INTERNATIONAL STANDARD

ISO 22718

First edition
2006-02-01

Cosmetics — Microbiology — Detection of *Staphylococcus aureus*

Cosmétiques — Microbiologie — Détection de Staphylococcus aureus

Reference number
ISO 22718:2006(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

Foreword ... iv

Introduction ... v

1 **Scope** ... 1

2 **Normative references** ... 1

3 **Terms and definitions** ... 1

4 **Principle** ... 2

5 **Diluents and culture media** ... 2
5.1 **General** ... 2
5.2 **Diluent for the bacterial suspension (tryptone sodium chloride solution)** ... 3
5.3 **Culture media** ... 3

6 **Apparatus and glassware** ... 6

7 **Strains of microorganisms** ... 6

8 **Handling of cosmetic products and laboratory samples** ... 6

9 **Procedure** ... 7
9.1 **General recommendation** ... 7
9.2 **Preparation of the initial suspension in the enrichment broth** ... 7
9.3 **Incubation of the inoculated enrichment broth** ... 7
9.4 **Detection and Identification of *Staphylococcus aureus*** ... 7

10 **Expression of the results (detection of *Staphylococcus aureus*)** ... 8

11 **Neutralization of the antimicrobial properties of the product** ... 9
11.1 **General** ... 9
11.2 **Preparation of inoculum** ... 9
11.3 **Validation of the detection method** ... 9

12 **Test report** ... 10

Annex A (informative) **Other media** ... 11

Annex B (informative) **Neutralizers of antimicrobial activity of preservatives and rinsing liquids** ... 14

Bibliography ... 15

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 22718 was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

Introduction

Microbiological examinations of cosmetic products shall be carried out according to an appropriate microbiological risk analysis in order to ensure their quality and safety for consumers.

Microbiological risk analysis depends on several parameters such as:

— potential alteration of cosmetic products;

— pathogenicity of micro-organisms;

— site of application of the cosmetic product (hair, skin, eyes, mucous membranes, etc.);

— type of users (adults, children under 3 years).

For cosmetics and other topical products, the detection of skin pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* may be relevant. The detection of other kinds of micro-organism might be of interest since these micro-organisms (including indicators of faecal contamination e.g. *Escherichia coli*) suggest hygienic failure during manufacturing process.

Cosmetics — Microbiology — Detection of *Staphylococcus aureus*

1 Scope

This International Standard gives general guidelines for the detection and identification of the specified micro-organism *Staphylococcus aureus* in cosmetic products. Micro-organisms considered as specified in this International Standard might differ from country to country according to national practices or regulations.

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 product to which this International Standard is applicable. Products considered to present a low microbiological risk include those with low water activity, hydro-alcoholic products, extreme pH values, etc.

The method described in this International Standard is based on the detection of *Staphylococcus aureus* in a non-selective liquid medium (enrichment broth), followed by isolation on a selective agar medium. Other methods may be appropriate dependent on the level of detection required.

NOTE For the detection of *Staphylococcus aureus*, subcultures can be performed on non-selective culture media followed by suitable identification steps (e.g. using identification kits).

Because of the large variety of cosmetic products within this field of application, this method may not be appropriate for some products in every detail (e.g. certain water immiscible products). Other International Standards (ISO 18415 [10]) may be appropriate. Other methods (e.g. automated) may be substituted for the tests presented here provided that their equivalence has been demonstrated or the method has been otherwise validated.

2 Normative references

The following referenced documents are indispensable for the application 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 21148:2005, *Cosmetics — Microbiology — General instructions for microbiological examination*

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

3 Terms and definitions

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

3.1
product
portion of an identified cosmetic product received in the laboratory for testing

3.2
sample
portion of the product (at least 1 g or 1 ml) that is used in the test to prepare the initial suspension

3.3
initial suspension
suspension (or solution) of the sample in a defined volume of an appropriate enrichment broth

3.4
sample dilution(s)
dilution(s) of the initial suspension

3.5
specified micro-organism
aerobic mesophilic bacteria or yeast that is undesirable in a cosmetic product and is recognized as a skin pathogen species that may be harmful for human health or as indication of hygienic failure in the manufacturing process

3.6
Staphylococcus aureus
Gram-positive cocci, mainly joined in grape-like clusters, smooth colonies generally pigmented in yellow

NOTE 1 The main characteristics for identification are: growth on specific selective medium, catalase positive, coagulase positive.

NOTE 2 *Staphylococcus aureus* is an opportunistic pathogen bacterium for humans that can be also present on the skin of healthy people without causing disorder for them. It is undesirable in cosmetic products due to its potential pathogenicity.

