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Child use and care articles — Method for determining the release of N-nitrosamines and N-nitrosatable substances from elastomer or rubber teats and soothers

bsi.

National foreword

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Artikel für Säuglinge und Kleinkinder - Verfahren zur Bestimmung der Abgabe von N-Nitrosaminen und N-nitrosierbaren Stoffen aus Babysaugern aus Elastomeren und Gummi

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European foreword

This document (EN 12868:2017) has been prepared by Technical Committee CEN/TC 252 “Child use and care articles”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2017, and conflicting national standards shall be withdrawn at the latest by July 2017.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 12868:1999.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

Compared to EN 12868:1999, this document contains the following significant changes:

— The common practice to perform at least double determinations has been made a requirement, including the preparation of samples.

— Sample preparation has been defined and simplified, reducing this source of interlaboratory variation.

— The pre-boiling and migration steps have been separated for the determination of N-nitrosamines and of N-nitrosatable substances, allowing use of the same vessels and avoiding the possible loss of migrated substances. Amounts of sample have been adjusted, increasing the sample mass for the determination of N-nitrosatable substances.

— Extraction of N-nitrosamines from the aqueous migrates has been restricted to one method, reducing interlaboratory variability. A rinsing step has been introduced to avoid variability due to possible loss of analytes.

— The calculation of results has been revised including a repeatability requirement for multiple determinations and taking into account state of the art analytical procedures.

— The confirmation of N-nitrosamines and application of analytical tolerances have been clarified including a N-nitrosamine specific adjustment as suggested by the validation trial.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

It has been shown that feeding teats and soothers made of elastomer or rubber may release N-nitrosamines and substances capable of being converted into N-nitrosamines (N-nitrosatable substances). The Scientific Committee for Food of the European Union has given the opinion that N-nitrosamines and N-nitrosatable substances may endanger human health owing to their toxicity [5]. Hence in 1993, the European Commission issued Directive 93/11/EEC [1] controlling rubber and elastomeric teats and soothers releasing these substances. The Directive also provided basic rules for determining the release of these substances and criteria for the method of analysis to be adopted.

The purpose of this European Standard is to provide a detailed analytical method for the identification and determination of N-nitrosamines and N-nitrosatable substances released from teats and soothers in order that compliance with the requirements of Directive 93/11/EEC may be tested.

This method has been validated.

The testing laboratories should take special care to observe occupational health and safety standards. Persons using this European Standard should be familiar with normal laboratory practice. This European Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This European Standard specifies the method for determining N-nitrosamines and N-nitrosatable substances released from elastomer or rubber teats in contact with artificial saliva salt solution for testing compliance with Directive 93/11/EEC.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

EN ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
teat
flexible elastomeric part designed to be placed in the mouth

3.2
soother
article which includes a teat and which is intended to satisfy the non-nutritive sucking need of children

Note 1 to entry: Soothers are also known as pacifiers or babies' dummies.

3.3
feeding teat
any teat that permits a child to obtain food or drink

3.4
elastomer
material which undergoes substantial, elastic (fully reversible) deformation when put under stress and consisting of three-dimensional networks of cross-linked flexible polymers

Note 1 to entry: The cross-links are chemical bonds generated by curing in rubbers (like natural rubber or synthetic rubber including silicones) or physical, thermo-reversible fixation points in thermoplastic elastomers (TPE) or the combination of both (TPEV).

3.5
rubber
types of elastomer

3.6
N-nitrosamine
substance characterised by the N-nitroso functional group, N-NO, usually formed by the reaction of an amine with a nitrosating agent, e.g. nitrite, at acidic pH

3.7
N-nitrosatable substance
substance which when released into the artificial saliva salt solution (5.5) and submitted to the conditions of step 8.3.3 undergoes nitrosation to form a N-nitrosamine

3.8
N-nitroamine
substance characterized by the N-nitro functional group bonded to an amine, N-NO_2, also called N-nitramine

3.9
ready to use product
product intended to be used without the need to clean before first use, but may be reusable

4 Principle

N-nitrosamines and N-nitrosatable substances are migrated into a nitrite-containing artificial saliva salt solution under specified conditions. Two migrations are carried out: migration A for the determination of N-nitrosatable substances (determined as N-nitrosamines from migrate A after nitrosation) and migration B for the determination of N-nitrosamines (from migrate B). After extraction from the migrate and concentration, the concentrates are examined for N-nitrosamines by gas chromatography employing a chemiluminescence detector (TEA). The N-nitrosamines and N-nitrosatable substances released are expressed as N-nitrosamines in milligram per kilogram of the sample.

5 Reagents

Unless otherwise specified, all chemicals shall be of analytical grade and free of N-nitrosamines and N-nitrosatable substances (see 8.8).

5.1 Distilled water, or water of equivalent purity conforming to at least grade 3 of EN ISO 3696.

5.2 Ammonia hydroxide aqueous solution, CAS 1336-21-6, c(NH_4OH) = 0,1 mol/l.

5.3 Hydrochloric acid (CAS 7647-01-0).

5.3.1 Aqueous solution, c(HCl) = 0,1 mol/l.

5.3.2 Aqueous solution, c(HCl) = 1,0 mol/l.

5.4 Sodium hydroxide (CAS 1310-73-2).

5.4.1 Aqueous solution, c(NaOH) = 0,1 mol/l.

5.4.2 Aqueous solution, c(NaOH) = 1,0 mol/l.

5.5 Artificial saliva salt solution.

Table 1 — Salts and their masses for 1 l of artificial saliva salt solution

Salts	CAS	Mass (g) [a]
Sodium hydrogen carbonate	144–55–8	4,2 ± 0,021
Sodium chloride	7647–14–5	0,5 ± 0,0025
Potassium carbonate	584–08–7	0,2 ± 0,001
Sodium nitrite	7632–00–0	0,03 ± 0,001
[a] Tolerances are ± 0,5 % of the mass, except for the sodium nitrite		

Prepare the artificial saliva salt solution by dissolving the salts given with the appropriate masses in Table 1 in (950 ± 5) ml of water (5.1).

The artificial saliva salt solution shall have a pH of (9,0 ± 0,1). If necessary adjust by adding 0,1 molar hydrochloric acid solution (5.3.1) or 0,1 molar sodium hydroxide solution (5.4.1) drop by drop. Transfer into a 1 l volumetric flask and dilute to the mark with water (5.1).

The artificial saliva salt solution has limited stability and shall not be used after more than 5 days.

5.6 **Ethanol** (CAS 64-17-5), **absolute**

5.7 **Dichloromethane** (CAS 75-09-2)

5.8 Glass wool, washed with the dichloromethane (5.7)

5.9 Diatomaceous earth

NOTE 1 Examples for suitable diatomaceous earth are Extrelute® or Toxelut® pH9,0 or Chromabond XTR®.

NOTE 2 To remove any N-nitrosamines the diatomaceous earth can be heated for 1 h to 200°C, cooled and washed with dichloromethane (5.7), or can be calcined, e.g. for 4 h at 550 °C.

5.10 Sea sand, acid washed and calcined

5.11 Purified nitrogen

5.12 Boiling chips

6 Apparatus

6.1 Normal laboratory apparatus.

Amber glassware and / or glassware protected from light by wrapping in aluminium foil shall be used to avoid degradation of N-nitrosamines.

To avoid loss of N-nitrosamines or N-nitrosatable substances, flasks shall be closed with ground glass stoppers.

The migration flasks shall be treated with ammonia solution (5.2), rinsed with water (5.1) and dried, prior to use in the tests.

NOTE This is to avoid uncontrolled nitrosation which could result from direct contact of the sample with acidic surfaces

6.2 **Oven**, maintained at a temperature of (40 ± 2) °C.

6.3 Columns for solid phase extraction (SPE columns).

Columns shall have capacities which when prepared in accordance with 8.5 allow the complete absorption of the entire amount of aqueous migrate (see 8.6).

NOTE 1 Suitable columns are normally glass columns with a length of approximately 450 mm and an internal diameter of (18 ± 2) mm or of length of approximately 300 mm and an internal diameter of (26 ± 2) mm. Columns may be equipped with a stoppered outlet (e.g. polytetrafluoroethylene stopper) to help adjusting the eluent flow.

NOTE 2 Alternatively to self-prepared columns, suitable pre-filled SPE columns for single use are commercially available. They can be used provided that they are free from N-nitrosamines and of sufficient capacity.

6.4 Sintered glass frits for columns (6.3).

6.5 Evaporator flask, glassware for concentration step (8.7) or any suitable automated evaporator capable to reduce the volume of Extract A or B (60 ml or above, see 8.6) to (0,9 ± 0,1) ml.

6.6 Water bath, capable of maintaining temperatures in the range 40 °C to 60 °C.

6.7 GC glass vials with septa free from N-nitrosamines.

6.8 UV lamp suitable for confirmation of N-nitrosamines according to 10.1 a). Illumination of the highest concentration of the calibration solutions in a UV transparent vial within a reasonable time (1–3 h) shall degrade N-nitrosamines and significantly decrease the intensity of their peaks.

NOTE Longwave (365 nm) UV lamps have been shown to significantly decrease the intensity of peaks from the highest concentrated calibration standard solution, but midrange and shortwave UV lamps are generally also suitable.

6.9 UV transparent glass vials to be used for confirmation of N-nitrosamines according to 10.1 a).

6.10 pH-meter with minimum (±0,2) pH-relative accuracy.

6.11 Chemiluminescence detector (Thermal Energy Analyser, TEA, see A.7).

6.12 Gas Chromatography (GC).

The GC system shall separate the N-nitrosamines named in this standard, such that their peak areas can be compared with that due to the internal standard (see 7.4). It shall also separate N-nitroamines (3.8) from the named N-nitrosamines.

