BRITISH STANDARD

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Workplace atmospheres — Determination of airborne endotoxins

The European Standard EN 14031:2003 has the status of a British Standard

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National foreword

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Workplace atmospheres - Determination of airborne endotoxins

Atmosphères des lieux de travail - Détermination des endotoxines en suspension dans l'air

Arbeitsplatzatmosphäre - Bestimmung von luftgetragenen Endotoxinen

This European Standard was approved by CEN on 21 November 2002.

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Contents

Foreword

This document (EN 14031:2003) has been prepared by Technical Committee CEN/TC 137 "Assessment of workplace exposure", the secretariat of which is held by DIN.

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of an identical text or by endorsement, at the latest by July 2003, and conflicting national standards shall be withdrawn at the latest by July 2003.

Annexes A and B are informative.

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Introduction

Endotoxins are integral components of the outer membrane of gramnegative bacteria which are composed of proteins, lipids, and lipopolysaccharides. The term 'endotoxin' refers to the toxin as present on the bacterial cell wall. Lipopolysaccharides of gram negative bacteria refer to a class of pure lipid carbohydrate molecules (free of protein and other cell wall components) that are held responsible for most of the biological properties characteristic of bacterial endotoxins. In annex A a brief overview is given with respect to physical and chemical properties of endotoxins and sources of exposure in the occupational environment.

Endotoxins are believed to play an important role in the development of organic dust related diseases in exposed workers.

It is important to assess occupational exposure to airborne endotoxins in a representative way to evaluate the exposure. There are, however, at present no generally accepted procedures for the measurement of environmental endotoxins, thus different practices continue to exist. Rigorous standardization with respect to sampling media, extraction media, analytical methods and storage conditions for endotoxins are needed to obtain results that are comparable between studies. By adhering to the recommendations outlined in this standard for choice of sampling, storage of samples, extraction and analytical procedures uncertainties in exposure assessment can be reduced and controlled, allowing comparable and representative measurements to be made.

1 Scope

This European Standard provides guidelines for the assessment of workplace exposure to airborne bacterial endotoxins. The standard provides methods for sampling, transportation, and storage of samples and determination of endotoxins.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate place in the text, and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references, the latest edition of the publication referred to applies (including amendments).

EN 481:1993, *Workplace atmospheres – Size fraction definitions for measurement of airborne particles.*

EN 1232, *Workplace atmospheres – Pumps for personal sampling of chemical agents – Requirements and test methods.*

EN 12919, *Workplace atmospheres – Pumps for the sampling of chemical agents with a volume flow rate of over 5 l/min - Requirements and test methods.*

3 Terms and definitions

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

3.1
bioaerosol
airborne particles with biological origin

NOTE See EN 13098:2000, 3.3, notes.

3.2
control standard endotoxin
standard that is traceable to the RSE

3.3
endotoxin
see EN 13098:2000.

3.4
endotoxinfree liquids
water and solutions with <0,2 EU/ml

NOTE Endotoxinfree and pyrogenfree are used interchangeable in the literature.

3.5
endotoxin unit
see EN 13098:2000

NOTE Endotoxin concentrations in samples are referenced to a defined reference standard endotoxin (RSE) and subsequently expressed in units based on the activity of the reference material in a LAL assay.

3.6
exposure (by inhalation)
situation in which a chemical or biological agent is present in air which is inhaled by a person

[EN 1540:1998]

3.7
inhalable particle size fraction
inhalable fraction which constitutes the mass fraction of total airborne particles which is inhaled through the nose and mouth

[EN 481:1993]

3.8
LAL-assay
functional assay to measure endotoxin concentrations

NOTE The method is highly sensitive and based on the activation of a clotting enzyme present in the lysate of hemolymph of the *Limulus polyphemus* (Horseshoe crab).

3.9
lipopolysaccharides
LPS present in gramnegative bacteria are water-soluble and stable molecules composed of lipid and polysaccharide

NOTE The lipid part of LPS is termed 'lipid A' and is essential for the toxic properties of LPS. The terms *'endotoxins'* and *'lipopolysaccharides'* are often used interchangeably in the scientific literature.

