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

*Tabac — Dosage des nitrosamines spécifiques du tabac — Méthode
d'extraction par solution tampon*

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

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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]).

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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-nitrosornicotine (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*

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3 Terms and definitions

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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-nitrosornicotine (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.

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- 5.1 **N-nitrosornicotine**, (NNN, CAS:¹⁾ 53759-22-1), $w \geq 98$ % (mass fraction).
- 5.2 **N-nitrosoanatabine**, (NAT, CAS: 71267-22-6), $w \geq 98$ %.
- 5.3 **N-nitrosoanabasine**, (NAB, CAS: 1133-64-8), $w \geq 98$ %.
- 5.4 **4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone**, (NNK, CAS: 64091-91-4), $w \geq 98$ %.
- 5.5 **N-nitrosodi-*n*-hexylamine**, (NDHA, CAS: 6949-28-6) (internal standard), $w \geq 98$ %.
- 5.6 **Citric acid**, anhydrous (CAS: 77-92-9), $w \geq 99,5$ %.
- 5.7 **Sodium hydrogen phosphate**, (Na_2HPO_4), anhydrous (CAS: 7558-79-4), $w \geq 99$ %.
- 5.8 **L-ascorbic acid**, (CAS: 50-81-7), $w \geq 99$ %.
- 5.9 **Dichloromethane**, (CAS: 75-09-02), $w \geq 99,9$ %.
- 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, $w \geq 99,995$ %.
- 5.12 **Helium**, (CAS: 7440-59-7), for carrier gas, $w \geq 99,995$ %.
- 5.13 **Oxygen**, (CAS: 7782-44-7), for generating ozone in the detector, $w \geq 99,6$ %.

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6 Apparatus

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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.

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 $-20\text{ }^{\circ}\text{C}$.

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 $(21,1 \pm 0,1)$ g citric acid (5.6), $(25,6 \pm 0,1)$ g sodium hydrogen phosphate (5.7) and $(7,0 \pm 0,1)$ 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 $4\text{ }^{\circ}\text{C}$ when not in use. The buffer extraction solution is clear and colourless with a pH value of $(4,3 \pm 0,2)$.

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 $2,0\text{ }\mu\text{g NDHA ml}^{-1}$ dichloromethane. This will yield a mass concentration of about $1,0\text{ }\mu\text{g NDHA ml}^{-1}$ in the final dilution (assuming complete recovery), sufficient for detection on the chromatographic system. Store the internal standard at about $-20\text{ }^{\circ}\text{C}$ when not in use. [ISO 22303:2008](https://standards.iteh.ai/catalog/standards/sist/2178d447-9c00-4bac-915b-441c70195b/iso-22303-2008)

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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 $-20\text{ }^{\circ}\text{C}$.

Table 1 — Approximate standard concentrations in dichloromethane ($\mu\text{g}\cdot\text{ml}^{-1}$)

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.					