BRITISH STANDARD

BS ISO 22303:2008

Tobacco — Determination of tobacco specific nitrosamines — Method using buffer extraction

ICS 65.160

BSI British Standards

National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/40, Tobacco and tobacco products.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Foreword

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ISO 22303 was prepared by Technical Committee ISO/TC 126, *Tobacco and tobacco products*.

Introduction

During the development of this International Standard, inter-laboratory tests were carried out using two different methods for the determination of tobacco specific nitrosamines; this method, using buffer extraction, and the method using alkaline dichloromethane 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 using alkaline dichloromethane extraction is described in Technical Specification ISO/TS 22304 (see Reference [1]).

INTERNATIONAL STANDARD ISO 22303:2008(E)

Tobacco — Determination of tobacco specific nitrosamines — Method using buffer extraction

1 Scope

This International Standard 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 use — 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 using a buffer solution. The aqueous portion of the buffer is absorbed into diatomaceous earth. The TSNAs are then eluted from the diatomaceous earth with methylene chloride and concentrated in a heated water bath using nitrogen. The TSNAs are separated and quantified by gas chromatography with chemiluminescent detection. Quantification is performed by an internal standard technique.

5 Reagents

Use only reagents of recognised analytical 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 **Citric acid**, anhydrous (CAS: 77-92-9), $w \geqslant 99{,}5$ %.

5.7 **Sodium hydrogen phosphate**, (Na_2HPO_4), anhydrous (CAS: 7558-79-4), $w \geqslant 99$ %.

5.8 **L-ascorbic acid**, (CAS: 50-81-7), $w \geqslant 99$ %.

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

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

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

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

5.13 **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 **Ultrasonic bath**, for sample extraction.

6.4 **GC column**, a fused silica capillary column of length 30 m and internal diameter 0,53 mm, coated with a 3,0 µm film of 100 % dimethylpolysiloxane.

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

6.5 **Flux calcined diatomaceous earth column**, capacity 100 ml.

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

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

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

6.9 **Glass tilting repeating dispensers**, constant volume, 10 ml and 50 ml.

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1) CAS: Chemical Abstract Service.

6.10 Glass Erlenmeyer flasks, capacity 125 ml.

6.11 Glass graduated cylinder, capacity 250 ml.

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

6.13 Sampling tube, capacity 300 ml.

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

7 Preparation of extraction solution and standards

7.1 Preparation of buffer extraction solution

The amounts, for the preparation of 2 l of buffer, are (21,1 ± 0,1) g citric acid (5.6), (25,6 ± 0,1) g sodium hydrogen phosphate (5.7) and (7,0 ± 0,1) g L-ascorbic acid (5.8). Distilled, deionized water is added to the mark and the mixture is stirred until all solids are dissolved. Buffer should be prepared weekly and stored at about 4 °C when not in use. The buffer extraction solution is clear and colourless with a pH value of (4,3 ± 0,2).

7.2 Preparation of internal standard

Prepare the internal standard by dissolving NDHA (5.5) in the dichloromethane (5.9) such that a sufficient response will be attained in the chromatogram. For example, when the sample mass is 1 g and the final dilution is 2 ml, make the internal standard to a mass concentration of about 2,0 µg NDHA ml^{-1} dichloromethane. This will yield a mass concentration of about 1,0 µg NDHA ml^{-1} in the final dilution (assuming complete recovery), sufficient for detection on the chromatographic system. Store 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 samples. At least four levels of the TSNAs should be used for calibration. When not in use, store standard solutions at about −20 °C.

Table 1 — Approximate standard concentrations in dichloromethane (µg·ml^{-1})

Level	NDHA	NNN	NAT	NAB	NNK
1	1,00	0,10	0,10	0,05	0,10
2	1,00	0,30	0,30	0,15	0,50
3	1,00	1,50	1,50	1,00	3,00
4	1,00	5,00	5,00	1,50	5,00
5	1,00	8,50	9,00	2,00	7,50
6	1,00	17,00	11,00	3,00	11,00
NOTE Based on 1 g sample, 2 ml final dilution.					

