BS EN
1104:2005

Paper and board intended to come into contact with foodstuffs — Determination of the transfer of antimicrobial constituents

The European Standard EN 1104:2005 has the status of a British Standard

ICS 67.250; 85.060

BSi
British Standards

Contents

Foreword

This European Standard (EN 1104:2005) has been prepared by Technical Committee CEN/TC 172 "Pulp, paper and board", 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of an identical text or by endorsement, at the latest by February 2006, and conflicting national standards shall be withdrawn at the latest by February 2006.

This European Standard supersedes EN 1104:1995.

With regard to EN 1104:1995 the following changes have been made:

a) description of the preparation of some reagents were partly modified;

b) more detailed description of the procedure was formulated;

c) editorial updating.

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

1 Scope

This European Standard specifies a method for the determination of transfer of antimicrobial constituents from paper and board materials and articles intended for food contact.

2 Normative references

The following referenced documents are indispensable for the application of this European Standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 186, *Paper and board — Sampling to determine average quality (ISO 186:2002)*

3 Terms and definitions

For the purposes of this European Standard, the following term and definition applies.

3.1
inhibition zone
zone formed around the test pieces, placed on nutrient medium, which has been seeded with a pre-selected test organism that releases water-soluble antimicrobial constituents.

4 Principle

A prepared nutrient medium is mixed with an appropriate inoculum and poured into Petri dishes. The test pieces are placed on the semi solid nutrient medium and then incubated. When incubation is terminated, the existence of an inhibition zone is an indicator of the release of antimicrobial constituents.

The test is performed with a bacterium, *Bacillus subtilis*, and with a fungus, *Aspergillus niger*.

NOTE The result is based on a visual inspection.

5 Apparatus

5.1 Punch Iron,

d = 10 mm to 15 mm, sterilizable.

5.2 Pressing device,

suitable for pressing the test pieces on the agar plate (e.g. Drygalski spatula).

5.3 Zone reading device,

to measure the diameter of inhibition.

NOTE Measuring the diameter of the inhibition zone is not compulsory

5.4 Ordinary microbiological laboratory apparatus

6 Reagents

6.1 Water,

freshly distilled or water purified by ion exchange and freshly boiled (deionised water).

6.2 Non ionic wetting agent,

for example polyoxyethylenesorbitane monooleate.

6.3 Nutrient medium for *Bacillus subtilis*

A typical formulation of the nutrient medium is:

— beef extract 3,0 g;

— tryptone (peptone of casein) 5,0 g;

— sodium chloride, pure 5,0 g;

— agar-agar 12,0 g

— water 1000,0 ml.

NOTE The addition of 10 mg/l $MnSO_4$ to the nutrient medium for *Bacillus subtilis* (6.7.1) will support the formation of spores.

Prepare the nutrient medium as follows:

Dissolve the components, or a ready-made medium of a comparable composition, in water by boiling.

The pH of the ready prepared nutrient medium shall be (7,2 ± 0,2) referred at a temperature of 45 °C.

Adjust the pH to (7,2 ± 0,2) as required either with NaOH approx. 0,01 *M* or HCl approx. 0,01 *M*.

Separate the nutrient medium into two parts.

Dispense one part in 300,0 ml portions into nutrient medium flasks or 600,0 ml flasks e.g. Roux flasks and stopper them with caps, e.g. Kapsenberg caps.

Use the other part for the preparation of the working culture media into test tubes.

Dispense 10,0 ml portions into 15 to 20 test tubes and seal them with stoppers, e.g. cellulose stoppers.

Sterilise flasks and test tubes for 15 min at (121 ± 1) °C. After sterilisation position the test tubes immediately in such a way that the nutrient medium solidifies with a sloping surface.

Store them at 4 °C to 8 °C not longer than 14 d.

Cool the nutrient medium flasks to approx. 45 °C for the preparation of the inoculating suspension of *Bacillus subtilis* (6.7) or allow to solidify.

Cool the flasks to solid.

6.4 Sabouraud modified mould nutrient medium for *Aspergillus niger*

A typical formulation of the Sabouraud modified mould nutrient medium is:

— tryptone (peptone of casein) 5,0 g

— peptone (peptone of meat) 5,0 g;

— D (+) glucose $C_6H_{12}O_6 \cdot H_2O$ 10,0 g;

— maltose $C_{12}H_{22}O_{11} \cdot H_2O$ 10,0 g;

— agar-agar 10,0 g to 15,0 g;

— water 1000,0 ml

Prepare the Sabouraud modified mould nutrient medium as follows:

Dissolve the components, or a ready-made medium of a comparable composition, by boiling.

The pH of the ready prepared nutrient medium shall be (5,4 ± 0,1) referred at a temperature of 45 °C.

Adjust the pH to (5,4 ± 0,1) as required either with NaOH approx. 0,01 *M* or HCl approx. 0,01 *M*.

Proceed as described in 6.3 dispensing the medium into nutrient medium flasks or flasks e.g. Roux flasks and test tubes and the sterilising and cooling procedures.