3.7
enrichment broth
non-selective liquid medium containing suitable neutralizers and/or dispersing agents and validated for the product under test

4 Principle

The first step of the procedure is to perform an enrichment by using a non-selective broth medium to increase the number of micro-organisms without the risk of inhibition by the selective ingredients that are present in selective/differential growth media.

The second step (isolation) of the test is performed on a selective medium followed by identification tests.

The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable micro-organisms [1]. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and validated [2], [3], [4].

5 Diluents 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 enrichment broth is used to disperse the sample and to increase the initial microbial population. It may contain neutralizers if the specimen to be tested has antimicrobial properties. The efficacy of the neutralization shall be demonstrated (see Clause 11). Information relative to suitable neutralizers is given in Annex B.

The following enrichment broth is suitable for checking the presence of *Staphylococcus aureus* in accordance with this International Standard provided that it is validated in accordance with Clause 11.

Other diluents and culture media may be used if they can be demonstrated to be suitable for use.

5.2 Diluent for the bacterial suspension (tryptone sodium chloride solution)

5.2.1 General

The diluent is used for the preparation of bacterial suspension used for the validation procedure (see Clause 11).

5.2.2 Composition

— tryptone, pancreatic digest of casein 1,0 g

— sodium chloride 8,5 g

— water 1 000 ml

5.2.3 Preparation

Dissolve the components in water by mixing whilst heating. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.3 Culture media

5.3.1 General

Culture media may be prepared using the descriptions provided below or from dehydrated culture media according to the instructions from the manufacturer. The instructions provided by the supplier of the media should be followed.

NOTE Ready to use media may be used when their composition and/or growth yields are comparable to those of the formulas given herein.

5.3.2 Agar medium for validation (see Clause 11) [soybean-casein digest agar medium (SCDA) or tryptic soy agar (TSA)]

5.3.2.1 Composition

— pancreatic digest of casein 15,0 g

— papaic digest of soybean meal 5,0 g

— sodium chloride 5,0 g

— agar 15,0 g

— water 1 000 ml

5.3.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.3.3 Enrichment broth

5.3.3.1 Eugon LT 100 broth

5.3.3.1.1 General

This medium contains ingredients which neutralize inhibitory substances present in the sample: lecithin and polysorbate 80, and dispersing agent: octoxynol 9.

5.3.3.1.2 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

5.3.3.1.3 Preparation

Dissolve the components, one after another, in boiling water – polysorbate 80, octoxynol 9 and egg lecithin until their complete dissolution. Dissolve the other components by mixing whilst heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to $7,0 \pm 0,2$ when measured at room temperature.

5.3.3.2 Other enrichment broths

Other enrichment broths may be used as appropriate (see Annex A).

5.3.4 Selective agar medium for isolation of *Staphylococcus aureus*

5.3.4.1 Baird Parker agar medium

5.3.4.1.1 Base medium

####### 5.3.4.1.1.1 Composition

— pancreatic digest of casein	10,0 g
— yeast extract	1,0 g

— meat extract 5,0 g

— sodium pyruvate 10,0 g

— L-glycine 12,0 g

— lithium chloride 5,0 g

— agar 12 g to 22 g [1)]

— water to a final volume of 950 ml

5.3.4.1.1.2 Preparation

Dissolve the components or the complete dehydrated base in the water by boiling. Transfer the medium in quantities of 100 ml to flasks or bottles of appropriate capacity. Sterilize the medium in the autoclave at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

5.3.4.1.2 Potassium tellurite solution

5.3.4.1.2.1 Composition

— potassium tellurite (K_2TeO_3) 1,0 g

— water 100 ml

5.3.4.1.2.2 Preparation

Dissolve the potassium tellurite completely in the water with minimal heating.

Sterilize by filtration using 0,22 µm pore size membranes. The solution may be stored at the maximum for one month at 3 °C ± 2 °C. Discard the solution if a white precipitate forms.

The solid should be readily soluble. If a white insoluble material is present in the water, the powder should be discarded.

5.3.4.1.3 Egg yolk emulsion (concentration approximately 20 % or according to the manufacturer's instructions)

If a commercial preparation is not available, prepare the medium as follows.

Use fresh hens' eggs, the shells being intact. Clean the eggs with a brush using a liquid detergent. Rinse them under running water, then disinfect the shells either by immersing them in 70 % (volume fraction) ethanol for 30 s and allow them to dry in the air, or by spraying them with alcohol followed by flame sterilization. Proceeding under aseptic conditions, break each egg and separate the yolk from its white by repeated transfer of the yolk from one half of the shell to the other. Place the yolks in a sterile flask and add four times their volume of sterile water. Mix thoroughly. Heat the mixture at 47 °C for 2 h and leave for 18 h to 24 h at 3 °C ± 2 °C to allow a precipitate to form. Aseptically collect the supernatant liquid in a fresh sterile flask for use.