7.4 Setting up of the gas chromatograph and detector

The GC (6.1) is set up for splitless injections and configured to operate with a chemiluminescence detector. A split liner is used in the split/splitless injection port. (The liner showed no indication of contamination after 50 sample injections.) It is recommended that a length of uncoated capillary tubing be used to connect the end of the analytical column to the detector to balance the flow or pull caused by the vacuum pump in the detector with the flow exiting from the analytical column. A 150 cm by 0,25 mm internal diameter uncoated capillary tubing for a 30 m by 0,53 mm internal diameter, and 3,0 µm film thickness analytical column should be sufficient. Connections to the analytical column may be made with universal press-tight connectors. The tubing is then inserted through the furnace and attached to the inlet of the detector.

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, to decrease runtime or to improve detection limit. 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 programme rate 1 (°C·min^{-1})	30
Column oven final temperature 1 (°C)	200
Column oven final time 1 (min)	0,00
Column oven programme rate 2 (°C·min^{-1})	3
Column oven final temperature 2 (°C)	235
Column oven final time 2 (min)	0,00
Column oven programme rate 3 (°C·min^{-1})	30
Column oven final temperature 3 (°C)	260
Column oven final time 3 (min)	8,00
Total run time (min)	24,82
NOTE Conditions may be adjusted to improve peak resolution or elution time.	

Table 3 — System parameters

Detector interface temperature (°C)	275
Detector pyrolyser temperature (°C)[a]	500
Ozone flow to detector (ml·min^{-1})	≈ 5,1
Injection mode	Splitless
Injector temperature (°C)	230
Injection volume (µl)	2
Helium pressure (psi)	7,0
Purge flow at 0,6 min (ml·min^{-1})	100
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

Accurately weigh (1,0 ± 0,01) g of tobacco into a 125 ml Erlenmeyer flask (6.10) and stopper the flask with a glass stopper. Add 50 ml of buffer extraction solution and sonicate for 1 h. The temperature of the water in the ultrasonic bath (6.3) should not exceed 40 °C. Allow the flask to cool for 10 min. Assemble a 100 ml capacity flux calcined diatomaceous earth column (6.5) above a 300 ml sampling tube (6.13). Pour the entire contents of the flask on to the head of the column. Rinse the flask with an additional 10 ml of buffer extraction solution and pour the contents on to the head of the column. After the liquid permeates about one-third of the length of the column and no liquid is standing at the head of the column, a 150 ml portion of dichloromethane (5.9) is added and allowed to penetrate until about 20 ml of liquid is visible above the solid surface of the column (do not let the column become dry). At this time, a portion of an additional 150 ml of dichloromethane is poured on to the column. Continue adding dichloromethane until the entire second 150 ml portion of dichloromethane has been added to the column. The dichloromethane is allowed to pass through the column (about 1 h) and is collected in the 300 ml sampling tube. The elution process is considered complete when the flow of dichloromethane from the column slows to less than one drop in 10 s and about 250 ml of dichloromethane has been collected. Add 1 ml of 2,0 $\mu g \cdot ml^{-1}$ NDHA to this collected solution.

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

7.6 Extract concentration

The sample tube containing the dichloromethane eluent that contains NDHA is placed in an eluent evaporator (6.2) and immersed in the water bath preheated to 38 °C. The pressure of the nitrogen gas flow is increased gradually to create a vortex in the liquid. The sample extract is concentrated to about 0,5 ml after which the sampling tube is removed from the apparatus. The contents of the sampling tube are transferred to a 2 ml volumetric flask. Two 0,5 ml portions of dichloromethane are used to rinse the sampling tube and each rinse is added to the sample. The sample is diluted to a final volume of 2 ml with dichloromethane. A portion is transferred to an autosampler vial and the vial is sealed using a cap with a septum. Figure A.2 is a chromatogram of a flue-cured tobacco extract. Samples may be stored overnight at about –20 °C prior to analysis.