3.10
reference standard endotoxin
purified LPS from *Escherichia coli* that serves as an international reference standard

4 Abbreviations

CSE control standard endotoxin

EU endotoxin units

LAL limulus amoebocyte lysate

LPS lipopolysaccharides

RSE reference standard endotoxin

5 Overall demands in endotoxin work

5.1 General

Environmental monitoring of endotoxins is performed by sampling inhalable aerosols. Aerosols are sampled on a filter using a pump to draw air through the filter. For assessing occupational exposure personal sampling shall be used for example when comparing with exposure guidelines. Area sampling can be used to identify sources of exposure.

5.2 Demands on operator

The operator that performs the sampling shall be trained in aseptic work conditions, have knowledge of sampling equipment and know how to carry out the sampling. The operator shall avoid contamination of the sample during all phases of sampling.

5.3 Demands on equipment

The pumps shall fulfil the requirements of the standards for pumps for personal and static sampling (EN 1232 and EN 12919). Personal samplers shall collect the inhalable aerosol fraction as defined by EN 481.

The filter holder or cassette should be rendered endotoxinfree. Prior to sampling, reusable samplers should be cleaned thoroughly so that contamination of the samples are avoided. Cleaning agents shall be removed by subsequent washing in endotoxinfree water. Disposable samplers do not need to be sterilised when one can assume the manufacturing process to be fairly endotoxinfree. Possible contamination shall be controlled by using blank filters (see 5.9).

5.4 Demands on glassware

All other materials used during sampling, transportation and storage shall be rendered endotoxinfree. Glassware can be rendered endotoxinfree by heating at least at 180 °C for 4 h.

5.5 Demands on extraction liquids, water and detergents

All liquids used shall be endotoxinfree.

5.6 Demands on filters

Binderfree glass fibre filters should be used for endotoxin sampling while these filters usually give the highest recoveries. Other types of filters can be used. If any other filter is used it shall be validated toward filters of glass fibre.

NOTE Several types of filters (cellulose esters, polyvinylchloride, polytetrafluoroethylene and polycarbonate) are commonly used but some material adsorb endotoxins and thereby give false low values.

5.7 Transportation of samples

After sampling filters shall be prepared for transport by sealing in a container or sampler cassette. The sample shall be transported in dry conditions, preferably with a dehumidifier. Samples can be transported at room- or outside temperature but conditions for transport shall be documented and include transportation time, temperature etc. If transportation time is over 24 h the samples shall be transported in a condition that prevents the filters from getting moist during transportation, i.e. with a dehumidifier or frozen.

5.8 Storage of samples at the laboratory

Samples shall never be repeatedly frozen and thawed, as this may effect the detectable endotoxin content of the sample. Endotoxins are very stable when frozen. If the sample is not extracted within a few days after arrival to the laboratory the sample shall be stored at approximately –20 °C or below.

5.9 Test for contamination

To test for contamination a series of at least 2 blank filters shall be included in each sampling session. Except for the actual sampling, blank filters shall be treated equal to the other filters that are used for sampling.

5.10 Sampling documentation

A list of sampling operations shall be documented to obtain comparable and reliable concentration values of endotoxins. The sampling document shall include at least the following parameters:

- name of the organization and operator performing the sampling;
- date of sampling;
- a unique identifier code for the sample;
- name and address of the plant where sampling was carried out, or a unique identifier to preserve confidentiality;
- type of sampling (personal or static) and in case of personal sampling: name and function of the person who was involved in the sampling, or a unique identifier to preserve confidentiality; or in case of static sampling: location of sampling;
- type of sampler and type of filter used;
- start and end time of sampling;
- flow rate before and after sampling period;
- sampled volume;
- details about sample transportation and storage (temperature, duration, humidity);
- other relevant observations from the operator.

6 Extraction

6.1 General

The following paragraphs describe a standard method for the extraction of endotoxins from filters. If other equipment and methods are used they shall be validated against this described method.

6.2 Equipment

A standard shaker apparatus able to vigorously shake the sample in extraction fluid should be used. In addition a standard table centrifuge is needed that is able to centrifuge at 1 000 *G*. Endotoxinfree glass or polypropylene tubes shall be used throughout the extraction procedure.

6.3 How to extract

The loaded filter shall be transferred aseptically into an endotoxinfree container. Extraction shall be performed in endotoxinfree water using a method validated to maximise extraction. After extraction the pH should be measured to be compatible with the performance of the assay and in accordance with the recommendations of the manufacturer of the assay kit. After extraction, samples shall be centrifuged for 15 min at 1 000 *G*. Supernatant shall be collected for immediate analysis and the remainder stored frozen in at least three aliquots in case subsequent analysis is required. Frozen and thawed samples may give up to 25 % lower values.

