

SLOVENSKI STANDARD

SIST EN 1657:2006

01-junij-2006

Nadomešča:

SIST EN 1657:2001

Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za vrednotenje fungicidnega delovanja ali delovanja kemičnih razkužil in antiseptikov na kvasovke v veterini - Preskusna metoda in zahteve (faza 2, stopnja 1)

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in the veterinary area - Test method and requirements (phase 2, step 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire - Méthode d'essai et prescriptions (phase 2, étape 1)

Ta slovenski standard je istoveten z: EN 1657:2005

ICS:

11.080.20	Dezinfektanti in antiseptiki	Disinfectants and antiseptics
11.220	Veterinarstvo	Veterinary medicine

SIST EN 1657:2006

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EUROPEAN STANDARD

EN 1657

NORME EUROPÉENNE

EUROPÄISCHE NORM

December 2005

ICS 11.080.20; 71.100.35

Supersedes EN 1657:2000

English Version

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in the veterinary area - Test method and requirements (phase 2, step 1)

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire - Méthode d'essai et prescriptions (phase 2, étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 28 July 2005.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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Foreword

This European Standard (EN 1657:2005) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, 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 June 2006, and conflicting national standards shall be withdrawn at the latest by June 2006.

This European Standard supersedes EN 1657:2000.

It was revised to include the results of a collaborative trial (ANDISTAND), to correct obvious errors and ambiguities, to harmonize the structure and wording with other quantitative suspension tests of CEN/TC 216 existing or in preparation, and to improve the readability of the standard and thereby make it more understandable.

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.

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Introduction

This European Standard specifies a suspension test for establishing whether a chemical disinfectant or antiseptic has a fungicidal or yeasticidal activity in the fields described in the scope.

This laboratory test takes into account practical conditions of application of the product, including contact time, temperature, test organisms and interfering substances, i.e. conditions which may influence its action in practical situations.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each utilization concentration of the chemical disinfectant or antiseptic found by this test corresponds to defined experimental conditions. However, for some applications the recommendations of use of a product may differ and therefore additional test conditions need to be used.

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1 Scope

This European Standard specifies a test method and the minimum requirements for fungicidal or yeasticidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation when diluted with hard water or — in the case of ready-to-use-products — with water. Products can only be tested at a concentration of 80 % or less, as some dilution is always produced by adding the test organisms and interfering substance.

This European Standard applies to products that are used in the veterinary area – i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in the food chain following death and entry to the processing industry.

NOTE 1 The method described is intended to determine the activity of commercial formulations or active substances under the conditions in which they are used.

NOTE 2 This method corresponds to a phase 2 step 1 test (Annex F).

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 12353, *Chemical disinfectants and antiseptics — Preservation of microbial strains used for the determination of bactericidal and fungicidal activity*

ISO 4793, *Laboratory sintered (fritted) filters — Porosity grading, classification and designation*

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3 Terms and definitions

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

3.1

product

chemical agent or formulation used as chemical disinfectant or antiseptic

3.2

fungicide

product that kills fungi (moulds and yeasts) and their spores under defined conditions

NOTE The adjective derived from "fungicide" is "fungicidal".

3.3

fungicidal activity

capability of a product to produce a reduction in the number of viable vegetative yeast cells and mould spores of relevant test organisms under defined conditions

3.4

fungistatic activity

capability of a product to inhibit the growth of fungi (moulds and/or yeasts) under defined conditions

3.5

yeasticide

product that kills yeasts under defined conditions

NOTE The adjective derived from "yeasticide" is "yeasticidal".

EN 1657:2005 (E)**3.6****yeastocidal activity**

capability of a product to produce a reduction in the number of viable yeast cells of relevant test organisms under defined conditions

4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction when diluted with hard water (**5.2.2.7**) or – in the case of ready-to-use products – with water (**5.2.2.2**) and tested in accordance with **Clause 5** under simulated low-level soiling (3 g/l bovine albumin solution – **5.2.2.8.2**) or simulated high-level soiling (10 g/l bovine albumin solution plus 10 g/l yeast extract – **5.2.2.8.3**) according to its practical applications and under the other obligatory test conditions (one or two selected test organisms, 10 °C, 30 min).

The fungicidal activity shall be evaluated using the following two test organisms:

- *Candida albicans* (vegetative cells);
- *Aspergillus niger* (spores).

