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SOUTH AFRICAN NATIONAL STANDARD

Cosmetics — Microbiology — Enumeration of yeast and mould

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Table of changes

Change No.	Date	Scope

Foreword

This South African standard was prepared by National Committee SABS/TC 217, *Cosmetics*, in accordance with procedures of the South African Bureau of Standards, in compliance with annex 3 of the WTO/TBT agreement.

This document was approved for publication in March 2020.

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INTERNATIONAL STANDARD

ISO 16212

Second edition
2017-06

Cosmetics — Microbiology — Enumeration of yeast and mould

Cosmétiques — Microbiologie — Dénombrement des levures et des moisissures

Reference number
ISO 16212:2017(E)

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

This second edition cancels and replaces the first edition (ISO 16212:2008), of which it constitutes a minor revision. The changes compared to the previous edition are as follows:

— in the Scope, "see ISO 29621" has been added and the reference has been added to the Bibliography;

— in the Scope, "used" has been changed to "substituted" and "validated" has been changed to "shown to be suitable";

— in 4.1, "validated" has been changed to "demonstrated";

— in 4.3, "by a valid method" has been changed to "as described in Clause 12" and "validated procedure" has been replaced by "described procedure";

— in 5.1, "specifications" has been changed to "instructions";

— in 5.2.3.1.2, "peptone" has been changed to "peptic digest of animal tissue";

— in Clause 7, "validation" has been changed to "suitability";

— in 9.3.2.1, "validated" has been changed to "demonstrated to be suitable";

— in 9.3.2.3, "prepared as validated" has been changed to "demonstrated to be suitable";

— in 11.2.1, "validated according to" has been changed to "demonstrated to be suitable for";

— in 12.3, "validation" has been changed to "suitability";

— in 12.3.2, instances of "validation" have been changed to "suitability test" and "validated" has been changed to "satisfactory";

— in 12.3.3, the first instance of "validation" has been changed to "suitability" and the second instance has been changed to "suitability test"; "validated" has been changed to "satisfactory";

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— in 12.3.4, the first instance of “validation” has been changed to “suitability” and the second instance has been changed to “suitability test”; “validated” has been changed to “satisfactory”;

— in Clause 13 f), “validation” has been changed to “suitability”;

— in A.1, B.1 and C.1, “validated” has been changed to “demonstrated to be suitable”.

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INTERNATIONAL STANDARD ISO 16212:2017(E)

Cosmetics — Microbiology — Enumeration of yeast and mould

1 Scope

This document gives general guidelines for enumeration of yeast and mould present in cosmetics by counting the colonies on selective agar medium after aerobic incubation.

In order to ensure product quality and safety for consumers, it is advisable to perform an appropriate microbiological risk analysis to determine the types of cosmetic products to which this document is applicable. Products considered to present a low microbiological risk (see ISO 29621) include those with low water activity or extreme pH values, hydro-alcoholic products, etc.

Because of the large variety of cosmetic products within this field of application, this method might not be suited to some products in every detail (e.g. certain water-immiscible products). Other methods (e.g. automated) can be substituted for the tests presented here provided that their equivalence has been demonstrated or the method has been otherwise shown to be suitable.

Yeast enumerated can be identified using suitable identification tests, for example, tests described in the standards listed in the Bibliography. Mould enumerated can be identified by other appropriate methods, if necessary.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 21148, *Cosmetics — Microbiology — General instructions for microbiological examination*

EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
yeast
single-cell fungus, which multiplies mainly vegetatively by budding, able to grow under the test conditions specified in this document

3.2
mould
mycelium forming microfungus, including spores and conidia, able to grow under the test conditions specified in this document

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3.3
product
portion of an identified cosmetic product received in the laboratory for testing

3.4
sample
portion of the *product* (3.3) (at least 1 g or 1 ml) that is used in the test to prepare the initial suspension

3.5
initial suspension
suspension (or solution) of the *sample* (3.4) in a defined volume of an appropriate enrichment broth

3.6
sample dilution
dilution of the *initial suspension* (3.5)

4 Principles

4.1 General

This method involves enumeration of colonies on a selective agar medium. The possible inhibition of fungal growth by the sample shall be neutralized to allow the detection of viable microorganisms[5]. In all cases and whatever the methodology, the neutralization of the antifungicidal properties of the product shall be checked and demonstrated[6][8][9].

