90	Linear
4,0	0,22	10	90	Linear
5,0	0,22	0	100	Linear
6,0	0,22	100	0	Linear
10,0	0,22	100	0	Linear

9.3 MS/MS parameters

9.3.1 General

The tandem mass spectrometer shall be operated in positive electrospray ionization mode (ESI) using multiple reaction monitoring (MRM) mode.

Suitable MS/MS parameters may vary with the make and model of instrument used, but the following parameters serve as guidelines.

- Gas 1 (Nebulizer gas): N₂, 345 kPa
- Gas 2 (Drying/Evaporation gas): N₂, 415 kPa

- Turbo ion spray temperature: 700 °C
- Interface temperature: on
- Curtain gas (CUR): N₂, 275 kPa
- Collision gas (CAD): N₂, 20 kPa
- Ion spray voltage (IS): 4 500 V

Inject each sample onto the LC-MS/MS and analyse as per the chromatographic conditions listed above.

9.3.2 Quantification and qualification transitions

The quantification is done by using multiple reaction monitoring (MRM) data of the transitions of the precursor ions and the product ions recommended in [Table 3](#).

Table 3 — Quantification and qualification transitions for TSNA

Name	Quantification transition <i>m/z</i>	Qualification transition <i>m/z</i>	Internal standard reference
NNK	208 > 122	208 > 79	NNK-d4
NNK-d4	212 > 126	Not applicable	Not applicable
NNN	178 > 148	178 > 105	NNN-d4
NNN-d4	182 > 152	Not applicable	Not applicable
NAT	190 > 160	190 > 79	NAT-d4
NAT-d4	194 > 164	Not applicable	Not applicable
NAB	192 > 162	192 > 133	NAB-d4
NAB-d4	196 > 166	Not applicable	Not applicable
^a The transitions are for guidance purposes only and the actual optimized values can vary from instrument to instrument.			

The performance of the system shall be sufficient to achieve chromatograms similar to those shown in [Annex B](#) (see [Figures B.1](#) and [B.2](#)). The dwell times need to be optimized to achieve accurate quantification, the number of data points across each peak should be at least 15.

9.4 System suitability

The system performance shall be evaluated for sensitivity, chromatographic performance, carry over, and any other criteria necessary to ensure sufficient performance of the LC-MS/MS system.

9.5 Calibration

Set the quantitation method to perform an internal standard linear calibration: 1/x² weighting is recommended. The calibration curve is a response of the area ratio of each analyte to the corresponding internal standard. The linear calibration should not be forced through the origin. Inject all calibration standards and then proceed to injecting the samples.

9.6 Calculation

All calibration standards and sample calculations utilize relative response factors. The relative response factor, RRF, for each injection is calculated using [Formula \(1\)](#):

$$RRF = \frac{A_A}{A_{IS}} \times C_{IS} \quad (1)$$

where

A_A is the area of the target analyte;

A_{IS} is the area of the corresponding internal standard;

C_{IS} is the concentration of corresponding internal standard.

The concentration, C , of the target analyte in a sample, in nanograms per gram, is determined using the calculated RRF for the sample, the slope and intercept obtained from the corresponding calibration curve, and [Formula \(2\)](#):

$$C = \frac{RRF - b_{Cal}}{a_{Cal}} \times \frac{V}{m} \quad (2)$$

where

RRF is the relative response factor;

b_{Cal} is the y-intercept from the calibration curve;

a_{Cal} is the slope from the calibration curve;

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

m is the amount of tobacco sample, in grams.

Depending on the TSNA concentration of the tobacco sample, the extract may require dilution in order to obtain a response covered by the calibration range. If no solid phase extraction step is involved, the sample extracts could be diluted with extraction solution containing internal standard with the same concentration as in the sample extraction solution. A dilution factor of 10 is sufficient for most samples. Alternatively, and always if a solid phase extraction step is involved, a lower sample weight can be used or the volume of extraction solution can be increased. When a larger volume of extraction solution is used, remember to increase the amount of internal standard as well in order to ensure that the response is in the calibration range; for example, if the volume of extraction solution is doubled, double the amount of internal standard as well. Increased extraction volume is preferred when portions of tobacco, such as pouches, are analysed. In all cases of dilution, remember to multiply the added dilution factor with the instrument test result.

The analyte concentration on a dry-weight basis, C_{dry} , is calculated using [Formula \(3\)](#):

$$C_{dry} = \frac{C_{wet} \times 100}{(100 - M)} \quad (3)$$

where

C_{wet} is the analyte concentration on a wet-weight basis;

M is the moisture or water content, in percent.

10 Repeatability and reproducibility

In 2009, an international collaborative study involving nine laboratories was conducted^[4]. This study included nine commercial tobacco products. Results were evaluated according to ISO 5725-2 to calculate the mean TSNA values, repeatability (r), reproducibility (R) and standard deviations for repeatability (s_r) and reproducibility (s_R).

