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Cosmetics — Microbiology — Detection of *Pseudomonas aeruginosa*

Cosmétiques — Microbiologie — Détection de Pseudomonas aeruginosa

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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 22717 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 the manufacturing process.

Cosmetics — Microbiology — Detection of *Pseudomonas aeruginosa*

1 Scope

This International Standard gives general guidelines for the detection and identification of the specified micro-organism *Pseudomonas aeruginosa* 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 *Pseudomonas aeruginosa* in a non-selective liquid medium (enrichment broth), followed by isolation on a selective agar medium. Other methods may be appropriate, depending on the level of detection required.

NOTE For the detection of *Pseudomonas aeruginosa*, 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 in every detail for some products (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
Pseudomonas aeruginosa
Gram-negative rod, motile; smooth colonies pigmented brown or greenish

NOTE 1 The main characteristics for identification are: growth on selective cetrimide agar medium, oxidase positive, production of diffusible fluorescent pigments and production of a soluble phenazine pigment (pyocyanin) in suitable media.

NOTE 2 *Pseudomonas aeruginosa* may be isolated from a wide variety of environmental sources, especially in water and has a very high potential to spoil many different substrates. It may produce infections of human skin or eye area. It is undesirable in cosmetic products for its potential pathogenicity and its capacity to affect the physico-chemical properties of the cosmetic formula.

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 of the test (isolation) 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 *Pseudomonas aeruginosa* 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 of 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 (soybean–casein digest agar medium or tryptic soy agar)

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 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 7,3 ± 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 that 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 *Pseudomonas aeruginosa*

5.3.4.1 Cetrimide agar medium

5.3.4.1.1 Composition

- pancreatic digest of gelatin 20,0 g
- magnesium chloride 1,4 g
- potassium sulfate 10,0 g
- cetrimide (cetyltrimethylammonium bromide) 0,3 g

— agar 13,6 g

— glycerin 10,0 ml

— water 1 000 ml

5.3.4.1.2 Preparation

Dissolve all solid components in the water, and add the glycerin. Heat, with frequent agitation, and boil for 1 min to effect dissolution.

Dispense in suitable flasks and sterilize 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.

5.3.5 Selective agar medium for confirmation of *Pseudomonas aeruginosa*

5.3.5.1 Pseudomonas agar medium for detection of pyocyanin (Pseudomonas agar P)

5.3.5.1.1 Composition

— pancreatic digest of gelatin 20,0 g

— anhydrous magnesium chloride 1,4 g

— anhydrous potassium sulfate 10,0 g

— agar 15,0 g

— glycerin 10,0 ml

— water 1 000 ml

5.3.5.1.2 Preparation

Dissolve all solid components in the water, and add the glycerin. Heat, with frequent agitation, and boil for 1 min to effect dissolution.

Dispense in suitable flasks and sterilize 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.

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:

Pseudomonas aeruginosa ATCC [1] 9027 (equivalent strain: CIP [2] 82118 or NCIMB [3] 8626 or NBRC [4] 13275 or KCTC [5] 2513 or other equivalent national collection strain).

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

The strain may be stored 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 and samples 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.

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 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 that is afterwards immersed in the enrichment broth, facilitates the neutralization of the antimicrobial properties of the product (see 11.3.)

1) ATCC = American Type Culture Collection.

2) CIP = Institut Pasteur Collection.

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

4) NBRC = National Biological Resource center.

5) KCTC = Korean Collection for type culture.

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 *Pseudomonas aeruginosa*

9.4.1 Isolation

Using a sterile loop, streak an aliquot of the incubated enrichment broth on the surface of cetrimide 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 — Morphological characteristics of *Pseudomonas aeruginosa* on selective medium

Selective medium	Characteristic colonial morphology of *Pseudomonas aeruginosa*
Cetrimide agar medium	Yellow-green pigment (pyocyanin), which fluoresces under UV light.

9.4.2 Identification of *Pseudomonas aeruginosa*

9.4.2.1 General

Proceed to the following tests, for the suspect colonies isolated on the cetrimide agar medium. The presence of *Pseudomonas aeruginosa* 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-negative rods.

9.4.2.3 Oxidase test

This test is described in ISO 21148.

Check for oxidase positive test.

9.4.2.4 Culture on Pseudomonas agar medium for detection of pyocyanin

Inoculate the surface of the Pseudomonas agar medium for detection of pyocyanin with suspect isolated colonies grown on cetrimide agar medium, so that individual colonies develop. Incubate at 32,5 °C ± 2,5 °C.

Check for bacterial growth after 24 h, 48 h and 72 h. *Pseudomonas aeruginosa* forms colonies surrounded by a blue to green zone due to pyocyanin formation or with a red to dark brown zone due to pyorubin production.

10 Expression of results

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

presence of *Pseudomonas aeruginosa* 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 *Pseudomonas aeruginosa* 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 the inoculum

Prior to the test, inoculate the surface of soybean casein digest agar (SCDA) or other suitable (non selective, non neutralizing) medium with *Pseudomonas aeruginosa*. 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, 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 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 on 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 into 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 on to the surface of a petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of cetrimide 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 diluted calibrated suspension (11.3.1.1) of bacteria contains between 100 CFU and 500 CFU per ml.

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

When growth is detected on the control plate (contaminated products), the neutralization and the detection method are validated if *Pseudomonas aeruginosa* 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 *Pseudomonas aeruginosa*.

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 *Pseudomonas aeruginosa*.

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 International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

Annex A
(informative)

Other enrichment broths

A.1 Soybean-casein-digest-lecithin-polysorbate 80 medium (SCDLP 80 broth)

A.1.1 Composition

— casein peptone	17,0 g
— soybean peptone	3,0 g
— sodium chloride	5,0 g
— dipotassium hydrogen phosphate	2,5 g
— glucose	2,5 g
— lecithin	1,0 g
— polysorbate 80	7,0 g
— water	1 000 ml

A.1.2 Preparation

Dissolve all of these components (or dehydrated complete medium) one after another in boiling water until their complete dissolution. 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 7,2 ± 0,2 when measured at room temperature.

A.2 D/E neutralizing broth (Dey/Engley neutralizing broth) [7]

A.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