4.2 Plate count

Plate count consists of the following steps.

— Preparation of poured plates, or spread plates, using a specified culture medium, and inoculation of the plates using a defined quantity of the initial suspension or dilution of the product.

— Aerobic incubation of the plates at 25 °C ± 2,5 °C for 3 d to 5 d.

— Counting of the number of colony-forming units (CFU) and calculation of the amount of yeast and mould per millilitre or per gram of product.

NOTE An alternative condition for incubation is 22,5 °C ± 2,5 °C for 5 d to 7 d using the culture medium without antibiotic.

4.3 Membrane filtration

Membrane filtration consists of the following steps.

— Transfer a suitable amount of the sample, prepared as described in Clause 12, in the filtration apparatus, wetted with a small volume of an appropriate sterile diluent. Filter immediately and wash according to the described procedure (see 12.3.4). Transfer the membrane filter onto the surface of the specified agar medium as specified in ISO 21148.

— Aerobic incubation of the membranes at 25 °C ± 2,5 °C for 3 d to 5 d.

— Counting of the number of colony-forming units (CFU) and calculation of the amount of yeast and mould per millilitre or per gram of product.

NOTE An alternative condition for incubation is 22,5 °C ± 2,5 °C for 5 d to 7 d using the culture medium without antibiotic.

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5 Diluents, neutralizers and culture media

5.1 General

General instructions are given in ISO 21148. When water is mentioned in this document, use distilled water or purified water as specified in ISO 21148.

The following diluents, neutralizers and culture media are suitable for enumeration of yeasts and moulds. Other diluents, neutralizers and culture media may be used if they have been demonstrated to be suitable for use.

5.2 Neutralizing diluents and diluents

5.2.1 General

The diluent is used to disperse the sample. It may contain neutralizers if the sample to be tested has antifungicidal properties. The efficacy of the neutralization shall be demonstrated before the determination of the count (see Clause 12). Information relative to suitable neutralizers is given in Annex D.

5.2.2 Neutralizing diluent

5.2.2.1 Fluid casein digest–soy lecithin–polysorbate 20 medium (SCDLP 20 broth)

5.2.2.1.1 Composition

Pancreatic digest of casein	20,0 g
Soy lecithin	5,0 g
Polysorbate 20	40 ml
Water	960 ml

5.2.2.1.2 Preparation

Dissolve the polysorbate 20 in 960 ml of water by mixing while heating in a water bath at 49 °C ± 2 °C. Add pancreatic digest of casein and soy lecithin. Heat for about 30 min to effect solution. Mix and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.2.2.2 Other neutralizing diluents

Other neutralizing diluents may be used as appropriate (see Annex A and Annex D).

5.2.3 Diluent

5.2.3.1 Fluid A

5.2.3.1.1 Composition

Peptic digest of animal tissue	1,0 g
Water	1 000 ml

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5.2.3.1.2 Preparation

Dissolve 1 g of peptic digest of animal tissue in water to make 1 l. Heat with frequent agitation. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

5.2.3.2 Other diluents

Other diluents may be used as appropriate (see Annex B).

5.3 Diluent for yeast suspension (tryptone sodium chloride solution)

5.3.1 Composition

Tryptone, pancreatic digest of casein	1,00 g
Sodium chloride	8,50 g
Water	1 000 ml

5.3.2 Preparation

Dissolve the components in the water by mixing while heating. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.4 Culture media

5.4.1 General

Culture media may be prepared as follows or from dehydrated culture media according to the manufacturer's instructions. Ready-to-use media may be used when their composition and/or growth yields are comparable to those of the formulae given herein.

