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Cigarettes — Determination of tobacco specific nitrosamines in mainstream cigarette smoke — Method using LC-MS/MS

Cigarettes — Dosage des nitrosamines spécifiques du tabac dans le courant principal de fumée de cigarette — Méthode par CL-SM/SM

Reference number
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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 19290:2016), 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 6.8) has been updated updated;

— the nomenclature of the deuterated nitrosamines in 6.1 to 6.8 has 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

Between 1999 and 2005, the CORESTA (www.coresta.org) Special Analytes Task Force studied the existing methodologies for the determination of Tobacco Specific Nitrosamines (TSNAs) in the mainstream smoke of cigarettes. Two main types of analytical methodologies had been proposed for this determination: GC-TEA (gas chromatography with a thermal energy analyser) and LC-MS/MS (liquid chromatography- tandem mass spectrometry). The Task Force decided in the first instance to develop a method using GC-TEA, because this methodology was the most widely used in laboratories at that time.

However, by 2009, it was ascertained that most laboratories applied an LC-MS/MS technique to measure yields of TSNAs. The Sub-Group (changed from Task Force) then investigated an LC-MS/MS method to complement the GC-TEA technique already available as CORESTA Recommended Method N° 63. Several such methods have been described in the literature and are referenced herein. A joint experiment was carried out in which 14 laboratories participated, using their in-house LC-MS/MS methodologies. The reproducibility data was better for LC-MS/MS than for GC-TEA and methodology was very similar across laboratories. In summary, cigarette mainstream smoke was collected on a Cambridge filter (CF) pad, an internal standard solution was added and, after extraction, an aliquot was separated and quantitatively analysed by LC-MS/MS. A general methodology was agreed, incorporating key learnings from the joint experiment.

This document was produced through a final collaborative experiment involving 20 laboratories from 12 countries. The method includes some notes to inform other laboratories that might wish to adopt it about some of the main features that need to be well controlled to provide data as robust and consistent as the repeatability and reproducibility data provided. Cigarettes were smoked using the smoking regime parameters given in ISO 3308 and statistical evaluations were made according to ISO 5725-2 recommendations.

No machine smoking regime can represent all human smoking behaviour:

— it is recommended that cigarettes also be tested under conditions of a different intensity of machine smoking than those specified in this document;

— machine smoking testing is useful to characterize cigarette emissions for design and regulatory purposes, but communication of machine measurements to smokers can result in misunderstandings about differences in exposure and risk across brands;

— smoke emission data from machine measurements may be used as inputs for product hazard assessment, but they are not intended to be nor are they valid as measures of human exposure or risks. Communicating differences between products in machine measurements as differences in exposure or risk is a misuse of testing using ISO standards.

Cigarettes — Determination of tobacco specific nitrosamines in mainstream cigarette smoke — 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 regulatory limitations prior to use.

1 Scope

This document specifies a method for the quantification of four tobacco specific nitrosamines (TSNAs) in the total particulate matter of mainstream cigarette smoke by using reversed phase high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS). The quantified TSNAs are: N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 3308, *Routine analytical cigarette-smoking machine — Definitions and standard conditions*

ISO 3402, *Tobacco and tobacco products — Atmosphere for conditioning and testing*

ISO 4387, *Cigarettes — Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine*

ISO 8243, *Cigarettes — Sampling*

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-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

[SOURCE: ISO 22303:2008, 3.1]

4 Principle

Cigarettes are smoked on a standard smoking machine. The mainstream smoke is trapped on a glass-fibre filter pad. After addition of an internal standard, the total particulate matter collected on the glass-fibre filter pad is extracted with 100 mM ammonium acetate solution using a shaker.

The extract is syringe filtered through a 0,45 µm PTFE column directly into an auto sampler vial.

The samples are subjected to reversed phase high performance liquid chromatography (HPLC) and quantified via tandem mass spectrometry (MS/MS).

5 Apparatus

Usual laboratory apparatus and equipment for use in preparation of samples and standards and in particular the following items. All glassware shall be cleaned before use to avoid any contamination.

5.1 Equipment needed to perform conditioning of cigarettes, in accordance with ISO 3402.

5.2 Equipment needed to perform marking for butt length of cigarettes.

5.3 Equipment needed to perform smoking of cigarettes, in accordance with ISO 3308.

5.4 Analytical balance, capable of measuring to at least four decimal places.

5.5 Centrifuge tubes, 50 ml.

5.6 Dispenser, of capacity 20 ml for extracting solutions.

5.7 Gas-tight syringes, of capacity 250 µl.

5.8 Automated volumetric pipette.

5.9 Shaker.

5.10 High performance liquid chromatograph coupled to tandem mass spectrometer (LC-MS/MS), consisting of:

5.10.1 Binary pump.

5.10.2 Autosampler.

5.10.3 Tandem mass spectrometer.

5.10.4 Data collection system.

5.10.5 LC column: XTerra MS C18®[1)], 2,5 µm, 2,1 mm × 50 mm or equivalent.

6 Reagents

Use only reagents of recognized analytical reagent grade.

1) XTerra MS C18 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 this product.

