INTERNATIONAL STANDARD

ISO 11930

First edition
2012-04-01

Cosmetics — Microbiology — Evaluation of the antimicrobial protection of a cosmetic product

Cosmétiques — Microbiologie — Évaluation de la protection antimicrobienne d'un produit cosmétique

Reference number
ISO 11930:2012(E)

COPYRIGHT PROTECTED DOCUMENT

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

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 11930 was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

Introduction

This International Standard is to be used in the overall evaluation of the antimicrobial protection of a cosmetic product.

The antimicrobial protection of a product can come from many sources:

— chemical preservation;

— inherent characteristics of the formulation;

— package design;

— manufacturing process.

This International Standard defines a series of steps to be taken when assessing the overall antimicrobial protection of a cosmetic product. A reference method for a preservation efficacy test (challenge test) along with evaluation criteria is also described in this International Standard.

The data generated by the risk assessment (see ISO 29621) or by the preservation efficacy test, or both, are to be used to establish the level of antimicrobial protection required to minimize user risk.

Cosmetics — Microbiology — Evaluation of the antimicrobial protection of a cosmetic product

1 Scope

1.1 General

This International Standard comprises:

— a preservation efficacy test;

— a procedure for evaluating the overall antimicrobial protection of a cosmetic product which is not considered low risk, based on a risk assessment described in ISO 29621.

This International Standard provides a procedure for the interpretation of data generated by the preservation efficacy test or by the microbiological risk assessment, or both.

1.2 Preservation efficacy test

This test is a reference method that is to be used to evaluate the preservation of a cosmetic formulation. It applies to cosmetic products in the market place.

This test is not required for those cosmetic products for which the microbiological risk has been determined to be low (see Annex A and ISO 29621).

This test is primarily designed for water-soluble or water-miscible cosmetic products and can require adaptation, for example to test products in which water is the internal phase. The test described in this International Standard involves, for each test micro-organism, placing the formulation in contact with a calibrated inoculum, and then measuring the changes in the micro-organism count at set time intervals for a set period and at a set temperature.

NOTE This test can be used as a guideline to develop an in-house method during the development cycle of cosmetic products. In this case, the test can be modified or extended, or both, for example to make allowance for prior data and different variables (microbial strains, media, incubation conditions exposure time, etc.). Compliance criteria can be adapted to specific objectives. During the development stage of cosmetic products, other methods, where relevant, can be used to determine the preservation efficacy of formulations.

1.3 Procedure for evaluating the antimicrobial protection of the cosmetic product

This procedure is based on careful consideration of the following points.

— Results of the preservation efficacy test. Not all cosmetic products will require a preservation efficacy test (see Annex A and ISO 29621).

— Formulation characteristics and data provided by the microbiological risk assessment (see ISO 29621). The analysis of the microbiological risk assessment is based on an overall approach. In particular, it integrates variables such as characteristics and composition of the formulation, its production conditions, the characteristics of the packaging in which the formulation will be delivered to the market place, recommendations for use of the cosmetic product and, when relevant, the area of application and the targeted user population (see Annex D).

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 16212, *Cosmetics — Microbiology — Enumeration of yeast and mould*

ISO 18415, *Cosmetics — Microbiology — Detection of specified and non-specified microorganisms*

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

ISO 21149, *Cosmetics — Microbiology — Enumeration and detection of aerobic mesophilic bacteria*

ISO 22716, *Cosmetics — Good Manufacturing Practices (GMP) — Guidelines on Good Manufacturing Practices*

ISO 29621, *Cosmetics — Microbiology — Guidelines for the risk assessment and identification of microbiologically low-risk products*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 21148 and the following apply.

3.1
cosmetic formulation
preparation of raw materials with a qualitatively and quantitatively defined composition

3.2
cosmetic product
finished cosmetic product that has undergone all stages of production, including packaging in its final container for shipment

3.3
antimicrobial protection of a cosmetic product
ability of a cosmetic product to overcome microbial contamination that might present a potential risk to the user

NOTE The overall antimicrobial protection includes preservation of the formulation, the specific manufacturing process and protective packaging.

