INTERNATIONAL STANDARD

ISO 21766

Second edition
2021-02

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

Reference number
ISO 21766:2021(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

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[7]. 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.

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

WARNING — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the applicability of any other restrictions prior to use.

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 ≥ 18,2 MΩ·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 C18[1)], 2,5 µm particle size, 2,1 mm × 50 mm, or equivalent.

6.3 Orbital shaker, wrist action shaker, or similar.

[1)] Waters XTerra® MS C18, 2,5 µm, 2,1 × 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 ± 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 ≥ 18,2 MΩ·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 TSNAs and deuterated internal standards in acetonitrile can be purchased at the required levels.