TECHNICAL SPECIFICATION

ISO/TS 22304

First edition
2008-07-15

Tobacco — Determination of tobacco specific nitrosamines — Method using alkaline dichloromethane extraction

Tabac — Dosage des nitrosamines spécifiques au tabac — Méthode d'extraction au dichlorométhane alcalin

Reference number
ISO/TS 22304:2008(E)

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Published in Switzerland

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Foreword

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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.

ISO/TS 22304 was prepared by Technical Committee ISO/TC 126, *Tobacco and tobacco products*.

Introduction

During the development of this Technical Specification, interlaboratory tests were carried out using two different methods for the determination of tobacco specific nitrosamines. These comprise the method discussed in this Technical Specification, using alkaline dichloromethane extraction, and the method using buffer extraction (see References [2], [3]).

These studies show that no differences occur between the results obtained by the two different methods (see Reference [4]). The method described in this Technical Specification could be preferable for those preferring a faster analysis using less solvent. The method using buffer extraction is described in ISO 22303 (voir Reference [1]).

Tobacco — Determination of tobacco specific nitrosamines — Method using alkaline dichloromethane extraction

1 Scope

This Technical Specification specifies the procedure for the determination of the tobacco specific nitrosamines (TSNAs): N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in ground leaf tobacco, manufactured tobacco and tobacco products. The determination is by means of gas chromatography.

2 Normative references

The following referenced documents are indispensable for the application 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 1042, *Laboratory glassware — One-mark volumetric flasks*

ISO 3696, *Water for analytical laboratory — Specification and test methods*

3 Terms and definitions

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

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)

4 Principle

TSNAs are extracted from ground tobacco samples with dichloromethane containing sodium hydroxide. The extract is eluted through a mixed bed of magnesium sulphate and sodium sulphate using dichloromethane, evaporated to near dryness and reconstituted in chloroform. The individual nitrosamines are then separated and quantified by gas chromatography with chemiluminescent detection. Quantification is performed by an internal standard technique.

5 Reagents

Use only reagents of analytical reagent grade.

SAFETY PRECAUTIONS — Nitrosamines are suspected carcinogens; therefore, appropriate safety precautions should be taken when preparing standards. Always wear laboratory gloves when handling standard solutions and making dilutions.

5.1 **N-nitrosonornicotine**, (NNN, CAS[1)]: 53759-22-1), $w \geqslant$ 98 % (mass fraction).

5.2 **N-nitrosoanatabine**, (NAT, CAS: 71267-22-6), $w \geqslant$ 98 %.

5.3 **N-nitrosoanabasine**, (NAB, CAS: 1133-64-8), $w \geqslant$ 98 %.

5.4 **4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone**, (NNK, CAS: 64091-91-4), $w \geqslant$ 98 %.

5.5 **N-nitrosodi-*n*-hexylamine**, (NDHA, CAS: 6949-28-6) (internal standard), $w \geqslant$ 98 %.

5.6 **Magnesium sulphate** ($MgSO_4$), anhydrous (CAS: 7487-88-9), (powder), $w \geqslant$ 98,0 %.

5.7 **Sodium sulphate** (Na_2SO_4), anhydrous (CAS: 7757-82-6), (granular), $w \geqslant$ 99,0 %, 2 mm (10 mesh) to 0,25 mm (60 mesh).

5.8 **Sodium hydroxide** (NaOH), pellets (CAS: 1310-73-2), $w \geqslant$ 97,0 %.

5.9 **Dichloromethane** (CAS: 75-09-02), $w \geqslant$ 99,9 %.

5.10 **Chloroform** (CAS: 67-66-3), $w \geqslant$ 99,9 %.

5.11 **Nicotine** (CAS: 54-11-5), $w \geqslant$ 99,0 %.

5.12 **Water**, (CAS: 7732-18-5), complying with grade 2 of ISO 3696 or better.

5.13 **Nitrogen**, (CAS: 7727-37-9), for eluent evaporator, $w \geqslant$ 99,995 %.