Should other than the N-nitrosamines named in this standard (Table 2) be found, the GC system shall be adaptable for their separation and identification.

N-nitrosamines which cannot be identified shall be confirmed according to 10.1 a) and be quantified and reported as given in 9.1.

NOTE Annex B provides an example for gas chromatography settings suitable to obtain the required separations.

7 Standard Solutions of N-nitrosamines

7.1 General

WARNING — Owing to their toxicity, some N-nitrosamines can be detrimental to human health. After use, the apparatus which has come into contact with N-nitrosamines should be carefully treated to destroy remains of N-nitrosamines, for example by rinsing with 15 % HBr (CAS 10035-10-6) / glacial acetic acid (CAS 64-19-7), UV light exposure or other suitable methods.

The concentration of N-nitrosamine standard solutions may change during storage due to UV-light, evaporation and/or adsorption. Therefore, amber glassware and/or glassware protected from light by wrapping in aluminium foil as well as ground glass stoppers shall be used. Any solutions shall be homogenized by rigorously shaking the containers before any liquid is transferred (some N-nitrosamines and N-nitrosatable substances adsorb at the internal surfaces of the vessels).

Certified N-nitrosamine standards and their mixtures can be purchased from several suppliers. Certification shall include storage and stability information as well as purity which shall be taken into account when calculating the quantities for the standard solutions.

Stock solutions (approximately 1 mg/ml) of N-nitrosamines shall be in ethanol (5.6) and stored in a freezer.

Internal standard solution and calibration standard solutions (7.4 and 7.3) shall be stored at temperature below 5°C in the dark and can be used for a maximum of 2 weeks.

7.2 N-nitrosamines identified in teats

The N-nitrosamines listed in Table 2 and / or the respective N-nitrosatable substances have been identified in teats and are relevant for testing and for calibration standards.

Table 2 — Names, abbreviated names and CAS numbers of N-nitrosamines relevant for this standard, and the necessary limits of quantification

Name	Abbreviated Name	CAS	LOQ (mg/kg)
N-nitrosodimethylamine	NDMA	62–75–9	0,001
N-nitrosodiethylamine	NDEA	55–18–5	0,001
N-nitrosodipropylamine	NDPA	621–64–7	0,001
N-nitrosodiisobutylamine	NDiBA	997–95–5	0,001
N-nitrosodibutylamine	NDBA	924–16–3	0,001
N-nitrosopiperidine	NPIP	100–75–4	0,001
N-nitrosopyrrolidine	NPYR	930–55–2	0,001
N-nitrosomorpholine	NMOR	59–89–2 or 67587–56–8	0,001
N-nitroso N-ethyl N-phenylamine	NEPhA	612–64–6	0,005
N-nitroso N-methyl N-phenylamine	NMPhA	614–00–6	0,005
N-nitroso-N,N-di(3,5,5-trimethylhexyl)amine also known as N-nitrosodiisononylamine	NDiNA	1207995–62–7	0,005
N-nitrosodibenzylamine	NDBzA	5336–53–8	0,005
NOTE N-nitrosamines are listed in order of elution as shown in Figure B.1			

The limits of quantification (LOQ) given in Table 2 shall be achieved by the laboratory.

NOTE N-nitrosodiethanolamine (NDELA, CAS 1116–54–7) has not yet been observed in baby teats. The analysis of NDELA according to this standard requires derivatisation [2].

7.3 Calibration solutions (for detector response)

Calibration solutions shall be prepared by mixing known amounts of the N-nitrosamines to be tested (Table 2). Alternatively, stock solutions of certified mixtures of these N-nitrosamines may be used.

A minimum of 4 different calibration levels with concentrations covering the range from 5 ng/ml to 500 ng/ml for each of the contained N-nitrosamines shall be prepared by dilution with ethanol (5.6) and addition of internal standard solution (7.4).

A calibration blank solution and each of the above calibration levels shall contain approximately 100 ng/ml of the internal standard (7.4).

NOTE For each calibration level e, concentrations of the internal standard and of the individual N-nitrosamines in the solution are recorded as $C_{int,\,e}$ and $C_{i,\,e}$, see 8.9.1

7.4 N-nitrosamine used as internal standard

N-nitrosodiisopropylamine (NDiPA, CAS 601-77-4) or a suitable alternative can be used as internal standard. The internal standard solution shall be free from other N-nitrosamines, and have a concentration of approximately 200 ng/ml in ethanol (5.6).

8 Procedure

8.1 General

The samples shall be kept at room temperature in closed containers and protected from light. Sample preparation shall avoid any contamination (including rubber gloves).

The determination of N-nitrosamines and N-nitrosatable substances releasing from teats according to this standard shall be at least in duplicate (see A.6), including the sample preparation steps. Separate samples A and B shall be prepared including separate pre-boiling. Also, they shall be migrated separately (see A.5).

The method recovery shall be (95 ± 30) % for each tested N-nitrosamine (Table 2) and for the internal standard (7.4).

It is recommended to re-check the method recovery for each tested N-nitrosamine at suitable intervals to document performance.

Any deviation from the following procedure shall be validated against the method of this standard for the whole range of the N-nitrosamines and N-nitrosatable substances tested (A7). However testing in duplicate, 8.2 to 8.4, the calculation of results (9), confirmation of N-nitrosamines (10) and analytical tolerances (11) shall be strictly followed.

Table 3 shows the procedures for both Sample A and B and for the Blank.

Table 3 — Flow Diagram of Testing Procedures

Sample of Soother teats or feeding teats or parts from whole elastomer soothers (8.2)		**Blank Test (8.8)**
Sample A	**Sample B**	
8.3 Sample A for determining N-nitrosatable substances	8.4 Sample B for determining N-nitrosamines	
8.3.1 Preparation and pre-boiling	8.4.1 Preparation and pre-boiling	
8.3.2 Preparation of Migrate A	8.4.2 Preparation of Migrate B	8.3.2 Preparation of Migrate Blank
8.3.3 Nitrosation of Migrate A and preparation of Solution A	8.4.3 Preparation of Solution B	8.3.3 Nitrosation of Migrate Blank and preparation of Solution Blank
8.5 Preparation of extraction columns for Solution A	8.5 Preparation of extraction columns for Solution B	8.5 Preparation of extraction columns for Solution Blank
8.6.1 Extraction of N-nitrosamines from Solution A to obtain Extract A	8.6.2 Extraction of N-nitrosamines from Solution B to obtain Extract B	8.6.1 Extraction of N-nitrosamines from Solution Blank to obtain Extract Blank
8.7.1 Concentration of N-nitrosamines in Extract A to obtain Concentrate A	8.7.2 Concentration of N-nitrosamines in Extract B to obtain Concentrate B	8.7.1 Concentration of N-nitrosamines in Extract Blank to obtain Concentrate Blank

8.2 Sample Preparation

Feeding teats shall be taken as they are from the packaging or detached from containers (usually baby bottles).

Soother teats shall be taken as a whole, including the bead. Therefore they shall be removed from the soother without cutting the teat, which may require destruction of the plastic parts of the soother. Any tool used to remove the teat from the soother shall not contaminate the teat.

Where the soother teat covers part of the shield, the teat including that part should also be removed without any damage as a whole.

When cutting of the teat is unavoidable, it shall be stated in the test report.

For whole elastomer soothers or soothers having different parts made of elastomers, the elastomer components shall be separated and tested as separate samples.

NOTE Different parts in whole elastomer soothers are for example teat, mouth shield and ring. These parts may have been manufactured by different technology or with different composition.

8.3 Sample A (for determination of N-nitrosatable substances)

8.3.1 Preparation and pre-boiling

For each determination from sample A, the sample weight shall be (5,5 ± 0,5) g (see A.4), using a balance capable of weighing with a precision of at least ± 0,01 g.

Cut the teats in half along the major axis to obtain nearly symmetrical pieces.

Weigh as many halves as necessary to make up a total weight of minimum 5,0 g. If the weight exceeds 6,0 g, cut the last half along the major axis in two parts to obtain nearly symmetrical pieces, and remove one piece. If necessary, repeat the same procedure with the smallest piece until reaching the required sample weight.

For feeding teats, each piece may be cut once more through the middle at right angles to the initial cut to ensure complete immersion.

For soother teats, no further cutting shall be undertaken.

Other elastomer parts of a soother shall be tested as separate samples. They shall be cut symmetrically along the major axis and also along the minor axis and if necessary be cut further as described above to match the required sample weight.

Note the weight of the sample(s) as $G_{(S)}$ in g.

For each determination from sample A, transfer the sample pieces to a beaker with (300 ± 30) ml of boiling water (5.1) and boil for 10 min. Remove them from the water with tweezers or tongs and shake off excess water. Transfer immediately into a flask as given in 8.3.2.

For ready to use products (see 3.9), the boiling step shall be omitted, which shall be noted in the test report.

The above amount of sample represents the necessary minimum. To achieve the necessary limits of detection, larger sample amounts may be used up to a maximum of 10 g. Ratios of sample to migrating solution shall be maintained and the volume of any glassware including separation columns, and the volume of reagents shall be proportionally increased during 8.3 to 8.6. See A.4.

8.3.2 Preparation of Migrate A (for determining N-nitrosatable substances) See A.2

Use a conical flask of a size (approximately 30 ml) suitable to hold the pieces of Sample A.

The sample shall remain completely immersed in the artificial saliva salt solution.

Place the sample pieces from 8.3.1 into the flask and add, by pipette, the volume of 4 ml per gram of sample ($G_{(S)}$ x 4 ml / g) of the artificial saliva salt solution (5.5).

For example, if $G_{(S)}$ = 5,7 g, the volume of artificial saliva salt solution shall be (22,8 ± 0,5) ml.

