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Footwear — Critical substances potentially present in footwear and footwear components — Determination of Nitrosamines

Chaussure — Substances critiques potentiellement présentes dans les chaussures et les composants de chaussure — Détermination des nitrosamines

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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This document was prepared by Technical Committee ISO/TC 216, *Footwear.*

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.

Footwear — Critical substances potentially present in footwear and footwear components — Determination of Nitrosamines

WARNING — The use of this document can involve hazardous materials, operations and equipment. It does not purport to address all of the safety or environmental problems associated with its use. It is the responsibility of users of this document to take appropriate measures to ensure the safety and health of personnel and the environment prior to application of the document.

1 Scope

This document specifies a method for the determination of the content of 12 kinds of Nitrosamines (see Annex A) in footwear and footwear components by using solvent extraction and Gas chromatography with mass selective detector (GC-MS).

This document is applicable to rubber in footwear materials.

NOTE ISO/TR 16178 defines which materials are concerned by this determination.

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 4787, *Laboratory glassware — Volumetric instruments — Methods for testing of capacity and for use*

3 Terms and definitions

No terms and definitions are listed in this document.

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/

4 Principle

Extract Nitrosamines in the sample with methanol using an ultrasonic bath. The extract is concentrated in a rotary vacuum evaporator and purified by passing through C_{18} solid-phase separation column. The Nitrosamines in test solutions are analysed by GC-MS, using full scan detection mode for qualitative analysis and selected ion monitoring (SIM) mode for quantitative analysis with an external standard solution.

5 Reagents and materials

Unless otherwise specified, all the reagents used are chromatographic grade.

5.1 Methanol, CAS number: 67-56-1.

5.2 C_{18} solid-phase extraction column, 500 mg/3 ml freshly made in lab or obtained from commercial sources.

5.3 Standard stock-solution, prepare stock solutions containing 200 mg/l of each Nitrosamine (see Annex A) in methanol (5.1) respectively. These solutions should be stored in amber glassware in a refrigerator at (−18 ± 3) °C. Under these conditions, the solutions can be used for 15 days.

NOTE Nitrosamines are susceptible to UV degradation, so extract and standard solutions cannot be exposed to sunlight or fluorescent light sources. Test solutions or standard solutions can be wrapped in aluminium foil or be stored in amber glassware in the dark at (−18 ± 3) °C.

5.4 Standard working-solutions, freshly prepared by mixing standard stock-solutions (5.3) and diluting to 10 mg/l with methanol (5.1). This solution should be stored in the absence of light at (−18 ± 3) °C.

5.5 Internal standard for chromatographic analysis, see 8.3.2.

6 Apparatus

The usual laboratory apparatus and amber laboratory glassware, according to ISO 4787, shall be used, in addition to the following.

6.1 Analytical balance, with a readability of at least 0,1 mg.

6.2 Conical flask, amber, which can be tightly sealed, (e.g. volume of 100 ml).

6.3 Ultrasonic bath, with the working frequency of 40 kHz ± 5 kHz.

6.4 Round-bottomed flask, amber, (e.g. volume of 25 ml, 100 ml).

6.5 Rotary vacuum evaporator or Nitrogen evaporator, with adjustable temperature, suitable for operation up to 40 °C.

6.6 Vortex mixer.

6.7 Volumetric flask, amber, (e.g. volume of 2 ml, 5 ml).

6.8 Solid phase extraction device, with a vacuum pump.

6.9 Centrifuge tube, amber, (e.g. volume of 2 ml).

6.10 Centrifuge, 2 000 × *g*.

6.11 Syringe filter, 0,45 µm, for example PTFE.

6.12 GC vial, amber, (e.g. volume of 2 ml).

6.13 Gas chromatograph with a mass selective detector (GC-MS).

Other chromatographic technics may be used, provided that they have been validated for this analysis.