5.14 **Helium**, (CAS: 7440-59-7), for carrier gas, $w \geqslant$ 99,995 %.

5.15 **Oxygen**, (CAS: 7782-44-7), for generating ozone in the detector, $w \geqslant$ 99,6 %.

6 Apparatus

Usual laboratory apparatus and, in particular, the following items.

6.1 **Gas chromatograph** (GC), with a chemiluminescence detector and autosampler (optional).

6.2 **Eluent evaporator**, for concentration of sample extract.

6.3 **Mixer, vortex type**, for sample extraction/mixing.

6.4 **GC column**, fused silica capillary column of length 30 m and internal diameter 0,53 mm, coated with a 1,5 µm film of 5 % phenyl methylpolysiloxane.

NOTE Other columns can be used provided that a satisfactory separation is achieved.

6.5 **Chromatography data acquisition system**, for measuring peak areas electronically.

6.6 **One-mark volumetric flasks**, complying with class A of ISO 1042.

6.7 **Disposable glass transfer pipettes**, length 229 mm.

1) CAS: Chemical Abstract Service.

6.8 Glass tilting repeating dispensers, constant volume, 10 ml.

6.9 Glass extraction test tubes, 20 mm ID × 150 mm.

6.10 Test tube caps for glass extraction test tubes, 18 thread to 415 thread.

6.11 Glass concentration test tubes, 16 mm ID × 100 mm.

6.12 Blank extraction column, reservoir capacity 14 ml.

6.13 Frits for blank extraction column, outer diameter 1,4 cm, pore size 20 µm.

6.14 Sample containers, borosilicate glass autosampler vials, capacity 2 ml, with PTFE-lined septum screwcap closures.

6.15 Refrigerating unit, for storing standards at −20 °C.

7 Preparation of solutions and standards

7.1 Preparation of sodium hydroxyde solution; w = 10 % (mass fraction)

Weigh about 10 g of NaOH pellets (5.8) in a 100 ml beaker. Add water to a total mass of 100 g and stir until dissolved.

7.2 Preparation of internal standard

Prepare the internal standard stock solution by dissolving NDHA (5.5) in dichloromethane (5.9) to yield a concentration of about 200 µg ml^{-1} NDHA. Further dilute this stock solution such that the concentration of NDHA is 0,40 µg ml^{-1}. This diluted solution in dichloromethane is used as part of the extractant (see Table 1). Store all solutions of the internal standard at about −20 °C when not in use.

7.3 Preparation of calibration standards

Table 1 lists typical concentrations of standards to be used for the analysis of tobacco. When not in use, store standard solutions at about −20 °C. At least four levels of the TSNAs should be used for calibration.

Table 1 — Standard concentrations in chloroform (µg·ml^{-1})

Level	NDHA	NNN	NAT	NAB	NNK
1	5,00	0,05	0,05	0,05	0,05
2	5,00	0,30	0,30	0,15	0,15
3	5,00	1,50	1,50	0,30	2,88
4	5,00	5,00	5,00	0,50	5,00
5	5,00	8,50	9,00	0,75	7,20
NOTE Depending on the final volume of the standard, 600 µl of nicotine should be added per 10 ml of chloroform, e.g. a 50 ml volume of standard should contain 3 ml of nicotine.					

7.4 Setting-up of gas chromatograph and detector

The GC (6.1) is set up for splitless injections and configured to operate with a chemiluminescence detector. A 4 mm internal diameter tube with a glass wool split liner is used in the split/splitless injection port. The GC column is connected to the ceramic pyrolyser tube of the chemiluminescence detector. Approximately 13 cm of the capillary GC column are inserted into the pyrolyser.

Table 2 lists typical temperatures and ramp rates used to separate the TSNAs. Table 3 gives recommended settings for the analysis. These conditions may be varied at the analyst's discretion to achieve better resolution, decrease run time or improve detection limit. The injection liner and septum should be changed after each autosampler run (about 100 injections), followed by two solvent (chloroform) injection runs.