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

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

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

6.4 4-(N-Methylnitrosamino)-1-(3-pyridyl)-1-butanone, (NNK), CAS-No: 64091-91-4, w ≥ 98 % (mass fraction)

6.5 Deuterated (N-Nitrosonornicotine), (NNN-d4), CAS-No: 66148-19-4, w ≥ 98 %, isotopic purity w ≥ 99 %.

6.6 Deuterated (N-Nitrosoanatabine), (NAT-d4), CAS-No: 1020719-69-0, w ≥ 98 %, isotopic purity w ≥ 99 %.

6.7 Deuterated (N-Nitrosoanabasine), (NAB-d4), CAS-No: 1020719-68-9, w ≥ 98 %, isotopic purity w ≥ 99 %.

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

6.9 Ammonium acetate, $w \geq 97$ %.

6.10 Acetonitrile, HPLC grade.

6.11 Methanol, HPLC grade.

6.12 Acetic acid, $w \geq 99{,}77$ %.

6.13 Deionized water, >18,8 MΩ.

6.14 Syringe filter, 0,45 µm polytetrafluoroethylene (PTFE) or equivalent.

6.15 Disposable syringes, 5 ml.

6.16 Autosampler vials (amber), caps and PTFE faced septa.

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 could interfere with the analytical process are removed from the work area.

7.2 Preparation of solutions

7.2.1 Extraction solution, 100 mM ammonium acetate solution.

Weigh 15,4 g ± 0,05 g of ammonium acetate. Put into a 2 000 ml volumetric flask and dilute to the mark with deionized water.

7.2.2 HPLC mobile phase A, 0,1 % acetic acid solution in water.

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

7.2.3 HPLC Mobile Phase B, 0,1 % acetic acid solution in methanol.

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

NOTE Extraction solution and mobile phases are stable for up to three months at room temperature.

7.3 Preparation of standards

7.3.1 General

For the preparation of standard solutions volumetric pipettes should be used.

7.3.2 Preparation of internal standard solutions

7.3.2.1 Primary solution

Weigh, to the nearest 0,1 mg, approximately 10 mg each of NNN-d4, NAT-d4, NAB-d4 and NNK-d4.

Put 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 solution

Transfer 5 ml of each primary solution of NNN-d4, NAT-d4 and NNK-d4 and 1 ml of NAB-d4 into a 100 ml volumetric flask. Dilute to the mark with acetonitrile and mix well.

The concentration in this solution is approximately 50 µg/ml of NNN-d4, NAT-d4 and NNK-d4 and 10 µg/ml of NAB-d4.

7.3.2.3 Working solution

Transfer 50 ml of the combined secondary solution into a 500 ml volumetric flask. Dilute to the mark with acetonitrile and mix well.

The concentration in this solution is approximately 5 µg/ml of NNN-d4, NAT-d4 and NNK-d4 and 1 µg/ml of NAB-d4.

7.3.3 Preparation of calibration standard solutions

7.3.3.1 Primary single TSNA solutions

Weigh, to the nearest 0,1 mg, approximately 10 mg each of NNN, NAT, NAB and NNK.

Put 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 TSNAs stock solution (I)

Transfer 4 ml of the primary single TSNA solutions of NNN, NAT and NNK and 1 ml of the primary single TSNA solution of NAB into a 100 ml volumetric flask. Dilute to the mark with acetonitrile and mix well.

The concentration in this solution is approximately 40 µg/ml of NNN, NAT and NNK and 10 µg/ml of NAB.

7.3.3.3 Mixed TSNAs stock solution (II)

Transfer 2 ml of the mixed TSNAs stock solution (I) into a 200 ml volumetric flask. Dilute to the mark with acetonitrile and deionized water mixed solution (30:70 volume fraction) and mix well.

The concentration in this solution is approximately 400 ng/ml of NNN, NAT and NNK and 100 ng/ml of NAB.

7.3.3.4 Working standard solutions

Prepare 7 working standard solutions that cover the concentration range of interest.

Add selected volumes of solutions listed in Table 1 in a 100 ml volumetric flask and dilute to the mark with deionized water.

These solutions have concentrations of approximately 50 ng/ml of NNN-d4, NAT-d4 and NNK-d4, 10 ng/ml of NAB-d4, from 0 ng/ml to 80 ng/ml of NNN, NAT and NNK and from 0 ng/ml to 20 ng/ml of NAB (see Table 2).

Each laboratory should establish the most suitable calibration range depending on the equipment used and the type of samples to be analysed. The standard preparation procedure is given as an example and is applicable for the range of the products in a collaborative study.

Table 1 — Preparation of working standard solutions for calibration

Solutions	S0 ml	S1 ml	S2 ml	S3 ml	S4 ml	S5 ml	S6 ml
Internal standard solution	1	1	1	1	1	1	1
Mixed TSNAs stock solution (II)	0	0,5	1	2	5	10	20
Ammonium acetate (100 mM)	10	10	10	10	10	10	10
Acetonitrile	10	10	10	10	8	7	4
Final volume	100	100	100	100	100	100	100

Table 2 — Concentration of each calibration standard

Concentrations	S0 ng/ml	S1 ng/ml	S2 ng/ml	S3 ng/ml	S4 ng/ml	S5 ng/ml	S6 ng/ml
NNN	0	2	4	8	20	40	80
NAT	0	2	4	8	20	40	80
NAB	0	0,5	1	2	5	10	20
NNK	0	2	4	4	20	40	80
NNN-d4	50	50	50	50	50	50	50
NAT-d4	50	50	50	50	50	50	50
NAB-d4	10	10	10	10	10	10	10
NNK-d4	50	50	50	50	50	50	50