The yeastocidal activity shall be evaluated using the following test organism:

- *Candida albicans* (vegetative cells).

Where indicated, additional specific fungicidal or yeastocidal activity shall be determined applying other contact times, temperatures and test organisms in accordance with **5.2.1** and **5.5.1.1** in order to take into account intended specific use conditions.

NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.

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5 Test method**5.1 Principle**

5.1.1 A sample of the product as delivered and/or diluted with hard water (or water for ready-to-use products) is added to a test suspension of fungi (yeast cells or mould spores) in a solution of an interfering substance. The mixture is maintained at (10 ± 1) °C for 30 min \pm 10 s (obligatory test conditions). At the end of this contact time, an aliquot is taken, and the fungicidal and/or the fungistatic activity in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving fungi in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using the vegetative cells of *Candida albicans* and the spores of *Aspergillus niger* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeastocidal activity) as test organisms (obligatory test conditions).

5.1.3 Additional and optional contact times and temperatures are specified. Additional test organisms can be used.

5.2 Materials and reagents

5.2.1 Test organisms

The fungicidal activity shall be evaluated using the following strains as test organisms:¹⁾

— *Candida albicans* ATCC 10231;

— *Aspergillus niger* ATCC 16404.

The yeasticidal activity shall be evaluated using only *Candida albicans*.

NOTE See Annex A for strain references in some other culture collections.

The required incubation temperature for these test organisms is $(30 \pm 1) ^\circ\text{C}$ (5.3.2.3).

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for five years.

5.2.2 Culture media and reagents

5.2.2.1 General

All weights of chemical substances given in this European Standard refer to the anhydrous salts. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

NOTE 1 To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

NOTE 2 For each culture medium and reagent, a limitation for use should be fixed.

5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralized water.

Sterilize in the autoclave [5.3.2.1 a)].

NOTE 1 Sterilization is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilized.

NOTE 2 If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

NOTE 3 See 5.2.2.7 for the procedure to prepare hard water.

1) The ATCC numbers are the collection numbers of strains supplied by the American Type Culture Collection (ATCC). This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

EN 1657:2005 (E)**5.2.2.3 Malt extract agar (MEA)**

Malt extract agar, consisting of:

Malt extract	30,0 g
Soya peptone, papaic digest of soybean meal	3,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization, the pH of the medium shall be equivalent to $5,6 \pm 0,2$ when measured at $(20 \pm 1) ^\circ\text{C}$.

NOTE In case of encountering problems with neutralization (5.5.1.2 and 5.5.1.3), it may be necessary to add neutralizer to the MEA. Annex B gives guidance on the neutralizers that may be used.

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride (NaCl)	8,5 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization, the pH of the diluent shall be equivalent to $7,0 \pm 0,2$ when measured at $(20 \pm 1) ^\circ\text{C}$.

5.2.2.5 Neutralizer

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The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. It shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.3. It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in 5.5.3.

NOTE Information on rinsing liquids that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.7 Hard water for dilution of products

For the preparation of 1 l of hard water, the procedure is as follows:

- prepare solution A: dissolve 19,84 g magnesium chloride (MgCl_2) and 46,24 g calcium chloride (CaCl_2) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1 a)]. Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month;

- prepare solution B: dissolve 35,02 g sodium bicarbonate (NaHCO_3) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;
- place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be $7,0 \pm 0,2$, when measured at $(20 \pm 1) ^\circ\text{C}$ (5.3.2.4). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

NOTE When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate (CaCO_3) in the test tube.

5.2.2.8 Interfering substance

5.2.2.8.1 General

The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 10 times its final concentration in the test.

The ionic composition (e.g. pH, calcium and/or magnesium hardness) and chemical composition (e.g. mineral substances, protein, carbohydrates, lipids and detergents) shall be defined.

NOTE The term "interfering substance" is used even if it contains more than one substance.

5.2.2.8.2 Low-level soiling (bovine albumin solution)

Dissolve 3,0 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of water (5.2.2.2).

Sterilize by membrane filtration (5.3.2.7), keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration of bovine albumin in the test procedure (5.5) is 3,0 g/l.

5.2.2.8.3 High-level soiling (mixture of bovine albumin solution with yeast extract)

Dissolve 50,0 g yeast extract powder in 150 ml of water (5.2.2.2) in a 250 ml volumetric flask (5.3.2.12) and allow foam to collapse. Make up to the mark with water (5.2.2.2). Transfer to a clean dry bottle and sterilize in an autoclave [5.3.2.1 a)]. Allow to cool to $(20 \pm 1) ^\circ\text{C}$.