The emulsion may be stored at 3 °C ± 2 °C for a maximum of 72 h.

---

1) Depending on the gel strength of the agar.

5.3.4.1.4 Complete medium

5.3.4.1.4.1 Composition

— base medium (5.3.4.1.1) 100 ml

— potassium tellurite solution (5.3.4.1.2) 1,0 ml

— egg-yolk emulsion (5.3.4.1.3) 5,0 ml

5.3.4.1.4.2 Preparation

Melt the base medium (5.3.4.1.1) then cool it to approximately 47 °C. Add, under aseptic conditions, the two other solutions (5.3.4.1.2 and 5.3.4.1.3), each of them previously warmed at 47 °C, mixing well after each addition.

5.3.4.2 Other selective agar media

Other selective agar media may be used (see Annex A).

6 Apparatus and glassware

The laboratory equipment, apparatus and glassware shall be as described in ISO 21148.

7 Strains of micro-organisms

For the validation of the test conditions, the following representative strain is used:

Staphylococcus aureus ATCC [2] 6538 (equivalent strain: CIP [3] 4.83 or NCIMB [4] 9518).

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

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 (3.1) and samples (3.2) 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 following procedure.

2) ATCC = American Type Culture Collection.

3) CIP = Institut Pasteur Collection.

4) NCIMB = National Collection of Industrial and Marine Bacteria.

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 in an appropriate solubilizing agent, the time which elapses between the end of the preparation and the moment the inoculum comes into contact with the enrichment broth shall not exceed 45 min, unless specifically mentioned in the established protocols or documents.

9.2 Preparation of the initial suspension in the enrichment broth

9.2.1 General

The enrichment is prepared from a sample (3.2) of at least 1 g or 1 ml of the well-mixed product under test, which is dispersed in at least 9 ml of enrichment broth.

Note, S, the exact weight or volume of the sample.

The method shall be checked to ensure that the composition (neutralizer eventually added) and the volume of the broth perform satisfactorily (see 11.3).

NOTE In some cases, and when possible, filtration of the cosmetic product through a membrane which is afterwards immersed in the enrichment broth facilitates the neutralization of the antimicrobial properties of the product (see 11.3).

9.2.2 Water-miscible products

Transfer the sample, S, of product to a suitable container containing an appropriate volume of broth.

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).

Disperse the sample within the solubilizing agent and add an appropriate volume of broth.

9.2.4 Filterable products

Use a membrane filter having a nominal pore size of not greater than 0,45 µm.

Transfer the sample, S, on to the membrane in a filtration apparatus (see ISO 21148). Filter immediately and wash the membrane using defined volumes of water and/or diluent.

Transfer and immerse the membrane into a tube or flask of suitable size containing an appropriate volume of broth.

9.3 Incubation of the inoculated enrichment broth

Incubate the initial suspension prepared in broth (see 9.2) at 32,5 °C ± 2,5 °C for at least 20 h (maximum 72 h).

9.4 Detection and Identification of *Staphylococcus aureus*

9.4.1 Isolation

Using a sterile loop, streak an aliquot of the incubated enrichment broth on the surface of Baird Parker Agar medium in order to obtain isolated colonies.

Invert the petri dish and then incubate at 32,5 °C ± 2,5 °C for at least 24 h (maximum 48 h).

Check for characteristic colonies (see Table 1).

Table 1 — Morphologic characteristics of *Staphylococcus aureus* on selective medium

Selective medium	Aspect of the colonies of *Staphylococcus aureus*
Baird Parker agar medium	Black, shiny colonies, surrounded by clear zones (2 mm to 5 mm)

9.4.2 Identification of *Staphylococcus aureus*

9.4.2.1 General

Proceed to the following tests, for the suspect colonies isolated on the Baird Parker agar medium. The presence of *Staphylococcus aureus* may be confirmed by other suitable, cultural and biochemical tests.

9.4.2.2 Gram's stain

This test is described in ISO 21148.

Check for Gram-positive cocci in clusters.

9.4.2.3 Catalase test

This test is described in ISO 21148.

Check for a catalase positive test.

9.4.2.4 Coagulase test

With an inoculating loop, transfer representative suspected well isolated colonies from the agar surface of the Baird Parker agar medium to individual sterile tubes, each containing 0,5 ml of mammalians, preferably rabbit or horse, plasma with or without suitable additives.

Incubate at 37 °C ± 2°C and examine the tubes at 3 h, 4 h, 6 h and up to 24 h if no coagulation appears within 6 h, unless otherwise specified by the manufacturer. A positive coagulation only appearing at 24 h shall be confirmed.