Close with a ground glass stopper and gently shake to ensure the pieces are completely immersed in the solution, and place the closed flask at (40 ± 2) °C for (24 ± 0,5) h in the oven (6.2).

NOTE Complete immersion can be aided by addition of glass beads to the flask.

After that time, remove the flask from the oven and vigorously shake 5 times by hand (see A.5).

Open the flask and with tweezers or tongs, one by one lift the pieces out of the migrate, shake off excess migrate into the flask and remove from the flask, leaving the migrate in the flask.

This is Migrate A.

Proceed immediately to 8.3.3.

8.3.3 Nitrosation of Migrate A (see A.3) and preparation of Solution A

Place the conical migration flask containing Migrate A in an oven (6.2) set at (40 ± 2) °C and reheat for (30 ± 1) min.

Remove from the oven and shake the flask vigorously 5 times by hand.

Open the flask and add (2,5 ± 0,1) ml of 1 molar hydrochloric acid solution (5.3.2) by pipette, close and shake the flask 5 times by hand to mix.

Return the flask to the oven (6.2) and leave for (30 ± 1) min at (40 ± 2) °C.

Immediately after the time indicated, remove the flask from the oven, add (5,0 ± 0,1) ml of 1 molar sodium hydroxide solution (5.4.2) by pipette and shake to make the solution alkaline and to stop the nitrosation reaction.

Add (0,5 ± 0,01) ml of the internal standard solution (7.4) by pipette, close with a ground glass stopper and shake. This is Solution A.

Solution A shall be protected from light and if stored overnight, shall be kept in a fridge. For longer periods of time it can be stored frozen in suitable containers.

8.4 Sample B (for determination of N-nitrosamines)

8.4.1 Preparation and pre-boiling

Prepare sample B in the same manner as given in 8.3.1.

With the prepared sample B proceed immediately to 8.4.2.

8.4.2 Preparation of Migrate B (for determining N-nitrosamines)

Prepare Migrate B in the same manner as given in 8.3.2.

With Migrate B proceed immediately to 8.4.3.

8.4.3 Preparation of Solution B

Add to Migrate B from 8.4.2 (0,6 ± 0,01) ml of 1 molar sodium hydroxide solution (5.4.2) by pipette and shake.

Add (0,5 ± 0,01) ml of the internal standard solution (7.4) and close.

This is Solution B.

Solution B shall be protected from light and if stored overnight, shall be kept in a fridge. For longer periods of time it can be stored frozen in suitable containers.

8.5 Preparation of extraction columns for Solutions A and B

The SPE columns used for solutions A and B are prepared identically.

The bottom of the column (6.3) is closed with glass wool (5.8) or by a sintered glass frit (6.4) and a total of (25 ± 0,5) g diatomaceous earth (5.9) is added.

Filling of the column with the diatomaceous earth shall be done in a way to ensure that the diatomaceous earth is compacted well enough to avoid collapsing and / or excess free volume when used, e.g. by filling the column in steps interrupted by frequently tapping its outside to attain a better uniform packing.

The prepared column shall have sufficient capacity to absorb the entire amount of solution A or B while maintaining a dry zone of 50 mm - 70 mm height above the bottom. Also after elution with

dichloromethane (8.6.1), the height of the remaining dry zone shall be sufficient to prevent any water from entering the extract. If necessary, adjust the amount of diatomaceous earth (5.9).

NOTE During elution with dichloromethane, the height of the dry zone shrinks further. This process is easily observed because of the different colour toning of the specimen-aqueous-soaked and of the dichloromethane-soaked diatomaceous earth. It is important to check that the dry zone capacity is not exhausted as otherwise the extract can contain water.

Cover the top of the column with a sintered glass frit (6.4) or with an approximately 1 cm thick layer of sea sand (5.10).

8.6 Extraction of N-nitrosamines

8.6.1 From Solution A

Stopper and shake the migration flask containing Solution A (8.3.3) vigorously 5 times by hand, and slowly transfer its content onto the extraction column (8.5).

Add (30 ± 0,5) ml dichloromethane (5.7) to the migration flask, stopper and shake vigorously 5 times by hand.

WARNING Pressure may occur when shaking the flask with dichloromethane! Make sure to carefully release the pressure to avoid spilling.

After (15 ± 1) min allowed for absorption of solution A by the diatomaceous earth slowly add the dichloromethane from the migration flask onto the top of the column. Collect the extract into the evaporator flask (6.5). With the aid of the PTFE stopper (6.3, Note 1) adjust the drip rate to allow steady addition of dichloromethane and to prevent the column from running dry.

Rinse the migration flask with a second portion of (30 ± 0,5) ml dichloromethane and add it to the column as described above. If needed to achieve higher recoveries this can be repeated increasing the amount of dichloromethane extract.

After the extract is collected, add (1 ± 0,01) ml of ethanol (5.6) to the evaporation flask (6.5).

This is Extract A.

8.6.2 From Solution B

Proceed in exactly the same manner as given in 8.6.1 to obtain Extract B.

8.7 Concentration of N-nitrosamines

8.7.1 In Extract A

8.7.1.1 General Procedure

Concentrate Extract A by evaporation (see 6.5) to a volume of (0,9 ± 0,1) ml.

Because of the volatility of some N-nitrosamines it is important that the volume does not fall below 0,8 ml in the concentration step.

Leave to equilibrate to room temperature and transfer to the GC glass vials (6.7).

This is Concentrate A.

The procedure in 8.7.1.2 is suitable to achieve the recovery condition given in 8.1 and is given as an example.

8.7.1.2 Example

Add two or three of the boiling chips (5.12) to the evaporator flask and concentrate the extract by evaporation to a volume of (5 ± 1) ml in the water bath (6.6), starting at (40 ± 2) °C and slowly raising the temperature to (60 ± 2) °C. After cooling rinse the walls of the evaporator flask with approximately 2 ml of dichloromethane (5.7). Further concentrate the extract to a volume of (0,9 ± 0,1) ml by gently passing a flow of nitrogen (5.11) over its surface.

8.7.2 In Extract B

Proceed in exactly the same manner as given in 8.7.1 to obtain Concentrate B.

8.8 Blank Test

A Blank test sample shall be prepared with the same volume of artificial saliva salt solution as used for Sample A but without the teats in the migration step and by following the procedures for Migrate A and Extract A as given in 8.3 to 8.7, see Table 3.

This yields the Concentrate Blank.

8.9 Analysis

8.9.1 Calibration function

Inject the appropriate amount of the calibration solutions (7.3) into the GC setup coupled with the chemiluminescence detector (6.11) and run the analysis under conditions which ensure the requirements set in 6.12 are met, for each calibration level and for the calibration blank.

Injection volumes (normally between 2 µL and 5 µL) should be chosen to unambiguously detect the lowest calibration points and to achieve the limits of quantification (LOQ) as given in Table 2.

Establish a calibration function for each N-nitrosamine tested:

For each N-nitrosamine i plot the measured peak areas ratio $A_{i,e}/A_{int,e}$ versus the corresponding concentration ratio $C_{i,e}/C_{int,e}$ for each calibration level e,

where

$A_{i,e}$ is the peak area of substance i, for calibration level e;

$A_{int,e}$ is the peak area of the internal standard, for calibration level e;

$C_{i,e}$ is the concentration of substance i, for calibration level e;

$C_{int,e}$ is the concentration of the internal standard, for calibration level e;

i is the index of the specific individual N-nitrosamines as shown in Table 2;

e is the index for concentration levels of calibration solutions according to 7.3 (e.g. 500 ng/ml, 100 ng/ml, 10 ng/ml, 5 ng/ml and calibration blank);

int is the index for the internal standard

The calibration function shall be linear in the range corresponding to the sample concentrations.

Determine the calibration function for each N-nitrosamine i by linear regression:

$$A_i/A_{int} = a_i \cdot C_i/C_{int} + b_i \quad (1)$$

where

a_i is the slope of the calibration curve of substance i;

b_i is the ordinate intercept of the calibration curve of substance i.

NOTE This calibration method is a standard tool in analytical chromatography software.

8.9.2 Determination of sample concentration

Inject the appropriate amount of test samples s, that is of Concentrate A (8.7.1), of Concentrate B (8.7.2) and of Concentrate Blank (8.8) into the GC setup coupled with the chemiluminescence detector (6.11) and run the analysis under conditions which ensure that the requirements set in 6.12 are met.

For each sample s determine the concentration of each substance i in mg/ml by using the calibration functions obtained in 8.9.1

$$C_{i,s} = [(A_{i,s}/A_{int,s} - b_i) / a_i] \times C_{int,s} \quad (2)$$

where

$A_{i,s}$ is the peak area of substance i, for sample s;

$A_{int,s}$ is the peak area of the internal standard, for sample s;

$C_{i,s}$ is the concentration of substance i, for sample s;

$C_{int,s}$ is the concentration of the internal standard, for sample s

NOTE This quantification method is a standard tool in analytical chromatography software.

The analysis shall be carried out within 24 h from preparation of the extract. If this is not possible, store the extracts and standards sealed and in the dark at a temperature of less than 5 °C and use within a maximum of 2 weeks.

If the result of a sample is outside the calibration range, the concentrate shall be diluted with ethanol (5.6). If dilution reduces the peak of the internal standard to below the LOQ, external calibration shall be used.

9 Calculation of results

9.1 General

For each sample s determine the mass concentration of every individual N-nitrosamine i and N-nitrosatable substance i determined as N-nitrosamine i in mg / kg of elastomer or rubber sample:

$$C_{i,s}[mg/kg] = C_{i,s}[mg/ml] \times V_s[ml] / G_s[kg] \quad (3)$$

where

$V_s[ml]$ is the final volume of the concentrate obtained from sample s, (0,9 ± 0,1) ml

$G_s[kg]$ is the weight of sample s (G_s in 8.3.1 or 8.4.1 converted to kg)

If the instrumental response observed for an individual N-nitrosamine is less than 3 times the background noise, it shall be recorded as “Not Detected” or “ND” and its amount treated as zero.