In 2015, a collaborative study was conducted involving 14 laboratories^[6]. This study included the analysis of the four CORESTA reference products. Results were evaluated in basic conformance with ISO 5725-2 and ISO/TR 22971.

In 2017, a collaborative study was conducted involving 18 laboratories^[2]. This study included the analysis of ground tobacco, ground cigarette fillers and ground cigar fillers. Results were analysed in basic conformance with ISO 5725-2 and ISO/TR 22971. The mean values, r , R , s_r and s_R for NNN, NAT, NAB and NNK are given in Tables 4, 5, 6 and 7, respectively, where the value of N is the number of laboratories used to determine the statistics after the removal of outliers.

Table 4 — Results overview for NNN

Sample type	N	Mean NNN (ng/g)	s_r (ng/g)	s_R (ng/g)	r (ng/g)	R (ng/g)
Nasal snuff ^[4]	8	1 089	25	133	69	372
Loose snus ^[4]	8	276	8	28	21	78
Chewing tobacco – Bits ^[4]	9	555	33	91	93	256
Chewing tobacco – Flake ^[4]	8	480	23	59	64	164
Pellet ^[4]	8	213	17	49	47	137
Chewing tobacco – Loose leaf ^[4]	8	511	19	39	52	109
Loose moist snuff ^[4]	9	2 171	33	369	94	1 034
Loose moist snuff ^[4]	8	2 496	63	261	175	730
Pouched snus ^[4]	9	694	39	93	108	259
CRP1 – Pouched snus ^[6]	12	671	26	36	72	102
CRP2 – Loose moist snuff ^[6]	13	1 823	45	102	127	286
CRP3 – Loose dry snuff powder ^[6]	13	8 249	219	445	612	1 247
CRP4 – Chewing tobacco, loose leaf ^[6]	11	1 948	51	96	143	270
1R6F Ground cigarette filler ^[2]	18	2 294	85	270	237	756
1R5F Ground cigarette filler ^[2]	17	3 221	104	407	292	1 141
RTDAC – Dark air cured ground tobacco ^[2]	17	3 947	131	613	367	1 717
RT2 – Flue cured ground tobacco ^[2]	16	117	6,1	19,1	17	54
NIST SRM 3222 Cigarette cut filler ^[2]	14	1 541	42	187	118	523
Flavoured ground cigar filler ^[2]	16	5 509	121	629	339	1 762
Dark air cured ground cigar wrapper and filler ^[2]	15	3 534	98	485	275	1 359

Table 5 — Results overview for NAT

Sample type	N	Mean NAT (ng/g)	S_r (ng/g)	S_R (ng/g)	r (ng/g)	R (ng/g)
Nasal snuff ^[4]	9	647	24	133	68	373
Loose snus ^[4]	7	176	4	44	12	122
Chewing tobacco – Bits ^[4]	9	305	27	111	75	311
Chewing tobacco – Flake ^[4]	8	123	7	33	19	91
Pellet ^[4]	8	171	16	51	45	142
Chewing tobacco – Loose leaf ^[4]	7	287	6	65	17	182
Loose moist snuff ^[4]	9	2 091	62	461	174	1 290
Loose moist snuff ^[4]	9	3 151	134	694	376	1 943
Pouched snus ^[4]	9	529	24	128	67	358
CRP1 – Pouched snus ^[6]	13	516	22	56	61	157
CRP2 – Loose moist snuff ^[6]	13	1 725	36	145	101	405
CRP3 – Loose dry snuff powder ^[6]	13	5 566	153	556	428	1 557
CRP4 – Chewing tobacco, loose leaf ^[6]	12	1 222	34	133	94	373
1R6F Ground cigarette filler ^[2]	18	2 093	78	215	219	602
1R5F Ground cigarette filler ^[2]	17	2 026	64	177	178	495
RTDAC – Dark air cured ground tobacco ^[2]	17	4 351	149	374	416	1 046
RT2 – Flue cured ground tobacco ^[2]	16	198	16	29	45	81
NIST SRM 3222 Cigarette cut filler ^[2]	14	48	3,3	10,7	9	30
Flavoured ground cigar filler ^[2]	16	2 813	83	219	233	614
Dark air cured ground cigar wrapper and filler ^[2]	15	1 740	91	172	255	481