3.4
preservation of a cosmetic formulation
set of means used to avoid microbial proliferation in a cosmetic formulation

EXAMPLES Preservatives, multifunctional compounds, hostile raw materials, extreme pH, low water-activity values.

3.5
reference method
method applied by interested parties to assess a product on the market and in case of dispute

3.6
development method
in-house method
method used during the development stage of a product before the product is put on the market

3.7
consumer
end user of a cosmetic product

4 Principle

The evaluation of the antimicrobial protection of a cosmetic product combines the following elements (see Annex A).

a) The characteristics of its formulation (see ISO 29621) or the results of the preservation efficacy test (if performed), or both.

   The preservation efficacy test is described in 5.1.

b) The characteristics of the cosmetic product in conjunction with the production conditions (see ISO 22716 and ISO 29621), the packaging materials and, if justified, recommendations for use of the product (see ISO 29621).

This International Standard describes a procedure for the interpretation of data generated by the preservation efficacy test (if appropriate) and by the microbiological risk assessment.

5 Preservation efficacy test

5.1 General

The evaluation of the preservation of a cosmetic formulation is based on inoculation of the formulation with calibrated inocula (prepared from relevant strains of micro-organisms). The number of surviving micro-organisms is measured at defined intervals during a period of 28 days. For each time and each strain, the log reduction value is calculated and compared to the minimum values required for evaluation criteria A or B (see Annex B).

When used as a reference method, procedures shall be strictly followed in order to avoid variability in results. To determine the preservation efficacy of a formulation during product development, other suitable development methods may be used (see 1.2).

Prior to the test, the microbiological quality of the product shall be determined in accordance with ISO 21149 and ISO 16212, or with ISO 18415.

NOTE The micro-organisms present in the test sample should not interfere with the recovery of the test organisms.

In the test, the neutralization of the possible antimicrobial activity of the tested sample shall be checked and demonstrated (see 5.5).

5.2 Materials, apparatus, reagents and culture media

General specifications and instructions are given in ISO 21148. When water is used in a formula, use distilled water or purified water as specified in ISO 21148:2005, 8.2.

5.2.1 Materials

Use usual microbiology laboratory equipment (see ISO 21148) and:

5.2.1.1 Glass beads, 3 mm to 4 mm in diameter.

5.2.1.2 Sintered glass filter, of porosity 2 (40 µm to 100 µm).

5.2.1.3 Roux flasks.

5.2.1.4 Sterile glass containers with closures, of suitable volumes.

5.2.1.5 Centrifuge, capable of a centrifugal force of 2 000 g.

5.2.2 Diluents, neutralizers and culture media

5.2.2.1 General

Unless otherwise specified, all reagents shall be equilibrated at ambient temperature before use. When available, ready-to-use reagents and media may be used.

5.2.2.2 Diluent

5.2.2.2.1 Composition

Pancreatic digest of casein	1,0 g
Sodium chloride	8,5 g
Water	1 000 ml

5.2.2.2.2 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.

5.2.2.2.3 **Polysorbate solution** (for preparation of *A. brasiliensis* spore suspension)

Prepare a solution of polysorbate 80 (0,5 g/l). Dissolve by mixing while heating until complete dissolution is achieved. Dispense the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

5.2.2.3 Neutralizer

5.2.2.3.1 General

The suitability and effectiveness of the neutralizing agent with respect to the test strains used and to the tested formulation shall be demonstrated as specified in 5.5.

The neutralizer described in 5.2.2.3.2 is frequently used. Examples of other suitable neutralizers are given in Annex C.

5.2.2.3.2 Eugon LT 100 liquid broth

5.2.2.3.2.1 General

This medium contains ingredients which neutralize inhibitory substances present in the sample (lecithin and polysorbate 80) and dispersing agent octoxynol 9 (Triton X100®[1)]). It may be prepared as described in 5.2.2.3.2.2, or from dehydrated culture medium, according to the manufacturer's instructions. A ready-to-use medium may also be used.