Analysis of Concentrate Blank shall yield “ND” except for the internal standard, otherwise determine source of contamination and redo all steps until a “ND” yield is obtained.

The recovery of the internal standard shall be (95 ± 30) % when calculated as the ratio between the absolute recovery (peak area / amount added) of the internal standard in the Concentrate Blank (8.8) and in the calibration blank solution (7.3).

Peaks of a N-nitrosamine which cannot be identified despite all the precautions laid down in 6.12 shall, by convention, be quantified as the N-nitrosamine from Table 2 which is supposed to elute prior to it, and be recorded as unidentified and quantified as (the given) N-nitrosamine.

EXAMPLE A peak of an unidentified N-nitrosamine is observed between the elution times of the peaks of NMOR and NEPhA (NMOR eluting before and NEPhA after the unidentified N-nitrosamine). This unidentified N-nitrosamine is quantified based on the calibration curve of NMOR and recorded as “unidentified, quantified as NMOR”.

9.2 Variability of results and calculation of means

9.2.1 Requirements for variability

For each individual N-nitrosamine, the difference between results of multiple determinations shall not be larger than the Repeatability Limit (r) when tested according to 9.2.2.

If all results are below the respective LOQ they are repeatable and a variability test is not necessary.

9.2.2 Test for variability

The variability of multiple results shall be tested as follows:

— Determine the difference (Diff $D_{i,s}$) between the multiple results obtained for $C_{i,s}$ [mg/kg] by subtracting the smallest result from the largest.

— Determine the mean of the multiple results ($x_{i,s}$).

— Multiply the mean ($x_{i,s}$) by a factor of 0,48 to give the Repeatability Limit (r) for these results (see Annex F).

— Compare the Repeatability Limit (r) with the difference (Diff D) between the multiple results.

If the difference between the multiple results is not larger than their Repeatability Limit (r), they are considered to be repeatable and valid.

If the difference between the multiple results is higher than the Repeatability Limit (r), all results for the concerned N-nitrosamines shall be rejected and a further determination in duplicate shall be carried out.

9.2.3 Calculation of means

Valid results shall be used to calculate the arithmetic means of the determinations.

If the results for an individual N-nitrosamine are all “not detected” the mean shall be recorded as ND and its amount treated as zero.

If for an individual N-nitrosamine the mean is below the respective LOQ, it shall be recorded as < LOQ and its amount treated as zero.

NOTE Annex C provides examples for variability testing of results and means formation.

9.3 Amount of total N-nitrosatable substances migrating from Sample A, analysed and expressed as N-nitrosamines from Concentrate A

After checking the repeatability of results (9.2), the mean of any individual N-nitrosatable substance determined from sample A shall be corrected by subtraction of the mean of the corresponding N-nitrosamine migrated from Sample B and determined from Concentrate B (see 9.4).

Calculate the total quantity of N-nitrosatable substances migrating from the sample by adding together the amounts of the individual N-nitrosamines determined in Concentrate A after the above corrections.

If the total quantity of N-nitrosatable substances exceeds 0,1 mg/kg, confirm the result according to Clause 10. If following the procedures of Clause 10 it is found that some peaks are false positive, the corresponding N-nitrosatable substances shall be recorded as “Not Detected” or “ND” and their amounts be treated as zero.

If NDiNA is confirmed and higher than 0,1 mg/kg adjust this result by subtracting from it 0,1 mg/kg (see Annex E).

Re-calculate the total quantity of N-nitrosatable substances accordingly.

NOTE Annex C provides examples for the calculation of results.

9.4 Amount of total N-nitrosamines migrating from Sample B, analysed and expressed as N-nitrosamines from Concentrate B

After checking the repeatability of results (9.2), calculate the total quantity of N-nitrosamines migrating from the sample by adding together the means of the individual N-nitrosamines determined in Concentrate B.

If the total quantity of N-nitrosamines exceeds 0,01 mg/kg, confirm the result according to Clause 10. If following the procedures of Clause 10 it is found that some peaks are false positive, the corresponding N-nitrosamines shall be recorded as “Not Detected” or “ND” and their amounts be treated as zero. Re-calculate the total quantity of N-nitrosamines accordingly.

10 Confirmation of N-nitrosamines

10.1 If applicable following 9.3 or 9.4 N-nitrosamines shall be confirmed in at least one of the following ways:

a) by filling comparable aliquots of the remaining Concentrate A and / or Concentrate B and of a calibration solution (7.3, at least medium concentration) in separate clear, UV transparent glass vials (6.9) and exposing them in parallel to UV radiation (6.8). The radiation dose shall ensure decomposition of N-nitrosamines so that upon subsequent analysis any peaks due to the presence of N-nitrosamines will disappear or be substantially reduced when compared to the initial analysis. If any of the peaks is not substantially reduced after irradiation indicates that the initial peak did not correspond to an N-nitrosamine.

   This method is suitable only for qualitative assessments. Parallel exposure of a calibration solution to the UV radiation is to ensure the effectiveness of the radiation source and dose. 3 h of illumination by a laboratory UV lamp with 365 nm wavelength and 8 Watts is normally sufficient to significantly decompose the N-nitrosamines present.

b) by the use of at least one other chromatography column with a stationary phase having a different polarity

c) by use of high resolution mass spectrometry (see Annex D and A.7).

While this standard requires the confirmation of N-nitrosamines only in case of results exceeding the limits (see 9.3 and 9.4), it is recommended to confirm any results which are doubtful or which do not match existing experience from the laboratory or from the sponsor of the sample, at least by method 10.1 a).

10.2 If it is found that a peak does not correspond to a N-nitrosamine after following at least one of the above procedures, it shall be deemed as false positive and disregarded.

11 Analytical tolerances

11.1 General

Any results for total N-nitrosatables or N-nitrosamines that exceed the limit values given in 9.3 and 9.4 after application of the N-nitrosamine specific adjustment and after confirmation according to Clause 10 shall be corrected by subtracting the analytical tolerances in 11.2 to give the corrected analytical result.

11.2 Analytical tolerances (see Annex F)

Analytical tolerance for the total quantity of N-nitrosatable substances	0,1 mg/kg.
Analytical tolerance for the total quantity of N-nitrosamines	0,01 mg/kg.

NOTE The analytical tolerances are required to take into account the inherent variability in measurement shown by the 2015 validation trial.

12 Compliance

A product shall comply with Directive 93/11/EEC if the total quantity of released N-nitrosamines is less than 0,01 mg/kg and if the total quantity of released N-nitrosatable substances is less than 0,1 mg/kg of elastomer or rubber, when obtained according to the present standard and after applying all applicable tolerances.

13 Test report

The work carried out by the testing laboratory shall be covered by a report which accurately, clearly comprehensible and unambiguously presents the test results and all other relevant information according to EN ISO/IEC 17025.

NOTE An example for results calculations and their representation is given in Annex C.

Additional to the information represented in C.3 the report shall also include the following information or facts:

— where the test was carried out (if different from the laboratory issuing the report);

— information on the competency of the laboratory to test rubber teats and soothers according to the present standard (for example, method accreditation);

— the sample weight used in every determination;

— if applicable, on cutting of the teats other than described in (8.2) and why it was unavoidable;

— if applicable the omission of the boiling step (8.3.1 and 8.4.1) for ready to use products;

— the individual N-nitrosamines tested for and the LOQs achieved by the laboratory;

— if applicable, how the N-nitrosamines have been confirmed (10.1);

— if applicable, how N-nitrosamine specific adjustment and / or analytical tolerances were applied;

- compliance statement with Directive 93/11/EEC and reference to this standard (EN 12868:2017);
- any deviation of the applied procedures to the method laid down in this standard and how it was validated (calibration, LOQs and recovery for internal standard and for each tested N-nitrosamine) see A.8;
- at least in case of the sample not complying with Directive 93/11/EEC, a photograph identifying the sample.

Annex A
(informative)

Rationales

A.1 General

This informative annex has been included with the purpose of providing the rationales for the inclusion of some of the requirements given in this standard.

The purpose of a rationale is to provide the underlying principles or reasoning for requirements and test conditions to aid the application of the standard. Also to provide additional information regarding any limitations or precautions that need to be kept in mind when applying the standard to products.

A.2 Migration Conditions (see 8.3.2)

Dynamic migration as a means of simulating the real use of the products was considered. It was felt that the static migration time of 24 h as set by the Directive is a worst case scenario which does not attempt to simulate exposure due to real use. Because interlaboratory variation is one of the big problems of EN 12868:1999 and because any mechanical agitation will overlay results with an additional source for variation, the static migration principle was maintained.

In addition, experiments have shown that compared to static migration for 24 h, dynamic migration yields only slightly increased results. This slight increase is outweighed by a disproportionate increase in the variability of results. It is also not easy to maintain a migration temperature of 40 °C in for example, a head over heels dynamic agitation device. Therefore it was decided to keep migration static.

A.3 Nitrosation conditions (see 8.3.3)

In EN 12868:1999 nitrosation was carried out for 30 min, but the temperature was not specified.

The technical committee discussed this question in detail and consulted the available information. It was decided that in the interest of reproducibility the temperature of nitrosation has to be specified and it was decided to set the temperature at 40 °C (close to body temperature).

Based on physiological observation, a longer nitrosation time could be deduced. However, experiments indicated that this would not generally increase the nitrosation yield for all N-nitrosamines. Therefore, in absence of better knowledge, it was decided to keep the nitrosation time at 30 min.