7 Preparation of test samples

Take a representative sample, cut into piece of less than 3 mm of length with appropriate tools.

8 Procedure

WARNING — Most Nitrosamines are potent carcinogens and every possible precaution shall be taken to avoid human exposure.

8.1 Extraction

Weigh 5,0 g (accurate to 0,01 g) samples into a conical flask (6.2). Add 30 ml of methanol (5.1) and extract in an ultrasonic bath (6.3) for (30 ± 2) min at room temperature. The temperature shall not exceed 40 °C. If the test sample is not sufficiently immersed in the extraction solvent, add more solvent and report the final volume for calculation of Nitrosamines amount.

Filter the extract into a round-bottomed flask (6.4).

Add 20 ml of methanol (5.1) into the conical flask (6.2) and repeat extraction again and combine the extracts.

Concentrate the methanol extract to less than 2 ml with rotary vacuum evaporator at 40 °C in a vacuum of 21,3 kPa to 16,3 kPa or nitrogen evaporator (6.5).

Remove the round-bottomed flask (6.4) from the evaporator (6.5) and shake vigorously with the vortex mixer (6.6) for 1 min (so that the adhesive substance stuck to the bottle wall can be dissolved in methanol).

After evaporation, transfer the remaining liquid and the residue quantitatively into a 5 ml amber volumetric flash (6.7) by using methanol (5.1) as rinsing solvent. Fill up the flask to the mark with methanol (5.1).

8.2 Purification

Pre-rinse the C_{18} column (5.2) with methanol (5.1). Accurately transfer 2,0 ml of the test solution (see 8.1) into the C_{18} column (5.2) and collect eluate. When the solution drains off (the surface of sample solution drops to filler's surface), add 2 ml of methanol (5.1) into C_{18} column (5.2) twice. Collect and combine all the eluate in a round-bottomed flask (6.4).

Concentrate this solution to approximately 1 ml (not to dryness) in a rotary vacuum evaporator or nitrogen evaporator (6.5) at no more than 40 °C. Then remove the remainder of methanol to dryness by a slow flow of inert gas. Dissolve the residue with methanol (5.1) and transfer it to an amber volumetric flask (6.7) and make up the volume with methanol (5.1).

Transfer this solution into an amber sample vial (6.12) after filtration with 0,45 µm syringe filter (6.11) and perform chromatographic analysis. If necessary (viscous solution), centrifuge this solution in centrifuge tube (6.9) before transferring.

8.3 Chromatographic analysis

8.3.1 The chromatography parameters for GC-MS

See an example in Annex B.

8.3.2 Qualitative and quantitative analysis by GC-MS

Inject an aliquot of standard working solution (5.4) and the test solution (see 8.2) separately into the column for determination of Nitrosamines. Analyse qualitatively by external standard method through selected characteristic ions (see Table A.1).

Within the linear range use at least five measurements at different concentrations. Example for calibration solutions, see Table 1.

Table 1 — Calibration solutions (examples)

Standard	L1	L2	L3	L4	L5
Volume of the standard working-solution (µl) (5.4)	50	100	250	500	1 000
Volume of solution (µl) (5.1)	950	900	750	500	0
Concentration of Nitrosamine (mg/l)	**0,5**	**1,0**	**2,5**	**5,0**	**10,0**

If the response value of test solution is out of the linear range of the detector, dilute the solution appropriately before measurement.

NOTE Under the above analysis conditions, GC-MS total ion chromatogram of 12 kinds of Nitrosamine standards is given in Annex C.

For N-nitroso-N-methylaniline, N-nitroso-N-ethylaniline and N-nitroso-diphenylamine, those decompose at injector temperature. If peaks of their characteristic ions in the chromatogram are found, these Nitrosamines shall be confirmed according to Annex D and E and reported in test report.

This analysis should also be performed using an internal standard for the quantification. The following two been proved as usable:

— N-nitroso-N,N,-dimethylamine-d6, CAS number: 17829-05-9;

— N-nitroso-N,N,-di-n-propylamine-d14, CAS number: 93951-96-3.