5.4.2 Sabouraud dextrose chloramphenicol agar medium (SDCA)

5.4.2.1 Composition

Dextrose	40,0 g
Peptic digest of animal tissue	5,0 g
Pancreatic digest of casein	5,0 g
Chloramphenicol	0,050 g
Agar	15,0 g
Water	1 000 ml

5.4.2.2 Preparation

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave

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at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

NOTE For known and non-contaminated products (with bacteria), the media are used without chloramphenicol.

5.4.3 Other media

Other media may be used as appropriate (see Annex C).

5.4.4 Agar medium for cultivation of reference strain: Sabouraud dextrose agar medium (SDA)

5.4.4.1 Composition

Dextrose	40,0 g
Peptic digest of animal tissue	5,0 g
Pancreatic digest of casein	5,0 g
Agar	15,0 g
Water	1 000 ml

5.4.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

6 Apparatus and glassware

The laboratory equipment, apparatus and glassware are described in ISO 21148.

7 Strain of microorganisms

For testing the efficacy of neutralizers, one yeast reference strain is used:

— *Candida albicans* ATCC[1)] 10231 or equivalent strain (IP[2)] 48.72 or NCPF[3)] 3179 or NBRC[4)] 1594 or KCTC[5)] 17205 or TISTR[6)] 5779) or other equivalent national collection strain.

The selected yeast strain being considered more susceptible to antifungicidal activity than moulds may be accepted as representative of fungi (yeast and mould) for the suitability of the methodology. However, in case of specific needs, the test for the efficacy of neutralizers may be performed with an additional mould reference strain, using a suitable protocol for the preparation of a calibrated inoculum (e.g. see EN 13624:2013, 5.4.1.4[3]).

The culture should be reconstituted according to the procedures provided by the supplier of the reference strain.

---

1) ATCC = American Type Culture Collection.

2) IP = Institut Pasteur.

3) NCPF = National Collection of Pathogenic Fungi.

4) NBRC = Biological Resource Center, NITE.

5) KCTC = Korean Collection for Type Cultures.

6) TISTR = Thailand Institute of Scientific and Technological Research.

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The strain may be kept in the laboratory in accordance with EN 12353.

8 Handling of cosmetic products and laboratory samples

If necessary, store products to be tested at room temperature.

Do not incubate, refrigerate or freeze products (3.3) and samples (3.4) before or after analysis.

Sampling of cosmetic products to be analysed should be carried out as described in ISO 21148. Analyse samples as described in ISO 21148 and according to the procedure described in Clause 9.

9 Procedure

9.1 General recommendation

Use sterile material, equipment and aseptic techniques to prepare the sample, initial suspension and dilutions. In the case of the preparation of the initial suspension, the time that elapses between the end of the preparation and the moment the inoculum comes into contact with the culture medium shall not exceed 45 min, unless specifically mentioned in the established protocols or documents.

9.2 Preparation of the initial suspension

9.2.1 General

The initial suspension is prepared from a sample of at least 1 g or 1 ml of the well-mixed product under test.

Note *S*, the exact mass or volume of the sample.

The initial suspension is usually a 1:10 dilution. Larger volumes of diluent may be required if high levels of contamination are expected and/or if antifungicidal properties are still present in the 1:10 dilution.

9.2.2 Water-miscible products

Transfer the sample, *S*, of product to an appropriate volume (e.g. 9 ml) of neutralizing diluent (5.2.2) or diluent (5.2.3).

Note *d*, the dilution factor.

9.2.3 Water-immiscible products

Transfer the sample, *S*, of product to a suitable container containing a suitable quantity of solubilizing agent (e.g. polysorbate 80 solution). Disperse the sample within the solubilizing agent and add an appropriate volume (e.g. 9 ml) of neutralizing diluent (5.2.2) or diluent (5.2.3).

Note *d*, the dilution factor.