NOTE The following method is recommended. For filters with a diameter of less than 50 mm a minimum of 5 ml of an extraction liquid should be used and a minimum of 10 ml for filters with a diameter of more than 50 mm. Samples are rocked/shaken for 1 h at room temperature, which has been shown to produce maximal extraction of endotoxin into suspension.

6.4 Storage

Supernatants of extracts shall be stored frozen at approximately –20 °C or below. The remainder of each aliquot shall after analysis be discarded. A second frozen aliquot will be used for a duplicate analysis but can give up to 25 % lower value. Extracts can be stored frozen in tightly sealed tubes for longer time periods (several years). Never repeatedly freeze and thaw an extract while this can affect the measurable endotoxin concentration in the fluid.

6.5 Extraction documentation

Documentation shall describe the extraction procedure (see 6.2, 6.3 and 6.4) and also document at least the following parameters:

- name of the organization and technician performing the extraction;
- date of extraction;
- unique identifier codes for the samples;
- extraction volume(s);
- number of aliquots per sample and volume per aliquot;
- other relevant observations from the technician.

7 Analytical methods

7.1 General

Kinetic chromogenic LAL-assays shall be used for the detection and quantification of endotoxin levels in the workplace atmosphere.

NOTE Other methods have been described for determination of chemical markers for endotoxin, e.g., GCMS, HPLC, but are not routinely applied.

7.2 Equipment

A kinetic reader that is able to read 96 wells plates and can be set at 37 °C is needed. Temperature in each well needs to be fixed at 37 °C ± 1 °C. A computer and software are needed to record the kinetic readings and to calculate the endotoxin concentrations.

NOTE Two distinctly different approaches exist to obtain the endotoxin concentration using a kinetic assay. The first is to calculate the concentration based on the maximum velocity (V_{max}) of the reaction. The reaction is followed over a prolonged time to facilitate this process. The second option is to calculate the concentration on the basis of only one measurement point, the

time elapsed till a certain optical density increase (most often OD = 0,2) is reached. The first approach (V_{max}-Method) is less sensitive to small analytical irregularities as can be induced by little air bubbles in the wells.

7.3 Procedure

7.3.1 Measurement process

Kinetic chromogenic LAL tests shall be used. Shake samples and LPS standard(s) vigorously for 3 min at high speed on a vortex mixer. This is important because aqueous endotoxins tend to adsorb on surfaces and forms agglomerates. Subsequently, samples (diluted if necessary: see 7.4 validation) or standards need to be mixed with an equal volume of LAL reagent and incubated at 37 °C ± 1 °C. Kinetic readings shall be recorded automatically each 30 s for a period of 50 min by a computer such that the maximum velocity of the kinetic reaction (V_{max}) can be used as an estimate of the endotoxin concentration. The reaction time required for the absorbance to increase 0,2 absorbance units may also be used as an estimate for the endotoxin concentration.

7.3.2 Calibration

New batches of LAL kits shall be calibrated in duplicate with at least two analytical blanks and eight calibration points for the range of 0,05 EU/ml to 50 EU/ml. For more narrow ranges the number of calibration point could be less than 8 but shall not be less than 4. New dilutions of the CSE shall be prepared and tested at four concentrations at intervals as specified by the supplier. This can be performed in a test run. Unknown samples shall be analysed at least with four concentrations of CSE. The blanks shall contain dilution medium which shall be the same medium as used for extraction. Prior to a routine test on unknown samples a validation protocol shall be performed as described in 7.4. Duplicate laboratory analyses shall be performed on the same sample on a different occasion using a new calibration curve and a second aliquot of the tested sample.

7.3.3 Calculation

Concentrations of samples shall be calculated by comparing V_{max} values of the sample with the calibration curve. Blanks shall not exceed the level of the lowest calibration point (0,1 EU/ml to 0,05 EU/ml). Extracts of blank filters shall not exceed the value of 0,2 EU/ml.