Test controls simultaneously with the suspected colonies according to the manufacturer recommendations.

Check for a coagulase positive test.

10 Expression of the results (detection of *Staphylococcus aureus*)

If the identification of the colonies confirms the presence of this species, express the result as:

Presence of *Staphylococcus aureus* in the sample, S.

If no growth after enrichment is observed and/or if the identification of the colonies does not confirm the presence of this species, express the result as:

Absence of *Staphylococcus aureus* in the sample, S.

11 Neutralization of the antimicrobial properties of the product

11.1 General

The different tests described below demonstrate that the micro-organism can grow in analysis conditions.

11.2 Preparation of inoculum

Prior to the test, inoculate the surface of soybean-casein digest agar (SCDA) or other suitable (non-selective, non-neutralizing) medium with *Staphylococcus aureus* (see Clause 7). 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 to obtain a calibrated suspension of about 1×10^8 CFU per ml. (e.g. using spectrophotometer) see ISO 21148:2005 (Annex C).

Use this calibrated suspension and its dilutions within 2 h.

11.3 Validation of the detection method

11.3.1 Procedure

11.3.1.1 In tubes of 9 ml of diluent prepare a dilution of the calibrated suspension in order to obtain a final count between 100 CFU per ml and 500 CFU per ml. To count the final concentration of viable micro-organisms in the diluted calibrated suspension, transfer 1 ml of the suspension into a petri dish and pour in 15 ml to 20 ml of the melted agar medium kept in a water bath at no more than 48 °C. Let solidify and then incubate at 32,5 °C ± 2,5 °C for 20 h to 24 h.

11.3.1.2 Prepare in duplicate the initial suspension in the conditions chosen for the test (at least 1 g or 1 ml of product under test, defined volume of enrichment broth) in a tube or flask. When using the membrane filtration method filter in duplicate at least 1 ml of product under test and transfer each membrane to a tube or flask containing the enrichment broth in the conditions chosen for the test.

11.3.1.3 Introduce aseptically 0,1 ml of the diluted calibrated suspension (11.3.1.1) of micro-organisms into one tube or flask (validation test). Mix, then incubate both tubes or flasks (validation test and non-inoculated control) at 32,5 °C ± 2,5 °C for 20 h to 24 h.

11.3.1.4 Perform an isolation for each tube or flask (validation test and non-inoculated control). Using a sterile loop, streak an aliquot (same conditions as in the test) of the incubated mixture onto the surface of a petri dish (diameter 85 mm – 100 mm) containing approximately 15 ml to 20 ml of Baird Parker agar medium. Incubate the plates at 32,5 °C ± 2,5 °C for 24 h to 48 h.

11.3.2 Interpretation of validation results

Check that the dituted calibrated suspension (11.3.1.1) of bacteria contains between 100 CFU per ml and 500 CFU per ml.

The neutralization and the detection method are validated if a growth characteristic of *Staphylococcus aureus* occurs on the validation plate and no growth occurs on the control plate.

When growth is detected on the control plates (contaminated products), the neutralization and the detection method are validated if *Staphylococcus aureus* is recovered on the validation plate.

Failure of growth on the validation plates indicates that antimicrobial activity is still present and necessitates a modification of the conditions of the method by an increase in the volume of nutrient broth, the quantity of product remaining the same, or by incorporation of a sufficient quantity of inactivating agent in the enrichment broth, or by an appropriate combination of modifications so as to permit the growth of *Staphylococcus aureus*.

If in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of broth it is still not possible to recover viable cultures as described above, indicate that the article is not likely to be contaminated with *Staphylococcus aureus*.

12 Test report

The test report shall contain the following information:

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

b) method used;

c) results obtained;

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

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

f) validation 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.

Annex A
(informative)

Other media

A.1 Other enrichment broths

A.1.1 Fluid soybean-casein digest medium

A.1.1.1 Composition

— pancreatic digest of casein 15,0 g

— papaic digest of soybean meal 5,0 g

— sodium chloride 5,0 g

— water 1 000 ml

A.1.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, heating if necessary. Sterilize in an autoclave at 121° C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

Dispense the medium into suitable containers.

A.1.2 D/E neutralizing broth (Dey/Engley neutralizing broth) [7]

A.1.2.1 Composition

— glucose 10,0 g

— soybean lecithin 7,0 g

— sodium thiosulfate pentahydrate 6,0 g

— polysorbate 80 5,0 g

— pancreatic digest of casein 5,0 g

— sodium bisulfite 2,5 g

— yeast extract 2,5 g

— sodium thioglycollate 1,0 g

— bromcresol purple 0,02 g

— water 1 000 ml