9.3 Counting methods

9.3.1 Dilutions for counting methods

Usually, the initial suspension is the first counted dilution. If needed, additional serial dilutions (e.g. 1:10 dilution) may be performed from the initial suspension using the same diluent (according to the expected level of contamination of the product).

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Generally, counting is performed using at least two Petri dishes, but it is possible to use only one Petri dish in case of routine testing, or if counts are performed on successive dilutions of the same sample or according to previous results.

9.3.2 Plate-count methods

9.3.2.1 Pour-plate method

In Petri dishes 85 mm to 100 mm in diameter, add 1 ml of the initial suspension and/or sample dilution prepared as demonstrated to be suitable (see Clause 12) and pour 15 ml to 20 ml of the melted agar medium (5.4.2) kept in a water bath at no more than 48 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly.

Mix the initial suspension and/or sample dilution with the medium, carefully rotating or tilting the plates sufficiently to disperse them. Allow the mixture in the Petri dishes to solidify on a horizontal surface at room temperature.

9.3.2.2 Surface spread method

In Petri dishes 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium (5.4.2) kept in a water bath at no more than 48 °C. If larger Petri dishes are used, the volume of the agar is increased accordingly.

Allow plates to cool and solidify, for example, in a microbiological cabinet or in an incubator. Spread over the surface of the medium a measured volume of not less than 0,1 ml of the initial suspension and/or sample dilution prepared as described in Clause 12.

9.3.2.3 Membrane filtration method

Use a membrane having a nominal pore size ≤ 0,45 µm.

Transfer a suitable amount of the initial suspension or of the sample dilution demonstrated to be suitable (preferably representing at least 1 g or 1 ml of the product) onto the membrane.

Filter immediately and wash the membrane (follow the suitability test procedure; see Clause 12).

Transfer the membrane onto the surface of the agar medium (5.4.2).

9.3.2.4 Incubation

Unless otherwise stated, invert the inoculated dishes and place them in the incubator set at 25 °C ± 2,5 °C for 3 d to 5 d or use the alternative condition (see Notes in 4.2 and 4.3). After incubation, the dishes shall, if possible, be examined immediately. Alternatively, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator at 5 °C ± 3 °C.

NOTE 1 In certain cases, where there is a potential for confusing particles from the product with counted colonies, it can be useful to prepare duplicate dishes containing the same sample dilutions and agar medium which are stored in the refrigerator for comparison with incubated dishes.

NOTE 2 An intermediate check can be performed where both yeast and mould are suspected.

10 Counting of colonies (plate counts and membrane filtration methods)

After incubation, count the colonies

— in Petri dishes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 11.2.3;

— on membranes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 11.2.3.

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11 Expression of results

11.1 Method of calculation for plate count

Calculate the number, *N*, of microorganisms present in the sample, *S*, using

— *m*, the arithmetic mean of the counts obtained from the duplicates [Formula (1)],

— *c*, the number of colonies counted on a single plate [Formula (2)], or

— *wm*, the weighted mean of the counts obtained from two successive dilutions [Formula (3)],

according to the following formulae:

$$N = m/(V \cdot d) \quad (1)$$

$$N = c/(V \cdot d) \quad (2)$$

$$N = wm/(V \cdot d) \quad (3)$$

where

m is the arithmetic mean of the counts obtained from the duplicates;

V is the volume of inoculum applied to each dish, in millilitres;

d is the dilution factor corresponding to the dilution made for the preparation of the initial suspension (see 9.2) or for the first counted dilution;

c is the number of colonies counted on a single plate.

The weighted mean of the colonies counted from two successive dilutions, $\bar{x}_c$, is given by

$$\bar{x}_c = \frac{\sum c}{\left(n_1 + 0,1 n_2\right)}$$

where

$\sum c$ is the sum of colonies counted on all the dishes retained from two successive dilutions;

n_1 is the number of dishes counted for the initial suspension (or for the first counted dilution);

n_2 is the number of dishes counted for the 1:10 dilution of the initial suspension (or for the second counted dilution).