Figure A.1 is a chromatogram of a standard analysed under the conditions given in Tables 2 and 3.

Table 2 — GC oven programme

Column oven initial temperature (°C)	100
Column oven initial time (min)	1,00
Column oven programm rate (°C min^{-1})	6
Column oven final temperature (°C)	240
Column oven final time (min)	3,00
Total run time (min)	23,33
NOTE Conditions may be adjusted to improve peak resolution or elution time.	

Table 3 — System parameters

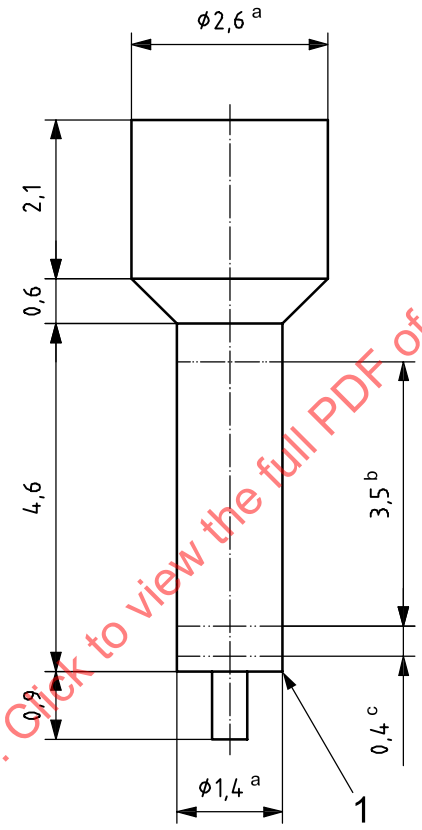
Detector interface temperature (°C) [a]	250
Detector pyrolyser temperature (°C)	550
Ozone flow to detector (ml min^{-1})	≈ 5,1
Injection mode	Splitless
Injector temperature (°C)	210
Injection volume (µl)	2
Helium flow (ml·min^{-1})	5,66
Inlet purge on (min)	2,5
Inlet purge off (min)	20
Autosampler sample pumps	6
Autosampler viscosity delay	2
Autosampler sample washes	0
Autosampler solvent [b] A washes	4
Autosampler solvent [b] B washes	4
[a] Detector temperature may be adjusted to improve detection limit.	
[b] Isopropyl alcohol.	

7.5 Tobacco extraction and isolation of TSNAs

NOTE Add water to dry (< 5 % moisture) samples to attain a moisture level of about 30 %.

Accurately weigh (1,5 ± 0,01) g of tobacco into a 20 mm × 150 mm test tube and cap. Fill blank fritted extraction columns (Figure 1) with a layer of sodium sulphate of about 0,4 cm topped with about 3,5 cm of magnesium sulphate. Do not tamp or compact the solid adsorbents.

Dimensions in centimetres



Key

1 one frit

a External diameter.

b $MgSO_4$ (about 2,5 g).

c Na_2SO_4 (about 0,75 g).

Figure 1 — Blank extraction column

7.6 Sample extraction

Dispense 10 ml of extraction solution (0,40 µg ml^{-1} NDHA in dichloromethane) into each test tube. Dispense 0,5 ml of a mass fraction of w = 10 % NaOH into each test tube. Screw on the test tube caps. Vortex each test tube for 90 s and then place in a rack to sit for 20 min. Vortex each test tube a second time for 90 s and then place in a rack for another 20 min. Vortex each test tube a third time for 60 s and then uncap. Slowly decant the liquid portion of the test tube contents through prepared blank extraction columns into 16 mm × 100 mm collection test tubes. Leave the majority of the tobacco sample behind in the test tubes.

NOTE 1 This is not a quantitative transfer step.