A.4 Weight of sample used (see 8.3.1)

In EN 12868:1999 it was required to pre-boil 40 g of elastomer or rubber sample and to use 10 g for the migration, subsequently dividing the migrate into 2 aliquots, followed by analysis procedures for the determination of N-nitrosatable substances and for N-nitrosamines.

For the current standard the technical committee decided a minimum amount of 2 × 5 g of elastomer or rubber samples requiring pre-boiling and to migrate each sample separately – for N-nitrosatable substances and for N-nitrosamines.

Therefore approximately 20 g of elastomer or rubber sample are needed for the double determinations of both N-nitrosatable substances and N-nitrosamines as required by this standard.

In doing so, the standard:

(a) accounts for the difficulty of always collecting a minimum of 40 g of sample (e.g. for the purpose of market surveillance).

(b) maintains the amount of sample necessary to achieve the required limits of determination for N-nitrosamines and

(c) increases the amount of sample effectively used to obtain results for N-nitrosatable substances, which improves their repeatability having proved more problematic than for N-nitrosamines.

It should also be noted that in both EN 12868:1999 and the current standard there is a provision for larger sample amounts at a maintained ratio of sample to artificial saliva salt solution and additions of reagents and solvents used (8.3.3, 8.4.3, 8.5, and 8.6). To use larger sample amounts, the capacity of the migration flasks, of the extraction columns and the amount of diatomaceous earth in 8.5 are increased accordingly to ensure that the entire migrate is used. This should assist laboratories unable to achieve the necessary limits of determination when using the minimum amount of sample.

A.5 Separate migrations (see 8.3.2 and 8.3.3)

Some N-nitrosamines and their precursors in particular have lower solubility in the artificial saliva salt solution and may also adhere at the surfaces of the vessel and sample. Depending on the elapsed time and on how the operator handles the vessel before transferring the solutions this may result in significant variations of yield.

To decrease those variations, the vigorous shaking step was prescribed before any operation carried out with the aqueous migrates and the rinsing step of the migration vessels with DCM was introduced.

For the same reasons the Technical Committee decided all the necessary steps with the aqueous medium to be performed in the same vessel which eliminates transfer of aqueous solutions, but requires 2 separate migration setups including pre-boiling.

Another advantage of the new procedure is that samples do not need to dry after the pre-boiling step, so that the whole course of sample preparation and analysis can be conveniently finished within 2 consecutive days, and that except for the nitrosation step, the method is exactly the same for both N-nitrosamines and for N-nitrosatable substances.

A.6 Duplicate tests (see 8.1)

It has become the norm particularly within experienced commercial laboratories to perform determinations of N-nitrosamines and N-nitrosatable substances at least in duplicate. Accordingly, this has been made a requirement. The Technical Committee believes this is a significant improvement, especially with the addition of a requirement for the variability of duplicate results, not only in terms of accuracy but also from a product safety point of view.

A.7 TEA and alternative detectors

The TEA detector is very selective and very sensitive for the detection of N-nitrosamines.

High resolution Mass Spectroscopic techniques are ideal to identify and/or confirm the structure of nitrosamines present and the current standard takes advantage of this fact.

However, care shall be taken when using only MS techniques as matrix effects may influence the quantitative results. The use of labelled internal standards for each substance is recommended but limited by availability. Also, selectivity may be problematic when not following the precautions laid down in Clause 10 and Annex D.

Annex D provides further information on using alternative methods.

A.8 Deviations (see 9.2, 9.3 and 11)

The 2015 validation trial and the experience from the practical application of EN 12868 have demonstrated that the N-nitrosamine specific adjustment (9.3), the analytical tolerances (11) and duplicate variability (9.2) are sufficient to describe the interlaboratory variability only if this standard is strictly followed. Therefore it is essential that any deviation from EN 12868 is validated.

To validate a deviation, it should be demonstrated that when compared to EN 12868:2017 it does not impair selectivity, LOQ, linearity of calibration range, recovery and repeatability and the possibility to confirm N-nitrosamines for each of the N-nitrosamines tested.

A.9 Main differences between this standard and EN 71-12 [3]

There are several important differences between the method of this standard and EN 71-12 (Safety of toys — Part 12: N-nitrosamines and N-nitrosatable substances, also see Annex D). These include:

— This method employs extraction of the aqueous migrates and gas chromatography as per Directive 93/11/EEC. It reduces adsorption losses of yield and potential reactions that can occur in aqueous media.

— This method avoids the transfer of aqueous migrate to other glass vessels (for nitrosation), which due to adsorption can lead to loss of N-nitrosatable substances and increased variability of results.

— This method includes a concentration step which achieves the necessary sensitivity.

— This method employs TEA detection which is N-nitrosamine– or N-NO –selective enabling the detection of “non-expected” N-nitrosamines.

— This method avoids some problems of quantification which are specific for MS (see A.7).

— This method introduces a variability criterion for acceptability of duplicate results.

— This method has been developed, optimized and validated for testing of baby teats, while EN 71-12 has to cover a wider range of products and also includes the determination of the content of substances from finger paints.

— This method introduces a N-nitrosamine specific adjustment (for NDiNA) and maintains the analytical tolerance of 100 %, which have both been validated.

— This method does not allow the determination of NDELA without a derivatisation step. NDELA, however, has not been found in baby teats to date, but it is a current issue in finger paints.

Annex B
(informative)

Suitable gas chromatographic method

As given in 6.12 each laboratory should ensure that adequate separation is achieved with their chosen system(s). The following set of conditions has been found suitable for the analysis of most of the N-nitrosamines found in baby teats and is given for guidance:

GC - Conditions

— Injector temp: 150 °C, 1 min 250 °C (100 °/min).

— Oven temp 60 °C 0,2 min / 82 °C (15 °/min) / 88 °C (1 °/min) /

140 °C (15 °/min) 7 min / 250 °C (25 °/min) 10 min.

— Column: DB-FFAP, 30,0 m, diam. 0,53 mm, film 1,5 µm.

— Pyrolyser temp: 500 °C.

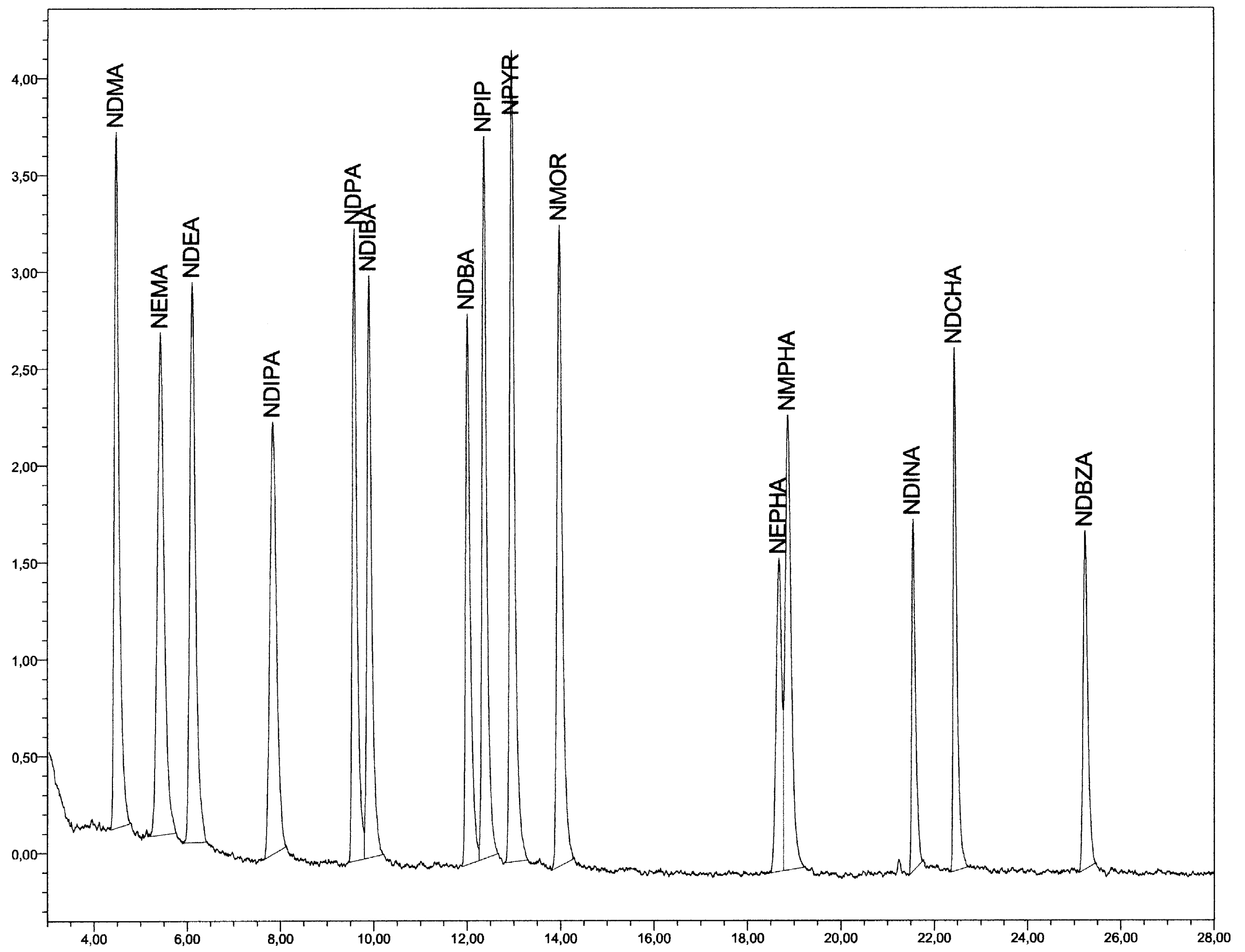
— Carrier gas: Helium at a flow rate of approximately 40 ml/min.