Table 6 — Results overview for NAB

Sample type	N	Mean NAB (ng/g)	s_r (ng/g)	s_R (ng/g)	r (ng/g)	R (ng/g)
Nasal snuff ^[4]	8	39	2	5	6	13
Loose snus ^[4]	8	15	1	2	3	6
Chewing tobacco – Bits ^[4]	8	16	2	3	5	8
Chewing tobacco – Flake ^[4]	8	48	2	8	7	22
Pellet ^[4]	8	19	2	5	5	13
Chewing tobacco – Loose leaf ^[4]	8	14	1	2	2	6
Loose moist snuff ^[4]	8	167	6	24	15	68
Loose moist snuff ^[4]	8	189	5	36	15	100
Pouched snus ^[4]	8	38	2	5	5	15
CRP1 – Pouched snus ^[6]	12	34	3	4	7	12
CRP2 – Loose moist snuff ^[6]	13	152	5	11	14	31
CRP3 – Loose dry snuff powder ^[6]	13	396	15	38	43	105
CRP4 – Chewing tobacco, loose leaf ^[6]	12	60	2	6	6	18
1R6F Ground cigarette filler ^[2]	18	101	5,8	14	16	39
1R5F Ground cigarette filler ^[2]	17	111	7,8	12	22	32
RTDAC – Dark air cured ground tobacco ^[2]	17	231	9,4	16	26	45
RT2 – Flue cured ground tobacco ^[2]	16	14	1,3	3,9	3,7	11
NIST SRM 3222 Cigarette cut filler ^[2]	14	7	1,3	2,0	3,7	5,5
Flavoured ground cigar filler ^[2]	16	188	7,3	19	21	53
Dark air cured ground cigar wrapper and filler ^[2]	15	134	8,4	15	24	42

Table 7 — Results overview for NNK

Sample type	N	Mean NNK (ng/g)	s_r (ng/g)	s_R (ng/g)	r (ng/g)	R (ng/g)
Nasal snuff ^[4]	9	482	16	47	46	131
Loose snus ^[4]	8	133	5	15	14	43
Chewing tobacco – Bits ^[4]	8	78	6	11	16	31
Chewing tobacco – Flake ^[4]	8	152	9	16	26	44
Pellet ^[4]	7	246	11	26	32	71
Chewing tobacco – Loose leaf ^[4]	8	94	5	9	14	25
Loose moist snuff ^[4]	9	729	20	180	56	505
Loose moist snuff ^[4]	9	583	27	133	75	372
Pouched snus ^[4]	8	265	5	26	14	73
CRP1 – Pouched snus ^[6]	13	205	9	17	25	48
CRP2 – Loose moist snuff ^[6]	13	437	12	29	33	82
CRP3 – Loose dry snuff powder ^[6]	13	4 138	178	389	498	1 088
CRP4 – Chewing tobacco, loose leaf ^[6]	12	440	22	39	61	109
1R6F Ground cigarette filler ^[2]	18	675	27	65	76	182
1R5F Ground cigarette filler ^[2]	17	781	27	64	76	179
RTDAC – Dark air cured ground tobacco ^[2]	17	1 908	129	223	360	624
RT2 – Flue cured ground tobacco ^[2]	16	107	6,5	18,8	18	53
NIST SRM 3222 Cigarette cut filler ^[2]	14	32	3,6	7,5	10	21
Flavoured ground cigar filler ^[2]	16	1 782	54	117	150	328
Dark air cured ground cigar wrapper and filler ^[2]	15	901	31	79	87	220

11 Test report

The test report shall state the yield of TSNAs in tobacco and tobacco products on an as-received basis (wet weight) in units of ng/g, and shall include all conditions not specified in this document that may affect the result. It shall also give all details necessary for the identification of each sample.

Annex A (informative)

Sample clean-up using solid phase extraction (SPE)

A.1 Reagents and supplies

A.1.1 Water deionized, resistivity $\geq 18,2 \text{ M}\Omega \cdot \text{cm}$ at 25 °C.

A.1.2 Methanol, HPLC grade.

A.1.3 Ammonium hydroxide, concentrated, reagent grade or better.

A.1.4 Formic acid, $w \geq 98 \%$.

A.1.5 Acetic acid, $w \geq 98 \%$.

A.1.6 25 mm syringe filter, 0,2 μm polyvinylidene fluoride (PVDF) or equivalent.

A.1.7 SPE cartridges, polymer reversed-phase sorbent 3 cm^3 (60 mg), or equivalent.

EXAMPLE Oasis® HLB ²⁾.

A.2 Reagent preparation

A.2.1 Wash No. 1: 4,5 % methanol and 0,5 % ammonium hydroxide in water

Combine approximately 100 ml of deionized water, 45 ml of methanol and 5 ml of concentrated ammonium hydroxide in a 1 l volumetric flask. Dilute to volume with deionized water and mix solution well.

A.2.2 Wash No. 2: 0,01 % formic acid in water

Add 0,100 ml of formic acid ($w \geq 98 \%$) to a 1 l volumetric flask containing approximately 500 ml of deionized water. Dilute to volume with deionized water and mix well.

A.2.3 Eluting solvent: 30 % water and 0,1 % acetic acid in methanol

Combine approximately 200 ml of methanol, 300 ml of deionized water, and 1 ml of acetic acid in a 1 l volumetric flask. Dilute to volume with methanol and mix well.

A.3 SPE procedure

Perform sample extraction as described in 8.2 a) to d).

- a) Filter approximately 4 ml of sample directly into labelled disposable culture tubes using a 25 mm, 0,2 μm PVDF syringe filter.

2) Oasis® HLB is a product of the Waters Corporation and 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 the product named. Equivalent products may be used if they can be shown to lead to the same results.