Rinse the filtration columns, using 2 ml of dichloromethane, into the collection test tubes. Place the collection tubes containing the eluent in an eluent evaporator (6.2) at 42 °C for 40 min using nitrogen (5.13) to evaporate the solvent. Reconstitute the evaporated extract residue with about 0,7 ml of chloroform (5.10) and vortex. Transfer the extracts to sample containers and cap. Figure A.2 is a chromatogram of a flue-cured tobacco extract. Samples may be stored overnight in a freezer (< 0 °C) prior to analysis.

NOTE 2 It is suggested that a tobacco control sample (monitor) be analysed within each set of samples. The data obtained from the monitor tobacco could then be used to create monitor control charts for quality control purposes.

8 Data analysis and calculation of results

GC calibration is achieved by analysing the calibration standards in duplicate using bracket calibration where the standards are analysed prior to and after the extracting of the samples. Results are calculated using an internal standard technique. The data are plotted using the area response ratio of area $TSNA_{STD}$ to area $NDHA_{STD}$ as the dependent variable (y axis) and the concentration ratio of $TSNA_{STD}$ amount to $NDHA_{STD}$ amount for each of the standards as the independent variable (x axis), yielding a linear equation for each TSNA standard of the form:

$$y = mx + b$$

where

m is the slope;

b is the y-intercept.

This linear least-squares regression of the instrument responses versus standard amounts should result in a calibration correlation coefficient, R^2, of greater than 0,990 0 for each analyte.

The calibration curve generated is used to determine the concentration of the TSNAs in the sample. The mass fraction of TSNAs in the sample, w_{TSNA}, expressed in micrograms per gram, is given by the following equation:

$$w_{TSNA} = \frac{m_{NDHAS} \times r_s}{m_s}$$

where

m_{NDHAS} is the mass of NDHA in the sample, expressed in micrograms;

r_s is the amount ratio of the sample;

m_s is the mass of the sample, expressed in grams.

The amount ratio of the sample is taken from the calibration curve at the given area ratio for the sample. Figure A.3 illustrates a typical calibration curve obtained from NNN at seven concentration levels.

9 Repeatability and reproducibility

An international collaborative study was conducted including sample types of leaf, cigarette cut filler and moist snuff. Twelve laboratories reported results with the following mean repeatability, r, and reproducibility, R, over a wide analyte concentration range as indicated in Table 4.

Table 4 — Mean, repeatability and reproducibility standard deviations for tobacco samples (µg g^{-1} dry-mass basis)

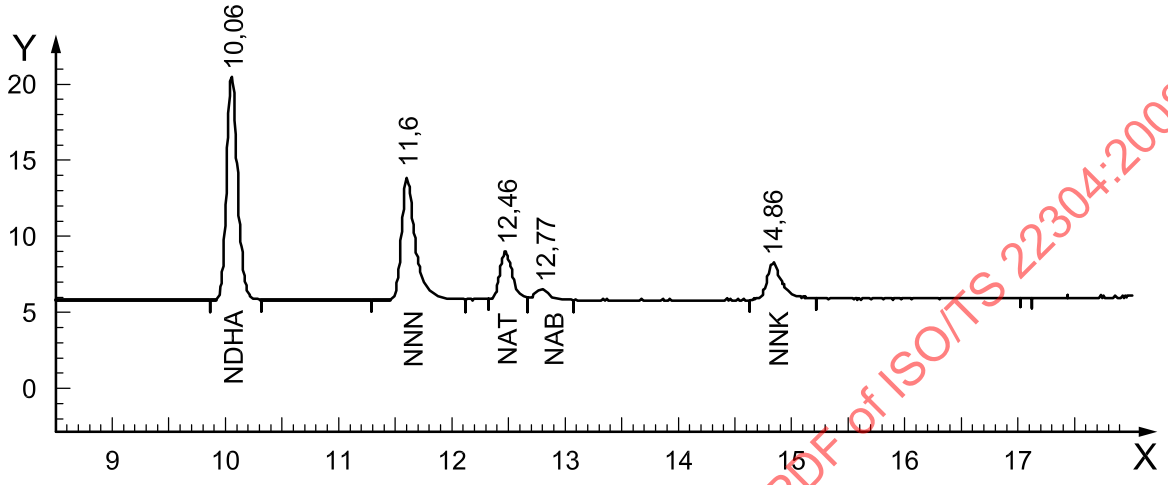
Sample	NNN			NAT			NAB			NNK		
	Mean	r	R	Mean	r	R	Mean	r	R	Mean	r	R
Turkish	0,24	0,02	0,06	0,12	0,02	0,06	0,03	0,01	0,02	0,10	0,01	0,09
Low TSNA, flue-cured	0,27	0,02	0,06	0,41	0,03	0,07	0,04	0,00	0,01	0,28	0,02	0,07
Flue-cured	0,76	0,02	0,10	1,02	0,02	0,10	0,10	0,01	0,07	1,56	0,03	0,36
1R4F tobacco	2,10	0,07	0,28	1,74	0,06	0,12	0,09	0,01	0,03	0,98	0,05	0,22
Composite comm. cig. tob.	2,56	0,04	0,30	2,02	0,04	0,14	0,12	0,01	0,04	1,31	0,05	0,31
Reference snuff	3,20	0,06	0,49	2,84	0,05	0,28	0,19	0,02	0,04	1,11	0,03	0,28
Burley	8,17	0,14	0,94	4,06	0,08	0,25	0,21	0,02	0,04	0,67	0,03	0,15
Burley stem	9,06	0,45	2,20	2,84	0,16	0,24	0,14	0,01	0,04	1,19	0,08	0,46