— Coupling: Direct coupling between GC column and pyrolysis oven.

The corresponding GC chromatogram was recorded by TEA and is shown in Figure B.1. It was obtained from a calibration solution of 15 N-nitrosamines (at 200 ng/ml) incl. the internal standard, at an injection volume of 5 µL and subjected to automated integration.

The abbreviations shown on the peaks are those given in Table 2, except for NDCHA (N-nitrosodicyclohexylamine, CAS 947-92-2) – elution time 23 min.

While all other peaks are well separated, there occurs an overlay of NEPhA and NMPhA. Hence, for testing samples that show peaks eluting near to NEPhA and NMPhA the above conditions would need to be adjusted to achieve better separation of these peaks.



Key

y-axis intensity (relative units)

x-axis elution Time (minutes)

Figure B.1 — GC Chromatogram of a calibration solution recorded by TEA

Annex C
(normative)

Example of results calculation and test report

C.1 General

By using an example of analytical results, this Annex and the Tables included illustrate how to treat results and to undertake the necessary mathematical and logical operations which are normative.

The example is an artificial example chosen to include most of the possibilities which may occur during analysis and which require special attention.

C.2 Example for variability testing and means calculation for analytical results

The requirements for variability testing and means formation of analytical results obtained according to Clause 8 are described in Clause 9. Tables C.1 and C.2 illustrate the operations to be carried out for sample A and B, respectively. They are not meant to form part of the test report.

The first columns, labelled “NA” list the N-nitrosamines given in 7.2, Table 2 as tested. Where analysis had to be repeated for a N-nitrosamine, these results are also included in this example as NDMA (2) and NDBA (2). For sample A, determinations for NDMA failed the variability test and were repeated. For sample B, this was the case for NDBA. “NMOR” identifies results for an N-nitrosamine which was impossible to identify and which due to its elution time being between those of NMOR and NEPhA was quantified as NMOR (see 9.1).

The limits of quantification which the laboratory obtained for the given N-nitrosamines are listed in the column “LOQ”. The requirement for LOQs is given in 7.2, Table 2.

In the example, double determinations D1 and D2 were carried out and the tables list results from each determination. More determinations would have required additional columns. Non-detectable or “ND” results are treated as zero (9.1) in the following calculations.

Calculations are done separately for each individual N-nitrosamine. The columns “Mean”, “Diff D” and “(r)” are used to test the variability of results D1 and D2 according to 9.2.2, “Mean” being their arithmetical average, “Diff D” the difference between the highest and the lowest determination, and “(r)” their repeatability limit which is 0,48 times their “Mean”.

If “Diff D” ≤ “(r)”, the variability test is passed and results of these determinations are valid. When all results are < LOQ they are valid and a variability test is not necessary.

Valid results are used to obtain the final means given in the last column “Final Mean”. Here “invalid” identifies results that failed the variability test, which are followed by valid results determined in a repeated analysis. Means equalling zero are given as “ND” and means below the limit of quantification as “< LOQ”.

The final means are used for further calculation as given in C.3.

Table C.1 — Example for variability testing and means calculation for Sample A

Sample A: N-nitrosamines after nitrosation (mg/kg)							
NA	**LOQ**	**D1**	**D2**	**Mean**	**Diff D**	**(r)**	**Final Mean**
NDMA	0,001	0,0260	0,0520	0,0390	0,0260	0,0187	invalid
NDMA (2)	0,001	0,0240	0,0360	0,0300	0,0120	0,0144	0,0300
NDEA	0,001	0,0006	0,0009	0,0008			< LOQ
NDPA	0,001	ND	ND	0			ND
NDiBA	0,001	ND	0,0009	0,0005			< LOQ
NDBA	0,001	0,0081	0,0052	0,0067	0,0029	0,0032	0,0067
NPIP	0,001	ND	ND	0			ND
NPYR	0,001	ND	ND	0			ND
NMOR	0,005	0,0150	0,0230	0,0190	0,0080	0,0091	0,0190
NMPhA	0,005	ND	ND	0			ND
NEPhA	0,005	ND	ND	0			ND
NDiNA	0,005	0,1960	0,1560	0,1760	0,0400	0,0845	0,1760
NDBzA	0,005	0,0050	0,0060	0,0055	0,0010	0,0026	0,0055
"NMOR"	0,001	0,0560	0,0670	0,0615	0,0110	0,0295	0,0615

Table C.2 — Example for variability testing and means calculation for Sample B

Sample B: N-nitrosamines (mg/kg)							
NA	**LOQ**	**D1**	**D2**	**Mean**	**Diff D**	**(r)**	**Final Mean**
NDMA	0,001	0,0009	0,0013	0,0011	0,0004	0,0005	0,0011
NDEA	0,001	0,0001	0,0002	0,0002			< LOQ
NDPA	0,001	ND	ND	0			ND
NDiBA	0,001	0,1400	0,1500	0,1450	0,0100	0,0696	0,1450
NDBA	0,001	0,0100	0,0200	0,0150	0,0100	0,0072	invalid
NDBA (2)	0,001	0,0010	0,0009	0,00095	0,0001	0,0004	< LOQ
NPIP	0,001	ND	ND	0			ND
NPYR	0,001	ND	ND	0			ND
NMOR	0,005	0,0070	0,0100	0,0085	0,0030	0,0041	0,0085
NMPhA	0,005	ND	ND	0			ND
NEPhA	0,005	ND	ND	0			ND
NDiNA	0,005	ND	ND	0			ND
NDBzA	0,005	0,0030	0,0040	0,0035	0,0010	0,0017	< LOQ
"NMOR"	0,001	0,0020	0,0030	0,0025	0,0010	0,0012	0,0025

C.3 Results calculation and results table for test report

The information given in Table C.3 is indispensable for the unambiguous and comprehensible representation of the results in the test report.

The test report shall list the N-nitrosamines tested, the corresponding limit of quantification achieved by the laboratory and the number of determinations done. It shall confirm or demonstrate that determinations were subjected to the variability test and are valid according to 9.2.2.

The information and results of calculation provided in Table C.3 shall form part of the test report including the information given in the footnotes when applicable. While the example represents just one possible form of how this information can be represented, provision of this information is normative.

Table C.3 is summarizing the results of operations to be carried out to obtain the results for N-nitrosatable substances and for N-nitrosamines, which are explained in the following.

Calculations are done separately for each individual N-nitrosamine. Results given as “ND” and “<LOQ” are both treated as zero.

To obtain the results for N-nitrosatable substances, the final means taken from Table C.1 (sample A) are corrected by subtracting the final means of the N-nitrosamines from Table C.2 (sample B). The test report shall clearly state if values given for individual N-nitrosatable substances results have been corrected for N-nitrosamines.

The results for N-nitrosamines are the final means from Table C.2 (sample B).

To obtain the total N-nitrosatable substances, the individual N-nitrosatables are summed. The same is carried out for the total N-nitrosamines.

If totals for N-nitrosatable substances or for N-nitrosamines exceed 0,1 mg/kg or 0,01 mg/kg, respectively, or if there is doubt as to their correctness, the N-nitrosamines determined in sample A or B shall be confirmed in accordance with Clause 10. Information on confirmation and method used shall be included in the test report.

In the example, all N-nitrosamines are confirmed with the exception of NDiBA in both sample A and sample B. Accordingly, the determinations of NDiBA as given in Tables C.1 and C.2 are identified as false positives and their results set as “ND” in Table C.3 (see 9.4).

NOTE Information on false positive determinations may be given in the final test report.

In the example, N-nitrosatable NDiNA exceeds 0,1 mg/kg (0,176 mg/kg in Table C.1) and was confirmed in accordance with Clause 10 (see above). Hence, the NDiNA result shall be adjusted by subtraction of 0,1 mg/kg (see 9.3) which yields 0,076 mg/kg and appropriate information on the adjustment shall be given in the test report.

The results for total N-nitrosatable substances and for total N-nitrosamines shall be given in the test report.

If the total for N-nitrosatable substances and / or for N-nitrosamines exceed 0,1 mg/kg and / or 0,01 mg/kg, respectively, the analytical tolerances according Clause 11 shall be applied. The test report shall give both the result(s) before and after the analytical correction(s).

Table C.3 — Example of Final results and their representation

Nitrosamines tested	LOQ [mg/kg]	N-nitrosatable substances [a] after deduction of equivalent N-nitrosamine [mg/kg]	N-nitrosamines [a] [mg/kg]
NDMA	0,001	0,029	0,001
NDEA	0,001	< LOQ	< LOQ
NDPA	0,001	ND	ND
NDiBA	0,001	ND	ND
NDBA	0,001	0,007	< LOQ
NPIP	0,001	ND	ND
NPYR	0,001	ND	ND
NMOR	0,001	0,011	0,009
NMPhA	0,005	ND	ND
NEPhA	0,005	ND	ND
NDiNA	0,005	0,076 [b]	ND
NDBzA	0,005	0,006	< LOQ
"NMOR"[c]	0,001	0,059	0,003
SUM		0,188	0,013
Result after applying the analytical tolerance		0,088	0,003
Total Value allowed (93/11/EEC)		0,1	0,01
Compliance to 93/11/EEC		**PASS**	**PASS**

[a] double determinations, validity confirmed according to EN 12868:2017, 9.2.2.
[b] NDiNA nitrosatable substance after NDiNA specific adjustment
[c] "NMOR" unidentified, quantified as NMOR
Results were confirmed according to EN 12868:2017, 10.1 method a.

C.4 Other Information for test report

Other information for the test report shall be given in accordance with Clause 13.

NOTE this information does not affect the representation of results and does not require an example.