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 by 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:

$$y = ax + b$$

where

a 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 typical calibration curves obtained for the TSNA standards at six concentration levels.

9 Repeatability and reproducibility

An international collaborative study was conducted including sample types of leaf, cigarette cut filler, and moist snuff. Nine 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)

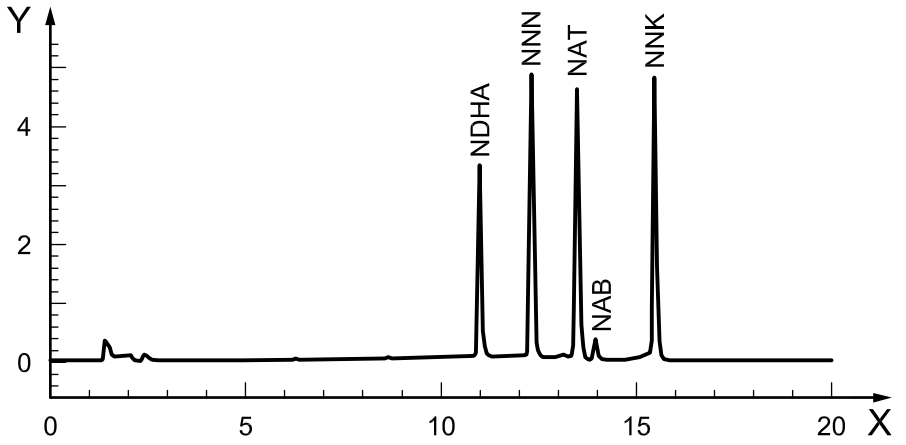
Sample	NNN			NAT			NAB			NNK		
	Mean	r	R	Mean	r	R	Mean	r	R	Mean	r	R
Turkish	0,25	0,01	0,04	0,12	0,01	0,07	0,06	0,01	0,04	0,07	0,01	0,06
Low TSNA flue-cured	0,29	0,01	0,04	0,44	0,02	0,03	0,05	0,01	0,04	0,28	0,02	0,07
Flue-cured	0,82	0,04	0,08	1,06	0,09	0,11	0,13	0,01	0,09	1,62	0,14	0,26
1R4F tobacco	2,38	0,05	0,22	2,04	0,04	0,11	0,14	0,01	0,04	1,04	0,04	0,11
Composite cigarette tobacco	2,69	0,07	0,22	2,12	0,07	0,12	0,15	0,01	0,04	1,31	0,04	0,14
Reference snuff	3,41	0,10	0,40	3,09	0,09	0,25	0,22	0,01	0,06	1,21	0,03	0,16
Burley	8,71	0,26	0,90	4,36	0,09	0,24	0,23	0,02	0,06	0,62	0,02	0,11
Burley stem	9,15	0,31	0,92	3,08	0,13	0,28	0,16	0,01	0,06	1,09	0,08	0,18

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 manufacturer, country of manufacture and product name shall be specified. For all tobacco samples, the type of sampling procedure shall be specified as to the date and location of purchase.