6.5 Nutrient medium for inhibition test with *Bacillus subtilis*

The composition of the nutrient medium for inhibition test with *Bacillus subtilis* shall be as follows:

— tryptone (peptone of casein) 3,45 g;

— peptone (peptone of meat) 3,45 g;

— sodium chloride, pure 5,1 g;

— agar-agar 13,0 g;

— water 1000,0 ml.

Prepare the nutrient medium as follows:

Dissolve the components, or a ready-made medium of a comparable composition, in water by boiling.

The pH of the ready made nutrient medium shall be (6,0 ± 0,1) referred at a temperature of 45 °C. Adjust the pH to (6,0 ± 0,1) as required either with NaOH approx. 0,01 *M* or HCl approx. 0,01 *M*.

Dispense portions of nutrient medium flasks or test tubes and stopper them with caps. e.g. Kapsenberg caps and sterilise for 15 min at (121 ± 1) °C.

Cool the flasks to below 60 °C for the preparation of the inoculation medium (8.2.2) or allow to solidify.

6.6 Salt peptone solution

The composition of the salt peptone solution shall be as follows:

— peptone (peptone of meat) 1,0 g;

— sodium chloride, pure 8,5 g;

— water 1000,0 ml.

Prepare the salt peptone solution as follows:

Dissolve the components in water of pH between 6 and 7. Dispense equal volumes into three flasks, stopper with caps, e.g. Kaspensberg caps and sterilise for 15 min at (121 ± 1) °C.

The solution shall be used within 8 d if stored at room temperature and within 14 d when stored at 4 °C to 8 °C.

6.7 Test micro-organisms

6.7.1 General

The following are used:

Bacillus subtilis DSM 347 (ATCC 6633) and *Aspergillus niger* DSM 1957 (ATCC 6275) or other corresponding strains.

As different strains may also have different sensitivity, a comparative examination would have to be performed when using other strains than ATTC 6633 and ATTC 6275.

Working cultures of *Bacillus subtilis* are obtained by inoculating onto the test tubes (6.3) and incubating for 7 d at 30 °C. After incubation the test tubes are stored at 4 °C to 8 °C.

Working cultures of *Aspergillus niger* are obtained by inoculating onto the test tubes (6.4) and incubating for 5 d at 25 °C. After incubating the test tubes are stored at 4 °C to 8 °C.

6.7.2 Preparation of inoculating spore suspension of *Bacillus subtilis*

Transfer aliquots of about 15,0 ml of the liquefied nutrient medium (6.3) cooled to approximately 45 °C to 10 sterile Petri dishes (d = 90 mm) and allow to solidify.

The nutrient medium in flasks (6.3) is ready for inoculation.

Wash off the colonies of ten test tubes with the working culture of *Bacillus subtilis* (6.7.1) with 2,0 ml to 3,0 ml sterile salt, peptone solution (6.6). Spread the washings over the surface of the ten Petri dishes (each dish is incubated from a separate tube) or all the washings over the surface of the Roux flask.

Incubate for 7 d at 30 °C. Wash off the colonies from the Petri dishes with 3,0 ml salt peptone solution (6.6) and the flask with 30,0 ml salt peptone solution (6.6). Bring the suspension over into a sterile flask by using a sterile funnel and close the flask with a sterile stopper.

Heat the solution with occasional shaking, for 30 min in a water bath at approx. 85 °C in order to kill the vegetative forms. After heating transfer the spore suspension to a sterile centrifuging flask of 40,0 ml and centrifuge for 10 min at 10000 g. Eliminate the liquid. Wash the residue with 30,0 ml salt peptone solution (6.6) and centrifuge again. Repeat the washing 3 times. Suspend the spores in 20,0 ml of the salt peptone solution (6.6).

The spore suspension may be stored at 4 °C to 8 °C not longer than 4 weeks.

NOTE The spore suspension is also commercially available.

6.7.3 Preparation of inoculating spore suspension of *Aspergillus niger*

Transfer aliquots of about 15,0 ml of the liquefied modified Sabouraud medium (6.4), cooled to approx. 45 °C to at least 5 sterile Petri dishes (d = 90 mm) and allow to solidify.

The nutrient medium in flask (6.4) is ready for inoculation.

Inoculate the *Aspergillus niger* strain from the working cultures (6.7.1) with an inoculation loop onto the Petri dish. Each Petri dish is inoculated from a separate tube. The flask is incubated from at least five test tubes.

Incubate for 8 d to 10 d at 25 °C. Transfer the conidia with an inoculating ring moistened with salt peptone solution (6.6) to a sterile test tube containing 10,0 ml of salt peptone solution (6.6) mixed with 0,01 ml of a non ionic wetting agent (6.2) and seal with a sterile stopper.

Shake the dispension well before using. The inoculating suspension may be stored at 4 °C to 8 °C not longer than 4 weeks.

6.7.4 Concentrations of spores for inhibition test

Dilute the spore suspension such that the concentration of spores in the agar is:

— *Bacillus subtilis*: 10^4 spores per ml test agar;

— *Aspergillus niger*: 10^5 conidia per ml test agar.