9 Expression of results

9.1 Calculation of results

The content of each Nitrosamine in the sample is calculated by Formula (1):

$$X_i = \frac{A_i \times c_i \times V}{A_{is} \times m} \quad (1)$$

where

X_i is the content of Nitrosamine *i* in the sample;

A_i is the peak area of Nitrosamine *i* in the test solution, in area units;

A_{is} is the peak area of Nitrosamine *i* in the standard working solution, in area units;

c_i is the concentration of Nitrosamine *i* in the standard working solution, in µg/ml;

V is the volume of the test solution (see 8.1), in ml;

m is mass of the sample (see 8.1), in g.

Results are rounded to 0,1 mg/kg. When the result exceeds 100 mg/kg, express the result to the nearest 1 mg/kg.

For determination using internal standard method. Set up the linear regression function by using the following ratio (A_i/A_{is}) and (C_i/C_{is}) with the help of Formula (2):

$$\frac{A_i}{A_{is}} = a \times \frac{C_i}{C_{is}} + b \quad (2)$$

where

A_i is the peak area of Nitrosamine *i* in the test solution, in area units;

A_{is} is the peak area of internal standard in the working solution, in area units;

c_i is the concentration of Nitrosamine *i* in the standard working solution, in µg/ml;

c_{is} is the concentration of internal standard in the working solution, in µg/ml

a is the slope of the linear function;

b is the ordinate intercept of the calibration curve. The units depend of the evaluation.

9.2 Limit of Quantification

This method is able to detect the Nitrosamines listed in Table A.1 with a limit of quantification of 0,5 mg/kg or lower. Results below 0,5 mg/kg should be reported as below limit of quantification (LOQ).

See results of the inter-laboratories tests in Annex F.

10 Test report

The test report shall include at least the following information:

a) a reference to this document, i.e. ISO 19577:2019;

b) all information necessary for complete identification of the sample tested;

c) test results;

d) information on confirmation and method used;

e) any deviation from this document.

Annex A
(informative)

Names of 12 kinds of Nitrosamines and the Standard selective GC-MS ions

Names of 12 kinds of Nitrosamines and the Standard selective ions see Table A.1.

Table A.1 — Names of 12 kinds of Nitrosamines and the Standard selective GC-MS ions

№	Substance	CAS No.	Molecular formula	Quantitative ion	Characteristic ions
1	N-nitroso dimethylamine	62-75-9	$C_2H_6N_2O$	74	74, 42, 43
2	N-nitroso methyl-ethylamine	10595-95-6	$C_3H_8N_2O$	88	88, 42, 56
3	N-nitroso diethylamine	55-18-5	$C_4H_{10}N_2O$	102	102, 42 56
4	N-nitroso pyrrolidine	930-55-2	$C_4H_8N_2O$	100	100, 41, 42
5	N-nitroso-N-methylaniline	614-00-6	$C_7H_8N_2O$	106	106, 107, 77
6	N-nitroso morpholine	59-89-2	$C_4H_8N_2O_2$	56	56, 86, 116
7	N-nitroso dipropylamine	621-64-7	$C_6H_{14}N_2O$	70	70, 43, 130
8	N-nitroso piperidine	100-75-4	$C_5H_{10}N_2O$	114	114, 42, 55
9	N-nitroso-N-ethylaniline	612-64-6	$C_8H_{10}N_2O$	106	106, 121, 77
10	N-nitroso dibutylamine	924-16-3	$C_8H_{18}N_2O$	84	84, 57, 41
11	N-nitroso-diphenylamine	86-30-6	$C_{12}H_{10}N_2O$	169	168, 169, 51
12	N-nitroso dibenzylamine	5336-53-8	$C_{14}H_{14}N_2O$	91	91, 65, 226
Other Nitrosamines may be analysed with this document, provided that they have been validated with this test method. In particular, their stability during the analysis shall be considered.					