Round off the result calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained. Note the number, *N*, obtained.

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11.2 Interpretation

11.2.1 The inherent variability of plate count should be taken into account. Two results should only be considered different if the difference exceeds 50 % or, when expressed logarithmically, the difference exceeds 0,3 log.

For a count to be precise, only plates or membranes with more than 15 colonies and less than 150 colonies shall be taken into account. Check that the counts are obtained from dilutions demonstrated to be suitable for the chosen method (see Clause 12).

11.2.2 Where the number of CFU is more than 15 and less than 150 on plates or membranes, express the result as follows:

— if S is at least 1 g or 1 ml, and V is at least 1 ml, the number of yeast and mould per millilitre or per gram of the sample = N/S;

— if S is less than 1 g or 1 ml and/or V is less than 1 ml, the number of yeast and mould in the sample (note the tested quantity of sample, taking into account S and V) = N;

where S is the mass or the volume of the sample (see 9.2).

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see examples).

11.2.3 Where the number of CFU is less than 15 on plates or membranes, express the result as follows:

— if S is at least 1 g or 1 ml, and V is at least 1 ml, the estimated number of yeast and mould per millilitre or per gram of the sample = N/S;

— if S is less than 1 g or 1 ml and/or V is less than 1 ml, the estimated number of yeast and mould in the sample = N;

where S is the mass or the volume of the sample (see 9.2).

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see examples).

11.2.4 Where no colony is observed, the result is reported as follows:

— there is less than $1/d \cdot V \cdot S$ of yeast and mould per gram or millilitre of the product (S is at least 1 g or 1 ml);

— there is less than $1/d \cdot V$ of yeast and mould in the sample S (note the tested quantity of sample, taking into account S and V) (S is less than 1 g or 1 ml);

where

d is the dilution factor of the initial suspension (see 9.2);

V is 1 (for counting with the pour-plate method and for membrane filtration) or 0,1 (for the spread plate method) (see example).

EXAMPLE 1 Two dishes for one dilution

S = 1 g; V = 1; counts obtained for the dilution 10^{-1}: 38 and 42

Formula (1) $N = m/(V \cdot d) = 40/(1 \cdot 10^{-1}) = 40/0{,}1 = 400$ or $4 \cdot 10^2$ yeast and mould per millilitre or per gram of the sample.

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EXAMPLE 2 One dish for one dilution

S = 1 g; V = 1; count obtained for the dilution 10^{-1}: 60

Formula (2) $N = c/(V \cdot d) = 60/(1 \cdot 10^{-1}) = 60/0{,}1 = 600$ or $6 \cdot 10^2$ yeast and mould per millilitre or per gram of the sample.

EXAMPLE 3 Two dishes for two dilutions

S = 1 g; V = 1; counts obtained for the dilution 10^{-2}: 235 and 282; for the dilution 10^{-3}: 31 and 39

Formula (3) $N = wm/(V \cdot d) = 235 + 282 + 31 + 39/1(2 + 0{,}1 \cdot 2) \cdot 10^{-2} = 587/0{,}022 = 26\,682$.

Rounding the result as specified above gives 27 000 or $2{,}7 \cdot 10^4$ yeast and mould per millilitre or per gram of the sample.

EXAMPLE 4 Two membrane filters for one dilution

S = 1 g; V = 1; counts obtained for the dilution 10^{-1}: 18 and 22

Formula (1) $N = m/(V \cdot d) = 20/(1 \cdot 10^{-1}) = 20/0{,}1 = 200$ or $2 \cdot 10^2$ yeast and mould per millilitre or per gram of the sample.

EXAMPLE 5 One membrane filter for one dilution

S = 1 g; V = 1; count obtained for the dilution 10^{-1}: 65

Formula (2) $N = c/(V \cdot d) = 65/(1 \cdot 10^{-1}) = 65/0{,}1 = 650$ or $6{,}5 \cdot 10^2$ aerobic yeast and mould per millilitre or per gram of the sample.