Measure the spore density of the inoculation suspension of *Bacillus subtilis* (6.7.2) by the conventional plate counting method on nutrient medium (6.3). Determine the spore density of the inoculation suspension of *Aspergillus niger* (6.7.3) using the conventional plate counting method on nutrient medium (6.4).

6.8 Positive controls

6.8.1 Penicillin, G 0,03 units

(commercially available).

6.8.2 Isothiazolinon-solution

Mixture of 5-chloro-2-methyl-4-isothiazolin-3-on and 2-methyl-4-isothiazolin-3-on with a concentration of active substance of 2,5 % (diluted 1 : 100).

NOTE This solution is commercially available.

7 Sampling and preparation of test pieces

Sample in accordance with EN ISO 186. Touch only the edges of the samples. Lay the samples immediately one above the other in a sterile sample vessel or wrap the samples in aluminium foil.

Take at least ten samples per unit. Punch at least 20 circular test pieces for each micro-organism out of the samples with a sterile punch iron (5.1). Transfer the test pieces immediately into a sterile vessel. Touch the test pieces only with sterile forceps or tweezers.

8 Procedure

8.1 Sterilisation

Sterilise the forceps or tweezers, punch iron, centrifuging flasks (packed in aluminium foil), conical flasks, test tubes with cellulose stoppers and vessels in the autoclave for 15 min at (121 ± 1) °C. Sterilise volumetric pipettes made of glass in pipette boxes in the hot air sterilizer for 2 h at (180 ± 1) °C.

8.2 Preparation of plates

8.2.1 General

Prepare at least three Petri dishes for each test.

8.2.2 *Bacillus subtilis*

Liquefy sterile test agar (6.5). Cool to below 60 °C. Add an amount of the inoculating spore suspension (6.7.2) resulting in a density of 10^4 spores per ml test agar. Distribute the suspension evenly by careful shaking. Dispense aliquots of precisely 15,0 ml into each sterile Petri dish (d = 90 mm). Lay three test pieces on the still semi solid nutrient medium using sterile forceps or tweezers. Press the test pieces down slightly with a suitable sterile device (5.2), ensuring that no air cushions are formed.

The side intended to come into contact with foodstuffs, should be placed face-downwards. Otherwise both sides shall be tested.

Prepare for each analysis a negative control (blank) nutrient agar plate without test pieces.

Prepare a positive control. Penicillin G 0,03 units (6.8.1) impregnated on a small paper sample (commercially available).

8.2.3 *Aspergillus niger*

Prepare plates as described in 8.2.2, but in this case with Sabouraud modified nutrient medium (6.4). This suspension should be used as the test agar and as inoculating suspension of *Aspergillus niger* (6.7.3). A density of 10^5 conidia per ml test agar should be reached.

Prepare for each analysis a negative control (blank) nutrient agar plate without test pieces.

Prepare a positive control. Isothiazolinon solution (mixture of 5-chloro-2-methyl-4-isothiazolin-3-on and 2-methyl-4-isothiazolin-3-on) (6.8.2) with a concentration of active substance of 2,5 %. The solution is diluted 1 : 100. From the diluted solution 50 µl is transferred with a pipette to a small filter paper (d = 1,2 cm), which is used as the control sample.

8.3 Incubation

The Petri dishes prepared as in 8.2.2 and 8.2.3 are stored for 2 h in a refrigerator at 4 °C to 8 °C to facilitate pre-diffusion.

Incubate Petri dishes prepared as in 8.2.2 and 8.2.3 at 30 °C and 25 °C respectively.

Arrange the plates in the refrigerator and in the incubator in such a way that the cover of the Petri dish is underneath preventing condensation water dripping onto the sample.

9 Evaluation

Evaluate the tests with the bacterium or the fungus after 3 d and 5 d respectively. Also evaluate the positive control.

NOTE 1 A preliminary inspection of the Petri dishes after 1 d and 2 d is useful.

Samples without evidence of an inhibition zone area are considered to contain no water-soluble antimicrobial substances. Evidence of an inhibition zone is given if there is no growth or a perceptible reduction of growth (approx. 20 % less than in the surrounding area) than in the surrounding area. Samples with inhibition zones (positive controls) should also be assessed. The diameter of the test disc shall be given.

NOTE 2 Samples overgrown are evaluated as free of inhibition zone.

NOTE 3 In case of interest it is recommended to measure the inhibition zone outside of the test piece with a magnifying glass and a measuring scale and to give the diameter of the test piece.

NOTE 4 If the negative control nutrient agar plates exhibit no growth the test is repeated with new inoculating suspension. If the positive controls show growth the test should be repeated.

10 Test report

The test report shall include the following information:

a) Reference to this European Standard;

b) date and place of testing;

c) identification of material tested including the diameter of the test piece;

d) transfer of antimicrobial constituents or no transfer of antimicrobial constituents;

e) any deviations from this European Standard;

f) every deviation from this specified test method which may have affected the results.