Annex D
(informative)

Alternative Methods

D.1 General

The Technical Committee is aware that the methods of LC-MS/MS and GC-MS are sometimes used to test soothers and teats for the release of N-nitrosamines and N-nitrosatable substances. In addition it is noted that the method of EN 71-12 [3] employs liquid chromatography in combination with MS/MS detection.

These methods may be used for the purpose of this standard provided that they have been validated against the method of this standard for each substance tested (A.8). Indeed results from some individual laboratories using either LC-MS/MS or GC-MS displayed validation in the 2015 validation trial. However, it was not possible to validate these methods overall due to the non-normality of their results (see Annex F).

Experience and the results from the 2015 validation trial suggest that the above alternative methods suffer similar problems of variability as EN 12868 when analyses include a wide range of N-nitrosamines and NDiNA.

Under D.2 and D.3 suitable sets of conditions are given. It is recommended to also follow the applicable procedures of the Guidance document on analytical quality control by DG SANTE/11945/2015 [4].

D.2 Liquid Chromatography (LC)

Ethanol is not a suitable solvent for N-nitrosamine standard solutions (Clause 7) for LC. Other solvents, e.g. methanol are therefore preferred for stock solutions. Concentration series of N-nitrosamine calibration solutions can be obtained by dilution with artificial saliva salt solutions (5.5). Such solutions are stable for up to 1 day.

The following LC conditions are just one example found suitable for the determination of N-nitrosamines when using mass detectors:

Chromatographic column:	e.g. Waters ACQUITY UPLC HSS T3 1,8 µm; 2,1x100 mm
Mobile phases:	A: 0,1 % HCOOH in Water
	B: 0,1 % HCOOH in Acetonitrile
Column oven temperature:	30 °C
Injection volume:	50 µl
Suitable HPLC-gradient:	See Table D.1

Table D.1 — Gradient profile for the given HPLC conditions

time [min]	flow rate [µl/min]	A [%]	B [%]
0	300	98	2
1,5	300	98	2
2,5	300	60	40
5	400	0	100
8	400	0	100
8,2	300	98	2
9	300	98	2

HPLC conditions are dependent on the chromatographic column. They have to be suitable to generate sufficient retention time for NDELA (if tested) and NDMA.

D.3 MS/MS conditions

The following MS/MS conditions and MRM transitions have been found suitable for the determination of N-nitrosamines when using liquid chromatography:

Ion source:	APCI
Polarity:	Positive
Minimum resolution:	0,7 Da
Suitable MRM-transitions:	See Table D.2

Table D.2 — Suitable MRM Transitions for MS/MS conditions

Compound	Q1 mass m/z	Q3 masses m/z	
NDMA	75,0	42,9	58,0
NDEA	103,1	75,0	47,0
NPIP	115,1	69,1	40,9
NMOR	117,0	86,9	85,9
NDPA/ NDiPA	131,1	89,1	43,1
NDELA	135,0	104,0	74,0
d8-NDELA	143,0	111,0	80,2
NDBA/ NDiBA	159,1	57,1	103,0
d6-NDMA	81,0	46,1	64,1
NDBzA	226,9	91,1	65,1
NEPhA	150,9	77,1	51,1
NMPhA	136,9	66,0	107,1
NDiNA	299,0	57,1	173,1

NOTE MRM-transitions can vary between different instruments. Suitable transitions need to be chosen for quantification and identification of N-nitrosamines. The given transitions were obtained using nitrogen as collision gas.

D.4 Confirmation and quantification of detected N-nitrosamines

N-nitrosamines detected by using an MS/MS detector are confirmed via their specific ratio of detected ions. Therefore, relative intensities of at least 3 ions expressed as a percentage of the intensity of the most intense ion, should correspond to those of the calibration standard solutions, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table D.3.

Table D.3 — Maximum permitted tolerances for relative ion intensities

Relative intensity (% of base ion intensity)	**Relative range of the response**
> 50 %	±20 %
> 20 % to 50 %	±25 %
> 10 % to 20 %	±30 %
≤ 10 %	±50 %

Table D.3 sets maximum permitted tolerances. When reaching these tolerances intensity ratios of more than 3 ions should be considered. Only if intensity ratios between all ions of sample and standard match, the substances are identical N-nitrosamines. If this is not the case, the substance from the sample is not a N-nitrosamine, even if retention times appear identical with that of the respective standard. Such findings should be deemed as false positive and disregarded.

For confirmed N-nitrosamines, quantification should consider the ion with the lowest matching relative intensity when compared to the standard. Calibration functions shall be linear in the detected concentration range.

When results for total N-nitrosatable substances or N-nitrosamines exceed the applicable limits, they should be confirmed as given in Clause 10.

Annex E
(informative)

Justification of an N-nitrosamine specific adjustment for NDiNA

The great achievement of EN 12868:1999 is to provide Commission Directive 93/11/EEC [1] with the necessary analytical method. Rubber teats and soothers that released carcinogenic substances in detectable amounts have since been eliminated from the EU market.

While all technically relevant nitrosamines were already included in EN 12868:1999, in the manufacture of teats at that time only accelerators based on volatile amines were used. Hence, validation of the 1999 method included only volatile N-nitrosamines.

To further minimize theoretical exposure to carcinogenic N-nitrosamines in the meantime producers substituted these accelerators by so called "nitrosamine safe" accelerators, which is also encouraged by the SCF opinion on nitrosamines in babies dummies and teats [5] and by the German TRGS 552 [6]. Nitrosamine safe means that either the high molecular weight and bulkiness of the nitrosatable substances dramatically decreases their accessibility thus restricting nitrosation or limiting the availability of their nitrosated amines and of their related alkylating substances, as in the case of NDiNA, or safe due to the chemical structure of the related nitrosamines only yielding stable carbenium ions which are weak alkylating substances and unable to damage RNA and DNA to the extent of causing carcinogenesis, like NDBzA. As a result their toxicology is non-carcinogenic.

In practice these nitrosamine safe accelerating systems are accompanied by minor amounts of so called co – accelerators and by latex stabilizers which still yield some volatile N-nitrosatable substances.

As a result contemporary products release complex mixtures of different N-nitrosatable substances and their analyses by EN 12868:1999 were of poor reproducibility. Collaborative trials organized by CEN TC 252 WG5 demonstrated that real interlaboratory variability for total N-nitrosatables would require an analytical tolerance of 0,4 mg/kg, that is 4 times the one provided by EN 12868:1999. This motivated the revision of the standard.

This revision of EN 12868 achieved an improvement in the method so that an analytical tolerance of 0,1 mg/kg (as originally provided by EN 12868:1999) is sufficient to cover interlaboratory variability when testing products that use today's complex accelerating systems. This is with the exception of higher amounts of NDiNA, for which the 2015 validation trial indicated that an overall tolerance of 0,2 mg/kg would be required (see F.4).

To retain the safety level as regards volatile carcinogenic N-nitrosamines it was decided to maintain the 0,1 mg/kg analytical tolerance of EN 12868:1999. However, in order not preclude the use of N-nitrosamine safe accelerators like NDiNA, the experts of WG5 agreed to the specific adjustment for higher amounts of nitrosatable NDiNA by subtracting 0,1 mg/kg. This adjustment is motivated by the following:

— Reproducible determination of NDiNA nitrosatable is impaired by its accumulation at any surfaces, which is different from other nitrosatables and significantly adds to variability.

— The molecular weight of NDiNA is 4 times higher than of NDMA, giving rise to a 4 times higher LOQ and variability in principle. It is therefore more relevant to keep the low analytical tolerance for low molecular weight nitrosamines than for high molecular weight ones.

— The significantly lower toxicity of non-carcinogenic NDiNA and of some other low volatile nitrosamines which can originate from nitrosamine safe accelerators advocates the use of these

accelerators instead of others. Use of nitrosamine safe accelerators should be encouraged rather than limited due to analytical circumstances.

— The 2015 validation trial showed the necessity of a nitrosamine specific adjustment only when there were higher amounts of NDiNA nitrosatable, that is over 0,1 mg/kg.

As a result, in providing the specific adjustment for increased amounts of non-carcinogenic NDiNA nitrosatable and by the numerous improvements achieving the analytical tolerance of 0,1 mg/kg for the complex test samples occurring today, the new revision of EN 12868 further secures the safety of the products in its scope.

Annex F
(informative)

Summary of the 2015 validation trial

F.1Outline

In April/May 2015, 37 laboratories worldwide were sent samples with sufficient material for duplicate analysis by the method given in prEN 12868 Rev April 2015 (E) for first enquiry.

Returned results were statistically analysed based on to ISO 5725-2 [7] and ISO/TR 22971:2005 [8].

Sample descriptions are shown in Table F.1

Table F.1 — Sample Descriptions for Validation Trial

Sample Number	Manufacturer Code	Type of samples
1	A	Latex Feeding Teats
2	B	Latex Soother Teats
3	C	Latex Soother Teats
5	C	Latex Soother Teats

The laboratories were not informed of the Manufacturer Code.

The designation for Sample 4 was used for a vial of N-nitrosamine standards in Ethanol to be used for calibration.

Laboratories were assigned at random an identification number (Lab 1, Lab 2 etc).

16 separate analyses were required per laboratory.

F.2Initial statistical analysis – N-nitrosatable substances

27 sets of results were returned. The results from 6 laboratories were rejected due to obvious violations of the protocol.

Total N-nitrosatable substances (after deducting N-nitrosamines) were examined for outliers using the Grubb's test. All the results of one laboratory were rejected and one result for two other laboratories.

Overall means of the total N-nitrosatable substances (after removal of outliers) and the reproducibility limits are given in Table F2.