Annex B
(informative)

The chromatography parameters for GC-MS

The test results depend on the equipment used, so it is impossible to provide the general parameters for chromatographic analysis. Operating parameters given below, as an example, have been proved to be feasible:

a) capillary column: DB-5MS 30 m × 0,25 mm × 0,25 µm, or equivalent;

b) temperature program: $38\,°C\,(4\ min) \xrightarrow{8\,°C/min} 83\,°C\,(4\ min) \xrightarrow{15\,°C/min} 300\,°C\,(6\ min)$;

c) injector temperature: 200 °C;

d) MS transfer line temperature: 280 °C;

e) carrier gas: helium (purity ≥99 %), flow rate: 1,0 ml/min;

f) ionization mode: EI;

g) ionization energy: 70 eV;

h) mass scan range: 35 m/z to 260 m/z;

i) injection mode: Splitless;

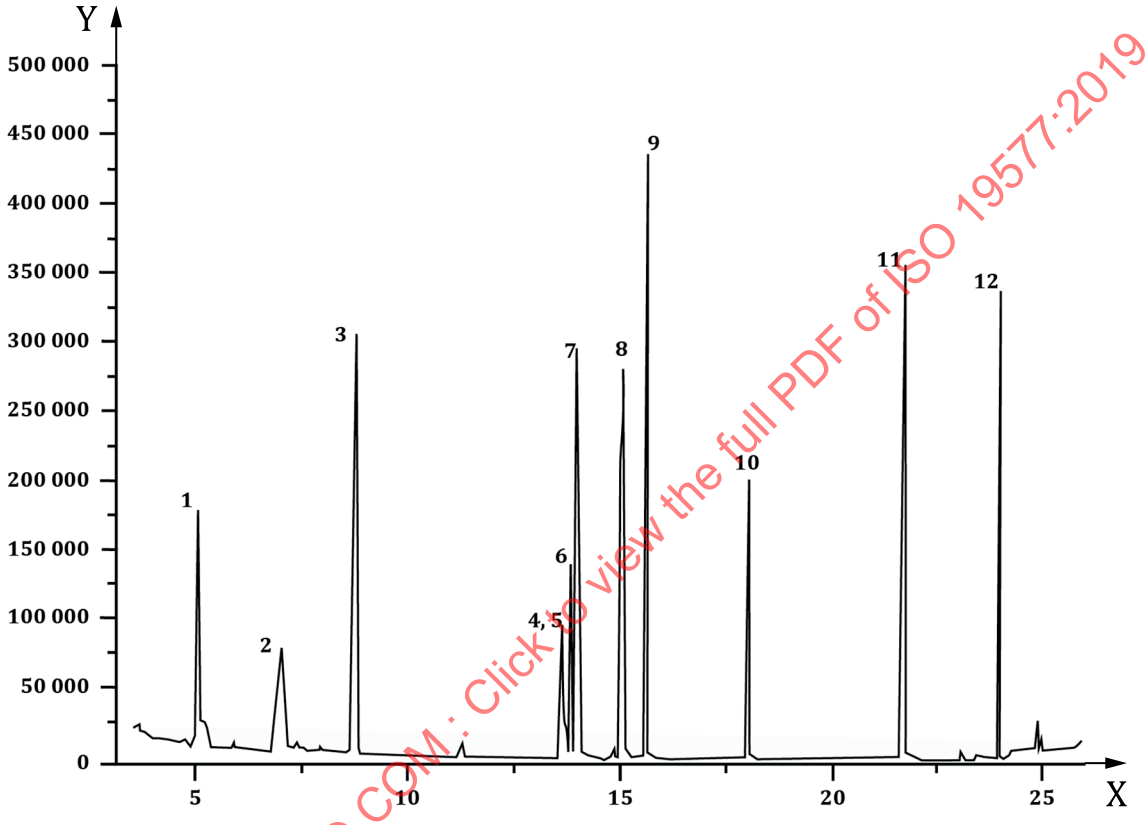
j) injection volume: 1 µl;

k) detection mode: Scan and SIM.