7.4 Validation

The method used should be validated at the laboratory with an international endotoxin assay standard. Inter-laboratory calibration or proficiency testing is recommended. Assays are at risk of being invalidated because for some samples other constituents present in the sample may interfere with the LAL assay, causing inhibition or enhancement. For example, deep coloration present in the extracted suspension could cause enhancement. It may be possible to dilute samples to remove such a problem. To determine whether inhibition or enhancement is affecting endotoxin assays performed on samples from occupational environments, one of the two procedures described in annex B shall be performed. If inhibition or enhancement is detected which exceeds acceptable levels, and measures to remove the problem are unsuccessful, the assay results shall be reported as invalid and the reasons stated. If some inhibition or enhancement is detected, but within accepted limits, it should be considered good practice to state this in the results report.

7.5 Analytical protocol

The documentation shall refer to the analytical method protocol (7.3 and 7.4) and also shall document at least the following parameters:

- name of the organization and technician performing the analysis;
- date of analysis;
- unique identifier codes for the samples;
- file name and path;

- lot number of LAL kit;
- lot number of reference endotoxin;
- conversion factor for weight versus EU's;
- reaction time and number of kinetic measurements;
- dilution at which each sample has been tested;
- concentrations of standards;
- number of replicates per sample;
- results validation protocol;
- an indication, if the analysis have been performed on frozen extracts;
- other relevant observations from the technician.

8 Expression of results

Endotoxin samples shall be referenced to the RSE. Since the RSE is too expensive and also exhaustible one has chosen to use a CSE, which is traceable to the RSE. The CSE is normally included in commercial LAL tests. Thus endotoxin results shall be expressed in EU per m^3.

9 Precision

When performing analysis of a sample the results obtained for the LAL-assay for duplicate extract analysis shall be less than 20 %.

10 Test report

The report shall include at least the following information:

- aim of the measurement(s);
- sampling strategy (including number of replicate samples);
- identification of the samples: in case of personal samples state function categories; in case of static samples state location(s) of sampling;
- documentation of sampling and extraction and analytical protocol according to clause 5, 6 and 7, respectively;
- coefficient of variation based on duplicate samples;
- results for blank filters as well as blanks;
- reference to this European Standard;
- interpretation and discussion of the results, including the results of the validation protocol.

Annex A
(informative)

Properties of endotoxins and sources of exposure

A.1 Identity and physical and chemical properties

A.1.1 Identity

Lipopolysaccharides (LPS) are amphipathic (i.e., soluble in water as well as lipids) macromolecules containing a lipid and a polysaccharide moiety. This group of macromolecules have been detected in such taxonomically remote groups of most gram negative bacteria as *Enterobacteriaceae*, *Pseudomonadaceae* and *Rhodospirillaceae* but have not been found in cell walls of gram positive bacteria, mycobacteria or fungi. Some blue green algae also contain lipopolysaccharides Inhalation studies, in which purified LPS has been used, have shown that the effects on the airways mimic those generally observed with occupational exposure to endotoxins. However, in occupational exposure (i.e., mainly by inhalation) it is most relevant to consider the biologically available LPS (referred to as endotoxin) as derived from aerosolised whole bacterial cells or cell components. The LAL assay measures biologically available endotoxins and therefore gives measurements relevant to occupational exposure assessment.

A.1.2 Physical and chemical properties

LPS are stable water soluble molecules composed of lipid and polysaccharide. The lipid moiety of LPS is termed 'lipid A' and is responsible for the toxic properties of LPS. The composition of Lipid A, an amphipathic and zwitterionic phosphoglycolipid, deviates significantly from other lipids in biological membranes. Among various bacterial species the composition of lipid A is remarkably constant. The hydrophilic polysaccharide moiety is composed of O-specific side chains and core sugars. The composition of O-specific chains varies considerably between bacterial species. Each bacterial processing industry, poultry slaughter houses, flax processing industry, animal feed industry, water sewage treatment and sewage composting plants, garbage handling facilities, organic waste composting facilities, wood chip composting and timber storing facilities, cotton mills, etc. Air humidifiers in buildings and recycled industrial process waters can also be an important source of airborne endotoxins exposure serotype synthesises unique LPS at least varying in the composition of the O-specific chain. The composition of the core is relatively constant and is identical or closely related for large groups of bacteria.

In the environment airborne endotoxins are usually associated with dust particles or aqueous aerosol with a broad size distribution.

