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Cigarettes — Determination of tobacco specific nitrosamines in mainstream cigarette smoke with an intense smoking regime — Method using LC-MS/MS

Cigarettes — Dosage des nitrosamines spécifiques du tabac dans le courant principal de la fumée de cigarette avec un régime de fumage intense — Méthode par CL-SM/SM

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Foreword

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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).

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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*.

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

In 2009 the CORESTA (www.coresta.org) Special Analytes Sub-Group focused on the development of a method for the determination of Tobacco Specific Nitrosamines (TSNAs) in mainstream cigarette smoke. The Sub-Group investigated a liquid chromatography- tandem mass spectrometry (LC-MS/MS) method to complement the gas chromatography with a thermal energy analyser (GC-TEA) technique already available as CORESTA Recommended Method N° 63. Several LC-MS/MS methods have been described in the literature and are referenced herein[3][4]. 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, mainstream cigarette smoke was collected on a glass fibre filter 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 CORESTA collaborative experiment, conducted in 2011, involving 20 laboratories from 12 countries[5][6]. Cigarettes were smoked with the intense smoking regime specified in Health Canada Official Method T-115 (equivalent to ISO 20778) and statistical evaluations were made according to the recommendations provided in ISO 5725[1].

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 International Standards.

Cigarettes — Determination of tobacco specific nitrosamines in mainstream cigarette smoke with an intense smoking regime — 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 the total particulate matter of cigarette mainstream smoke with the intense smoking regime specified in ISO 20778 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 3402, *Tobacco and tobacco products — Atmosphere for conditioning and testing*

ISO 8243, *Cigarettes — Sampling*

ISO 20778, *Cigarettes — Routine analytical cigarette smoking machine — Definitions and standard conditions with an intense smoking regime*

ISO 20779, *Cigarettes — Generation and collection of total particulate matter using a routine analytical smoking machine with an intense smoking regime*

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 routine analytical cigarette smoking machine according to ISO 20778. 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 syringe filter directly into an autosampler vial.

The samples are quantitated by LC-MS/MS.

5 Apparatus

In addition to the list provided below, usual laboratory apparatus and equipment are needed for preparation of samples and standards. All glassware shall be cleaned before use to avoid any contamination.

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

5.2 Extraction container, of approximately 50 ml.

5.3 Dispenser, of capacity 20 ml.

5.4 Gas-tight syringes, of capacity 250 µl.

5.5 Mechanical volumetric pipette.

5.6 Shaker.

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

5.7.1 Binary pump.

5.7.2 Autosampler.

5.7.3 Tandem mass spectrometer.

5.7.4 Data collection system.

5.7.5 LC column: Waters XBridge BEH C18®[1)], 2,5 µm, 2,1 mm × 50 mm or equivalent.

6 Reagents

Use only reagents of recognized analytical reagent grade.

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).

1) Waters XBridge BEH 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.

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

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

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* ≥ 97 % (mass fraction).

6.10 Acetonitrile, HPLC grade.

6.11 Methanol, HPLC grade.

6.12 Acetic acid, *w* ≥ 99,7 %.

6.13 De-ionized water, ≥ 18,2 MΩ·cm.

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 15,40 g ± 0,05 g of ammonium acetate. Put into a 2 000 ml volumetric flask and dilute to the mark with de-ionized water.

7.2.2 HPLC mobile phase A, 0,1 % (volume fraction) acetic acid solution in water.

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

7.2.3 HPLC Mobile Phase B, 0,1 % (volume fraction) 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 have been shown to be stable for up to three months when stored 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 internal standard 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 internal standard 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 internal standard 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 de-ionized 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 de-ionized 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

7.3.3.5 Storage

The above standard solutions have been shown to be stable for up to six months if refrigerated below 5 °C. Stability of all standard solutions shall be assessed by each laboratory.