Table F.2 — Mean data and calculations for total N-nitrosatable substances

Sample Number	Overall Mean (mg/kg)	Reproducibility Limit (R) (mg/kg)
1	0,096	0,101
2	0,134	0,267
3	0,065	0,110
5	0,066	0,115

The results making up the overall means for each sample showed a normal distribution.

The Reproducibility Limit (R) is defined in ISO/TR 22971 as 2,8 X S_R

Where S_R = Reproducibility standard deviation

The analytical tolerance for total N-nitrosatable substances given in EN 12868:1999 is 0,1 mg/kg which can be compared with the reproducibility limits given in Table F.2.

F.3Reproducibility Limit for N-nitrosatable substances

Overall total means for N-nitrosatable substances by method are shown in Table F.3

Table F.3 — Overall means for total N-nitrosatable substances by method

Sample Number	Overall Means (mg/kg)		
	GC-TEA	LC-MS/MS	GC-MS/MS
1	0,109	0,087	0,086
2	0,168	0,102	0,128
3	0,079	0,040	0,107
5	0,074	0,043	0,113

Detailed examination of the results within each method showed that only GC-TEA exhibited normal distribution for the 4 samples. LC-MS/MS and GC-MS/MS displayed normal distribution only for sample 1. For the normal distributed data reproducibility limits were calculated and are shown in Table F.4.

Table F.4 — Reproducibility limits for total N-nitrosatable substances by method

Sample Number	Reproducibility Limit (mg/kg)		
	GC-TEA	LC-MS/MS	GC-MS/MS
1	0,060	0,110	0,146
2	0,244		-
3	0,064		-
5	0,100		-

It is considered by the Technical Committee that reproducibility limits of 0,1 or lower, confirm the current analytical tolerance of 0,1 mg/kg for Nitrosatable substances in EN 12868:1999. Therefore the Technical Committee decided that for the GC-TEA results, Samples 1, 3 and 5 validate the new (2015) method. However, the results for Sample 2 required further exploration.

To do this, individual N-nitrosatable substances were considered. Table F.5 shows reproducibility limits for individual N-nitrosatable substances.

Table F.5 — Reproducibility limits (mg/kg) for individual N-nitrosatable substances

	Sample 1	Sample 2	Sample 3	Sample 5
NDMA	0,069	0,014	0,012	0,018
NDEA	0,045		0,015	0,022
NDBA				
NDiBA	0,056			
NDBzA	0,027	0,018	0,029	0,028
NDiNA		0,242	0,066	0,070

In Sample 2 the high reproducibility limit for total N-nitrosatable substances appears to be associated with the high reproducibility limit for NDiNA, particularly when compared with the reproducibility limits for the other individual N-nitrosatable substances.

F.4 Consideration of the NDiNA Reproducibility limit

The reproducibility limit for NDiNA from Samples 2, 3 and 5 were compared with the mean found by the TEA method and the following relationship was found:

Reproducibility Limit (mg/kg) = 1,5287 X NDiNA determined + 0,0105

The correlation of determination (R^2) for this relationship was 0,9944.

Examination of this relationship suggests that when determined NDiNA is in excess of 0,1 mg/kg, an analytical tolerance of at least 0,2 mg/kg would be more appropriate.

The Technical Committee came to the conclusion that the current validation trial demonstrated the necessity of a nitrosamine specific adjustment which however is to be applied only when there were higher amounts of NDiNA, that is over 0,1 mg/kg.

F.5 Initial statistical analysis – N-nitrosamines

Only a minority of Laboratories detected any N-nitrosamines above LOQs.

The results of 2 laboratories were removed due to outliers.

Only results from the TEA method showed normal distribution and these are given in Table F.6.

Table F.6 — Mean data and calculations for total N-nitrosamines

Sample Number	Overall Mean (mg/kg)	Reproducibility Limit (R) (mg/kg)
1	0,004	0,007
2	0,015	0,033
3	0,009	0,025
5	0,002	0,002

Although the reproducibility limits for sample 2 and 3 are somewhat elevated as compared with the analytical tolerance given in EN 12868:1999, the Technical Committee cognizant that only a minority of laboratories determined N-nitrosamines above LOQs decided to keep the analytical tolerance at 0,01 mg/kg.

There were insufficient data to statistically analyse individual N-nitrosamines.

F.6Variability between Determinations

For the first time, this version of EN 12868 requires that all determinations for N-nitrosatable substances and for N-nitrosamines are carried out at least in duplicate. The Technical Committee decided that these duplicate determinations should be subject to a test and requirement for variability.

To do this the repeatability limit (r) was utilized.

The repeatability limit (r) is defined in ISO/TR 22971 [8] as the value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be within a probability of 95 %.

For every duplicate determination the standard deviation was determined, which was multiplied by 2,8 to give the repeatability limit. This technique was used for all the duplicate determinations, after removal of outliers and a summary is shown in Table F.7.

Table F.7 — Summary of average repeatability limits for duplicate determinations of N-nitrosatable substances and N-nitrosamines

	N-nitrosatable substances		N-nitrosamines	
Substance	**Number of duplicate determinations**	**Average Repeatability Limit (mg/kg)**	**Number of duplicate determinations**	**Average Repeatability Limit (mg/kg)**
NDBA	9	0,000	2	0,001
NDBzA	56	0,004	17	0,003
NDEA	48	0,003	10	0,001
NDiBA	9	0,002	6	0,002
NDiNA	42	0,020	1	0,012
NDMA	57	0,005	18	0,001
NEPhA	3	0,000	3	0,000
NMOR	-	-	1	0,000
NMPhA	5	0,003	4	0,005
NPYR	1	0,000	1	0,000
Totals	230	0,007	63	0,002

Table F.7 shows that the repeatability limits for NDiNA, for both N-nitrosatable substance and N-nitrosamine are significantly higher than the other individual repeatability limits, again underlining the necessity to introduce a nitrosamine specific adjustment.

In addition average repeatability limits for N-nitrosatable substances are over three times higher than for N-nitrosamines.

The Technical Committee sought to establish a single requirement for the variability of multiple determinations, rather than having to set separate requirements for the variability of determinations for NDiNA, for N-nitrosatable substances and for N-nitrosamines

To do this the average repeatability limits were compared with the average mean results for individual N-nitrosatable substances and for N-nitrosamines shown in Table F.7. A statistically significant correlation was achieved with the formula:

Average Repeatability Limit (r) = 0,48 X mean N-nitrosatable substance/N-nitrosamine result.

The linear correlation of determination (R^2) for this relationship was 0,9312.

Therefore multiplying the mean of any pair of duplicate results (for both N-nitrosatables and for N-nitrosamines) by 0,48 provides a reliable repeatability limit for those results. This can be compared with the difference between the duplicate results. The difference between the two determinations should be lower than the repeatability limit.

This was tested for all duplicate TEA results and 91 % had differences between the two determinations lower than the repeatability limit.

The Technical Committee therefore decided to include this variability test into this standard.

F.7 Summary of Conclusions and implications for EN 12868

(a) It was concluded that the trial validated the method given in this standard, but only for TEA.

(b) This does not imply that some individual laboratories using LCMSMS or GCMSMS did not meet the necessary requirements, but that overall the results from these alternative techniques showed non-normal distribution – in the case of GCMSMS this is probably because of the low number of laboratories using this technique – see Table F.8.

Table F.8 — Methods used by laboratories (after removal of outliers)

Method Used	Number of Laboratories
TEA	8
LCMSMS	9
GCMSMS	3
Total	20

(c) The analytical tolerances set in EN 12868:1999 were confirmed at:

Analytical tolerance for the total quantity of N-nitrosatable substances 0,1 mg/kg.

Analytical tolerance for the total quantity of N-nitrosamines 0,01 mg/kg.

(d) Indeed the Technical Committee considers the method used for the validation trial (and given in this current standard) as a significant improvement on that specified in EN 12868:1999 because the whole range of N-nitrosamines identified in teats were tested (unlike some previous trials, for example the 1996 validation trial which analysed for only NDMA, NDEA, NDBA and NDBzA). Even so, the current method has achieved previous determined reproducibility limits.

(e) An N-nitrosamine specific adjustment for NDiNA of 0,1 mg/kg was found to be justified (see Annex E).

(f) Although detailed comparisons of repeatability limits between this and previous historical validation trials is not possible, there is a strong suggestion that the repeatability limits for duplicate determinations (see Table F.7) are in general lower. The Technical Committee considers this as another improvement of the current method and justifies the changes made and the detailed defining of sections of the analysis; including sample preparation and isolation of N-nitrosamines.

(g) Based on these repeatability limits, a reliable single variability test was introduced for duplicate determinations.

Bibliography

This European Standard incorporates references to EU Directives and other publications. These references are cited at the appropriate place in the text and the publications list hereafter.

[1] Commission Directive 93/11/EEC, Commission Directive of 15 March 1993 concerning release of N-nitrosamines and N-nitrosatable substances from elastomer or rubber teats and soothers

[2] BIA Arbeitsmappe 30. Lfg. IV/03 (German) about the derivatisation of NDELA for GC TEA

[3] EN 71-12:2013, *Safety of toys - Part 12: N-Nitrosamines and N-nitrosatable substances*

[4] Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed. SANTE/11945/2015

[5] *Opinion of the Scientific Committee for food on nitrosamines in babies dummies and teats of Dec. 10, 1987*

[6] Die Technischen Regeln für Gefahrstoffe (TRGS) 552 of May 2007

[7] ISO 5725-2:1994. Accuracy (trueness and precision) of measurement methods and results- Part2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

[8] ISO/TR 22971:2005. Accuracy (trueness and precision) of measurement methods and results- Practical guidance for the use of ISO 5725-2:1994 in designing and statistically analysing interlaboratory repeatability and reproducibility results.

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