A.2 Sources of occupational exposure

Because of their ubiquitous nature, bacterial endotoxins are found in various environments. Endotoxin levels are related to the occurrence of Gram negative bacteria which in turn are influenced by environmental conditions such as substrate availability, temperature, humidity etc. For occupational diseases, inhalation exposure is most relevant and has been studied in some detail over the last decade. Endotoxins can become airborne during manufacturing or handling of contaminated organic materials. Animal faeces and bacteria-contaminated plant materials contribute most in organic dust related endotoxin exposure. Endotoxin exposure is therefore most prevalent in agricultural and related industries such as pig, chicken, cow and horse farming, grain elevators, cotton industry, potato industry. In addition industrial oils and emulsions can also contain substantial amounts of endotoxins as a result of contamination with gram-negative bacteria. With exception of the latter, endotoxin exposure seems to be mainly associated with so called organic dust exposure (organic dust can vary from plant matter, to moulds and spores, mycotoxins, bacteria, their biochemical components (such as endotoxins), and excretions, enzymes and animal matter including parts of insects, rodents, birds and their excreta)

Annex B
(informative)

Principle of LAL-assays

B.1 General

This functional assay, which is highly sensitive, is based on the activation of a clotting enzyme (via factor C) present in the lysate of hemolymph of the *Limulus polyphemus* (horseshoe crab). Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the Limulus Amoebocyte Lysate (LAL). The initial rate of activation is determined by the concentration of endotoxin present.

The LAL-assay is adopted as the standard assay for endotoxin detection by the American Food and Drug Administration in 1980 and is normally performed in microtitre plates The LAL-method does not represent an absolute LPS measure but measures the portion of endotoxins that is biologically available to the assay. This biologically active portion measured with the LAL assay is expected to represent a relative toxicity measure. Kinetic LAL-assays are very sensitive and have a broad measurement range (0,01 (EU)/ml to 50 (EU)/ml). There are two type of assays available that are at present generally used with different detection methods; 1) the turbidimetric assay and 2) a chromogenic assay. The turbidimetric assay is based on the ability of endotoxins to induce gelation in LAL. As clot formation proceeds the reaction mixture becomes more turbid with the production of insoluble clotting enzyme. In the chromogenic assay, the natural LAL coagulogen is coupled with a synthetic colorless substrate that can be activated by the activated coagulase. The activated enzyme catalyses the splitting of p-nitroaniline (pNA) from the substrate which results in a yellow coloured solution that can be measured photometrically at 405 nm as shown below.

$$\text{1. Proenzyme} \xrightarrow{\text{endotoxins}} \text{Enzyme}$$

$$\text{2. Substrate} + H_2O \xrightarrow{\text{enzyme}} \text{Peptide} + \text{pNA}$$

Both assays can be performed as an endpoint test, where the measurement of optical density is made at the end of a fixed reaction time, or as a kinetic test where measurements take place continuously at defined intervals throughout a fixed reaction time or where the time elapse is measured to reach a required change in optical density. Since kinetic tests are more accurate and sensitive, these tests shall be used.

B.2 Detection of inhibition or enhancement of measured endotoxins using spiked samples

To detect inhibition or enhancement of the endotoxin assay, representative samples shall be spiked with a known amount of CSE endotoxin. The spike should be fully recovered (100 % $\pm$ 25 %). Therefore a dilution for routine analyses as described in 7.3 for the whole batch of samples shall be chosen such that no enhancement or inhibition effects occur, i.e., recovery of spike of <125 % and >75 % of the expected value.

B.3 Detection of inhibition or enhancement of measured endotoxins using parallel line assay

Representative samples shall be selected for each batch of samples and analysis performed as described in 7.3 on eight serial dilutions. The concentration, adjusted for the dilution factor, should give equal concentration estimates ($\pm$ 25 %) for each dilution when no inhibition or enhancement effects have occurred. Increased or decreased concentrations with increasing dilutions over a range of 10 to 10^3 fold dilution (sometimes higher dilutions are required) demonstrates that inhibition or enhancement effects have occurred. A dilution for routine analysis for the whole batch of samples shall be chosen such that no enhancement or inhibition effects occur, i.e., estimate results

at the chosen dilution should be <125 % and >75 % of adjusted concentrations from other dilutions. Addition of magnesiumchloride can also reduce inhibition or enhancement.

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