EXAMPLE 6 Two membrane filters for two dilutions

S = 1 g; V = 1; counts obtained for the dilution 10^{-1}: 121 and 105; for the dilution 10^{-2}: 15 and 25

Formula (3) $N = wm/(V \cdot d) = 121 + 105 + 15 + 25/1(2 + 0{,}1 \cdot 2) \cdot 10^{-1} = 266/0{,}22 = 1\,209$.

Rounding the result as specified above gives 1 200 or $1{,}2 \cdot 10^3$ yeast and mould per millilitre or per gram of the sample.

EXAMPLE 7 Two dishes for one dilution

S = 1 g; V = 1; estimated counts obtained for the dilution 10^{-1}: 28 and 22

Formula (1) $N = m/(V \cdot d) = 25/(1 \cdot 10^{-1}) = 25/0{,}1 = 250$ or $2{,}5 \cdot 10^2$ yeast and mould per millilitre or per gram of the sample.

The estimated number is 250 or $2{,}5 \cdot 10^2$ yeast and mould per millilitre or per gram of the sample.

EXAMPLE 8

S = 1 g; V = 1; estimated counts obtained for the dilution 10^{-1}: 0 and 0

Formula (1) $N = \leq 1/d \cdot V \cdot S, \leq (1/0{,}1) \cdot 1 \cdot 1 \leq 10$ yeast and mould per millilitre or per gram of the sample.

The estimated number is less than 10 yeast and mould per millilitre or per gram of the sample.

12 Neutralization of the antifungicidal properties of the product

12.1 General

The different tests described below demonstrate that the microorganisms can grow under the conditions of analysis.

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12.2 Preparation of inoculum

Prior to the test, inoculate the surface of the non-selective agar medium, Sabouraud dextrose agar (SDA), with *Candida albicans*. Incubate the plate at 32,5 °C ± 2,5 °C for 18 h to 24 h.

To harvest the culture, use a sterile loop, streak the surface of the culture and re-suspend in the diluent (see 5.2) to obtain a calibrated suspension of about 1×10^6 CFU/ml (e.g. determined using spectrophotometer); see ISO 21148.

Use this calibrated suspension and its dilutions within 2 h.

12.3 Suitability of counting methods

12.3.1 Principle

Mix the neutralized sample (initial suspension or sample dilution according to the antifungicidal activity or the low solubility of the product) with a dilution of the reference strain. Plate on a Petri dish or filter on a membrane. After incubation, check the nature of the colonies and compare the count with a control (without the sample).

If the count is less than 50 % (0,3 log) of the control, modify the procedure (diluents, neutralization agents or a combination of both; see Annex D). Failure of the inoculum to grow invalidates the test unless possible contamination of the product with this microorganism is unlikely.

12.3.2 Suitability test of the pour-plate method

Mix 9 ml of the initial suspension and/or the sample dilution(s) in neutralizing diluent (or other; see 5.2) with 1 ml of a suspension of microorganisms containing 1 000 CFU/ml to 3 000 CFU/ml. Transfer 1 ml in a Petri dish (preferably in duplicate) and pour 15 ml to 20 ml of the melted agar medium (5.4.2) kept in a water bath at no more than 48 °C. In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms, but without the sample.

After incubation for 3 d to 5 d at 25 °C ± 2,5 °C or using the alternative condition (see Notes in 4.2 and 4.3), count the colonies on the plates and compare the counts obtained for the test and for the control. The diluent and the counting method are satisfactory at the 1:10 dilution (when 1 ml of the initial suspension is used) if the suitability test count is at least 50 % of the control.

12.3.3 Suitability of the surface spread method

Mix 9 ml of the initial suspension in neutralizing diluent (or other; see 5.2) with 1 ml of a suspension of microorganisms containing 10 000 CFU/ml to 30 000 CFU/ml (or less if 0,5 ml or 1 ml is spread). Spread at least 0,1 ml on a solidified agar plate (5.4.2), preferably in duplicate. In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms, but without the sample.