10 Test report

The test report shall give the TSNA concentration as a mass fraction, in µg g^{-1}, based on the dry mass of the sample. For a straight-grade tobacco sample (not a commercial product or a tobacco blend), crop year, tobacco type and place of origin shall be specified. For a commercial tobacco product, the name of the manufacturer, country of manufacture and product name shall be specified. For all tobacco samples, the type of sampling procedure shall be specified as to date and location of purchase.

Annex A
(informative)

Typical chromatograms and calibration curves

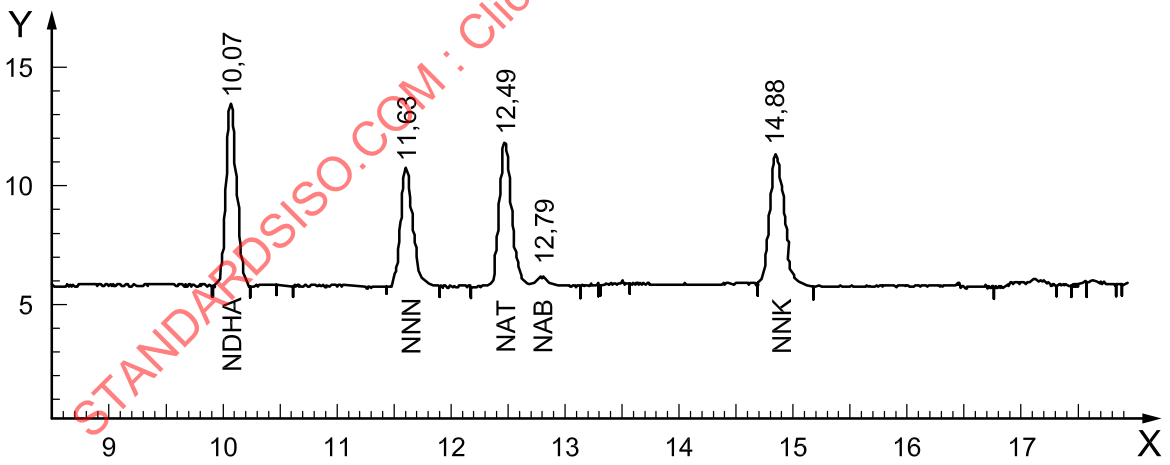


Key

X time (min)

Y response (mvolts)

Figure A.1 — Typical chromatogram of a standard



Key

X time (min)

Y response (mvolts)

Figure A.2 — Typical chromatogram of a tobacco sample (flue-cured) extract