Annex C
(informative)

GC-MS total ion current of Nitrosamine standard sample

GC-MS total ion current of Nitrosamine standard sample see Figure C.1.



Key

X-axis	Retention time, min
Y-axis	Abundance
1	62-75-9
2	10595-95-6
3	55-18-5
4	621-64-7
5	614-00-6
6	612-64-6
7	930-55-2
8	59-89-2
9	100-75-4
10	924-16-3
11	86-30-6
12	5336-53-8

Figure C.1 — GC-MS total ion current of Nitrosamine standard sample

Annex D
(normative)

Confirmation of detected Nitrosamines

D.1 General

Analysis of Nitrosamines using GC-MS is relatively simple and does not require complex procedures and expensive spectrometers. Most abundant mass spectral peaks are the molecular ion $[M]^{+\bullet}$ and the protonated molecular fragment ion $[M+H]^{+}$ in the electron impact ionization mode. However, for N-nitroso-N-methylaniline, N-nitroso-N-ethylaniline and N-nitroso-diphenylamine, that decompose at the injector temperature, the molecular and protonated molecular fragment ions of the product N-methylaniline, N-ethylaniline and diphenylamine are detected. Consequently, whenever N-methylaniline, N-ethylaniline and diphenylamine are found in a sample, further analysis should be performed to confirm corresponding Nitrosamines in at least one of the following ways.

D.2 Photodecomposition

Nitrosamines are confirmed via their susceptibility to UV rays. Purify another 2,0 ml of the remaining extraction solution (see 8.1) according to 8.2. Transfer the test solution into a UV transparent sample vial. The test vial is placed parallel to a high-pressure mercury lamp (λ_{max} = 365 nm) and subjected to the irradiation of UV rays. After sufficient illumination (at least 3 h), perform GC-MS analysis. The variation of peaks in chromatogram is applied for confirmation of the identity of detected Nitrosamines. As an effectiveness control, the UV irradiation of a similarly standard solution under the same conditions is also investigated.

Compare with the initial chromatogram without exposing to UV irradiation.

If the peaks corresponding to Nitrosamines disappear or the intensity of peaks is decreased significantly after UV irradiation, the presence of Nitrosamines is confirmed.

If the intensity of peaks is not decreased significantly after following the above procedures, this indicates that the initial peaks did not correspond to a Nitrosamine, and it shall be deemed as a false positive.

Annex E
(normative)

Alternative methods

E.1 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is recommended for the qualitative confirmation of Nitrosamines. After confirmation, determine quantitatively corresponding Nitrosamines with GC-MS.

HPLC conditions shall be suitable to generate sufficient retention time for Nitrosamines. The following HPLC conditions have been found suitable for the determination of Nitrosamines when using DAD detectors or equivalent:

Chromatographic column:	C_{18} 250 mm × 4,6 mm × 5,0 µm, or equivalent
Mobile phases:	Acetonitrile in Water (60/40)
Column temperature:	40 °C
Injection volume:	20 µl
Gradient	Isometric elution for 15 min

E.2 Gas Chromatography with Thermal Energy Analyzer (GC-TEA)

Examine Nitrosamines by gas chromatography employing a chemiluminescence detector (Thermal Energy Analyzer). The TEA detector is very sensitive to confirm the structure of Nitrosamines.

EN 12868:2017 provides further information on employing TEA detection.

E.3 Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS)

Liquid chromatography using tandem mass spectrometry (LC-MS/MS) has also been applied to determine Nitrosamines.

EN 71-12:2016 provides suitable sets of conditions for the testing of Nitrosamines.