After incubation for 3 d to 5 d at 25 °C ± 2,5 °C or using the alternative condition (see Notes in 4.2 and 4.3), count the colonies on the plates and compare the counts obtained for the test and for the control. The diluent and the counting method are satisfactory at the 1:10 dilution (when 1 ml of the initial suspension is used) if the suitability test count is at least 50 % (0,3 log) of the control count.

12.3.4 Suitability of the membrane filtration method

Mix to the volume of initial suspension or of the sample dilution used in the test (see 9.3.2.3) a suitable amount of a calibrated suspension of microorganisms corresponding to approximately 100 CFU.

Filter immediately the entire volume and wash the membrane using defined volumes of water (see 5.1), diluent (5.2.3) or neutralizing diluent (5.2.2). Transfer the membrane onto the surface of a suitable agar medium (5.4.2).

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In parallel, prepare a control under the same conditions as above, but without the product. Filter and wash the control under the same conditions.

After incubation for 3 d to 5 d at 25 °C ± 2,5 °C or using the alternative condition (see Notes in 4.2 and 4.3), count the colonies on the membranes and compare the counts obtained for the test and for the control. The membrane filtration method and the diluent are satisfactory if the suitability test count is at least 50 % (0,3 log) of the control count.

13 Test report

The test report shall contain the following:

a) all information necessary for the complete identification of the product;

b) the method used;

c) the results obtained;

d) all operating details for the preparation of the initial suspension;

e) the description of the method with the neutralizers and media used;

f) the suitability of the method, even if the test has been performed separately;

g) any point not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the results.

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Annex A
(informative)

Other neutralizing diluents

A.1 General

Any neutralizing diluent may be used to prepare the initial suspension if it has been checked and demonstrated to be suitable. The following neutralizing diluents are examples of suitable formulae. General information on neutralization is given in Annex D.

A.2 Eugon LT100 liquid broth

A.2.1 General

This medium contains ingredients that neutralize inhibitory substances present in the sample (lecithin and polysorbate 80) and dispersing agent (octoxynol 9).

A.2.2 Composition

Pancreatic digest of casein	15,0 g
Papaic digest of soybean meal	5,0 g
L-cystine	0,7 g
Sodium chloride	4,0 g
Sodium sulfite	0,2 g
Glucose	5,5 g
Egg lecithin	1,0 g
Polysorbate 80	5,0 g
Octoxynol 9	1,0 g
Water	1 000 ml

A.2.3 Preparation

Dissolve successively in hot water polysorbate 80, octoxynol 9 and egg lecithin until their complete dilution. Dissolve the other components by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

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A.3 Lecithin polysorbate (LP) diluent

A.3.1 Composition

Polypeptone	1,0 g
Egg lecithin	0,7 g
Polysorbate 80	20,0 g
Water	980 ml

A.3.2 Preparation

Mix and dissolve the ingredients by heating. Cool to 25 °C before dispensing the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

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Annex B
(informative)

Other diluents

B.1 General

Any diluent may be used to prepare the initial suspension if it has been checked and demonstrated to be suitable. The following diluents are examples of suitable formulae.

B.2 Buffered peptone solution (pH 7)

B.2.1 Composition

Meat peptone	1,0 g
Sodium chloride	4,3 g
Monopotassium phosphate	3,6 g
Disodium phosphate dihydrate	7,2 g
Water	1 000 ml

B.2.2 Preparation

Dissolve the ingredients in hot water. Mix and cool to 25 °C before dispensing the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

B.3 Phosphate buffer (pH 7,2)

B.3.1 Composition

Potassium dihydrogen phosphate	34 g
Water	500 ml

B.3.2 Preparation

Dissolve the ingredients in water in a 1 000 ml volumetric flask. Adjust the pH value to 7,2 ± 0,1 with about 175 ml of sodium hydroxide (4,3 g/100 ml). Then water is added to a final volume of 1 000 ml. The final concentration of the solution is 0,05 mol/l. It is the stock solution. Store it under refrigeration.