Pipette 25 ml of this solution into a 50 ml volumetric flask (5.3.2.12) and add 10 ml of water (5.2.2.2). Dissolve 5,0 g of bovine albumin fraction V (suitable for microbiological purposes) in the solution with shaking and allow foam to collapse. Make up to the mark with water (5.2.2.2), sterilize by membrane filtration (5.3.2.7), keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration in the test procedure (5.5) is 10,0 g/l yeast extract and 10,0 g/l bovine albumin.

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5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of the apparatus that will come into contact with the culture media and reagents or the sample, except those which are supplied sterile, by one of the following methods:

- a) by moist heat, in the autoclave [5.3.2.1 a)];
- b) by dry heat, in the hot air oven [5.3.2.1 b)].

5.3.2 Usual microbiological laboratory equipment ²⁾ and, in particular, the following:

5.3.2.1 Apparatus for sterilization:

- a) for moist heat sterilization, an autoclave capable of being maintained at (121_0^{+3}) °C for a minimum holding time of 15 min;
- b) for dry heat sterilization, a hot air oven capable of being maintained at (180_0^{+5}) °C for a minimum holding time of 30 min, at (170_0^{+5}) °C for a minimum holding time of 1 h or at (160_0^{+5}) °C for a minimum holding time of 2 h.

5.3.2.2 **Water baths**, capable of being controlled at (10 ± 1) °C, at (45 ± 1) °C (to maintain melted MEA in case of pour plate technique) and at additional test temperatures ± 1 °C (5.5.1).

5.3.2.3 **Incubator**, capable of being controlled at (30 ± 1) °C.

5.3.2.4 **pH-meter**, having an inaccuracy of calibration of no more than $\pm 0,1$ pH units at (± 1) °C.

NOTE A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (5.2.2.3).

5.3.2.5 **Stopwatch**

5.3.2.6 **Shakers**

- a) Electromechanical agitator, e.g. Vortex[®] mixer³⁾
- b) Mechanical shaker

5.3.2.7 **Membrane filtration apparatus**, constructed of a material compatible with the substances to be filtered.

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilization of hard water (5.2.2.7) and bovine albumin (5.2.2.8), and if the membrane filtration method is used (5.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the micro-organisms over the membrane and to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

2) Disposable sterile equipment is an acceptable alternative to reusable glassware.

3) Vortex[®] is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

- 5.3.2.8 **Refrigerator**, capable of being controlled at 2 °C to 8 °C.
- 5.3.2.9 **Graduated pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml, or calibrated automatic pipettes.
- 5.3.2.10 **Petri dishes (plates)**, of size 90 mm to 100 mm.
- 5.3.2.11 **Glass beads**, 3 mm to 4 mm in diameter.
- 5.3.2.12 **Volumetric flasks**.
- 5.3.2.13 **Fritted filter**, with porosity of 40 µm to 100 µm according to ISO 4793.
- 5.3.2.14 **Centrifuge** (2 000 g_N).
- 5.3.2.15 **Roux bottles or similar flasks**.

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 General

For each test organism, two different suspensions have to be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

5.4.1.2 Preservation and stock cultures of test organisms

The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.4.1.3 Working culture of test organisms

5.4.1.3.1 *Candida albicans* (yeast)

In order to prepare the working culture of *Candida albicans* (5.2.1), prepare a subculture from the stock culture (5.4.1.2) by streaking onto MEA (5.2.2.3) slopes or plates (5.3.2.10) and incubate (5.3.2.3). After 42 h to 48 h, prepare a second subculture from the first subculture in the same way and incubate for 42 h to 48 h. From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subcultures are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 72 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator (5.3.2.3) during the 72 h period.

Never produce and use a fourth subculture.

5.4.1.3.2 *Aspergillus niger* (mould)

For *Aspergillus niger* (5.2.1), use only the first subculture grown on MEA (5.2.2.3) in Roux bottles (5.3.2.15) and incubate for 9 d to 11 d. No further subculturing is needed.

5.4.1.3.3 Other test organisms (yeasts or moulds)

For additional test organisms, any departure from this method of culturing the yeast or the mould or of preparing the suspensions shall be noted, giving the reasons in the test report.