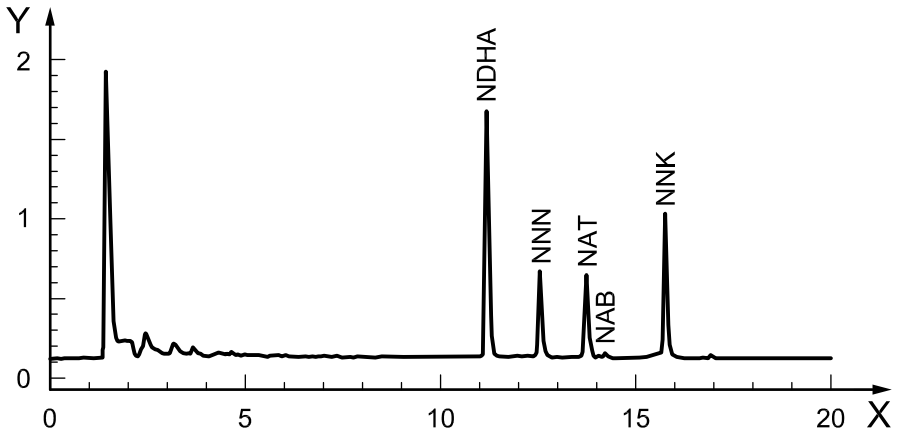
Annex A
(informative)

Typical chromatograms and calibration curves



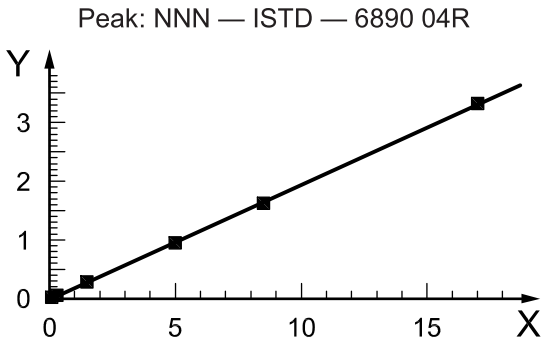
Key
X time in minutes
Y response in volts

Figure A.1 — Typical chromatogram of standard

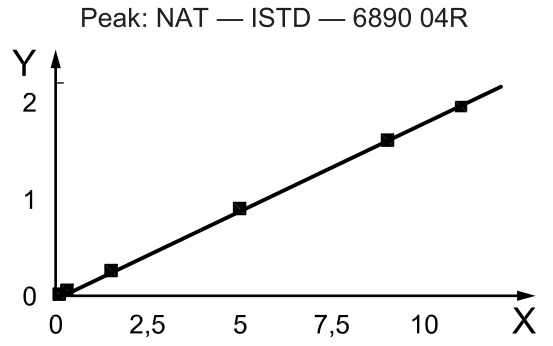


Key
X time in minutes
Y response in volts

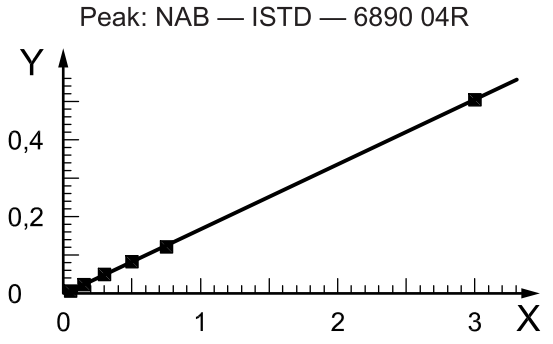
Figure A.2 — Typical chromatogram of a tobacco sample (Flue-cured) extract



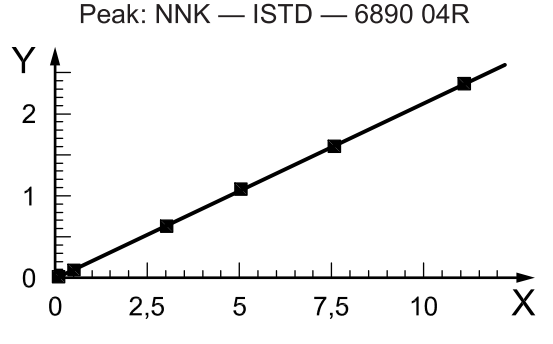
A. NNN, R^2 = 0,999 8



B. NAT, R^2 = 0,999 9



C. NAB, R^2 = 0,999 9



D. NNK, R^2 = 0,999 9

Key
X amount ratio
Y area ratio

Figure A.3 — Typical calibration curves for TSNAs

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