Before use, dilute the stock solution with water at a ratio of 1:800 and sterilize it in the autoclave at 121 °C for 15 min.

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Annex C
(informative)

Other culture media

C.1 General

Any culture medium may be used if it has been checked and demonstrated to be suitable. The following media are examples of suitable formulae.

C.2 Agar for counting

C.2.1 Potato dextrose agar medium with antibiotics

C.2.1.1 Composition

Potato extract	4,0 g
Glucose	20,0 g
Agar	15,0 g
Chloramphenicol	0,05 g
Water	1 000 ml

C.2.1.2 Preparation

Mix all the components and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

Alternatively, chloramphenicol may be replaced by use of 0,10 g of benzylpenicillin potassium and 0,10 g of tetracycline per litre of medium, added as sterile solution, just prior to use.

C.2.2 Glucose-peptone (GP) agar medium with antibiotics

C.2.2.1 Composition

Glucose	20,0 g
Yeast extract	2,0 g
Magnesium sulfate	0,5 g
Peptone	5,0 g
Monobasic potassium phosphate	1,0 g

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Agar	15,0 g
Chloramphenicol	0,05 g
Water	1 000 ml

C.2.2.2 Preparation

Mix all the components and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling, the pH shall be equivalent to 5,7 ± 0,1 when measured at room temperature.

Alternatively, chloramphenicol may be replaced by use of 0,10 g of benzylpenicillin potassium and 0,10 g of tetracycline per litre of medium, added as sterile solution, just prior to use.

C.3 Malt extract agar medium

C.3.1 Composition

Malt extract	30,0 g
Soya peptone, papaic digest of soybean meal	3,0 g
Agar	15,0 g
Chloramphenicol	0,05 g
Water	1 000 ml

C.3.2 Preparation

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 115 °C for 10 min. After sterilization, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

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Annex D
(informative)

Neutralizers of antifungicidal activity of preservatives and rinsing liquids

Table D.1

Preservative	Chemical compounds able to neutralize preservative's antifungicidal activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods)
Phenolic compounds: parabens, phenoxyethanol, phenylethanol, anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol Non-ionic surfactants	Polysorbate 80, 30 g/l + lecithin, 3 g/l. Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l. D/E neutralizing broth. Rinsing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Quaternary ammonium compounds Cationic surfactants	Lecithin, saponin, polysorbate 80, sodium dodecyl sulfate Ethylene oxide condensate of fatty alcohol	Polysorbate 80, 30 g/l + sodium dodecyl sulfate, 4 g/l + lecithin, 3 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. D/E neutralizing broth. Rinsing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Aldehydes Formaldehyde-release agents	Glycine, histidine	Lecithin, 3 g/l + polysorbate 80, 30 g/l + L-histidine, 1 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l + L-cysteine, 1 g/l. D/E neutralizing broth. Rinsing liquid: polysorbate 80, 3 g/l + L-histidine 0,5 g/l.
Oxidizing compounds	Sodium thiosulphate	Sodium thiosulphate, 5 g/l. Rinsing liquid: sodium thiosulphate, 3 g/l.
Isothiazolinones, imidazoles	Lecithin, saponin, amines, sulfates, mercaptans, sodium bisulfite, sodium thioglycollate	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Biguanides	Lecithin, saponin, polysorbate 80	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Metallic salts (Cu, Zn, Hg) Organo-mercuric compounds	Sodium bisulphate, L-cysteine Sulfhydryl compounds, thioglycolic acid	Sodium thioglycollate, 0,5 g/l or 5 g/l. L-cysteine, 0,8 g/l or 1,5 g/l. D/E neutralizing broth. Rinsing liquid: sodium thioglycollate, 0,5 g/l.
NOTE According to the pH of the cosmetic product, the pH of the neutralizer can be adjusted at a suitable value.		

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