5.2.2.3.2.2 Composition

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

1) Triton X100® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5.2.2.3.2.3 Preparation

Dissolve successively into boiling water polysorbate 80, octoxynol 9 and egg lecithin until they are completely dissolved. Dissolve the other components by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. Mix well after sterilization while the liquid is still hot to redissolve settled substances. After sterilization, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.2.2.4 Culture media

5.2.2.4.1 General

Culture media may be prepared as in 5.2.2.4.2, 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 in 5.2.2.4.2.1.

5.2.2.4.2 Culture medium for bacteria: tryptic soy agar (TSA) or soybean casein digest agar medium

5.2.2.4.2.1 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

5.2.2.4.2.2 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. Mix well after sterilization while the liquid is still hot to redissolve settled substances. After sterilization and cooling down, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.2.2.4.3 Culture medium for *C. albicans:* Sabouraud dextrose agar medium (SDA)

5.2.2.4.3.1 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

5.2.2.4.3.2 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.

5.2.2.4.4 Culture medium for *A. brasiliensis:* potato dextrose agar (PDA)

5.2.2.4.4.1 Composition

Potato infusion (see 5.2.2.4.4.2, Note 1)	200,0 g
Dextrose	20,0 g
Agar (see 5.2.2.4.4.2, Note 2)	20,0 g
Water	1 000 ml

5.2.2.4.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by 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.

NOTE 1 To prepare potato infusion, use a commercial dehydrated form or boil 200 g sliced, unpeeled potatoes in 1 l of water for 30 min. Filter through a cheesecloth, saving the effluent.

NOTE 2 Commercially available dehydrated medium powders which contain less than 20 g/l of agar can be supplemented with extra agar to the final concentration of 20 g/l if necessary.

5.3 Microbial strains

The test shall be run using the following strains as test micro-organisms:

— *Pseudomonas aeruginosa* ATCC®9027™[2] (equivalent strain: CIP®82.118™[3] or NCIMB®8626™[4] or NBRC®13275™[5] or KCTC®2513™[6] or other equivalent national collection strain);

— *Staphylococcus aureus* ATCC®6538™ (equivalent strain: CIP®4.83™ or NCIMB®9518™ or NBRC®13276™ or KCTC®3881™ or NCTC®10788™[7] or other equivalent national collection strain);

— *Escherichia coli* ATCC®8739™ (equivalent strain: CIP®53.126™ or NCIMB®8545™ or NBRC®3972™ or KCTC®2571™ or NCTC®12923™ or other equivalent national collection strain);

— *Candida albicans* ATCC®10231™ (equivalent strain: IP 48.72™[8] or NCPF® 3179™[9] or NBRC®1594™ or KCTC®7965™ or other equivalent national collection strain);

— *Aspergillus brasiliensis* (previously *A. niger*) ATCC®16404™ (equivalent strain: IP 1431 or IMI®149007™[10] or NBRC®9455™ or KCTC®6196™ or other equivalent national collection strain).

The culture should be reconstituted according to the procedures provided by the supplier of the reference strain. The strains should be stored in a laboratory conforming to EN 12353 or according to another suitable method.

---

2) ATCC®: American Type Culture Collection

3) CIP®: Collection de l'Institut Pasteur

4) NCIMB®: National Collection of Industrial Marine Bacteria

5) NBRC®: NITE Biological Resource Center, JP

6) KCTC®: Korean Collection for Type Cultures

7) NCTC®: National Collection of Type Cultures

8) IP: Institut Pasteur

9) NCPF®: National Collection of Pathogenic Fungi

10) IMI: International Mycological Institute, UK

5.4 Preparation and enumeration of inocula

5.4.1 General

To perform the tests, use the strains stored in the laboratory (see 5.3) to obtain the stock cultures and the working cultures.

The stock culture is a confluent culture obtained by streaking slant tubes or plates with the stored strain (single-use vial or bead). After incubation, the stock culture can be kept between 2 °C and 8 °C for two months and is used to obtain the working cultures.

The working culture, prepared when needed to perform a test, is used to obtain the calibrated suspension (inoculum).

The same growth conditions (agar media and incubation) are used for both stock cultures and working cultures (see 5.4.2 and 5.4.3).

NOTE 1 A limited number of serial subcultures and the use of confluent cultures instead of isolated colonies lower the risk of change in the susceptibility of strains. The standardization of growth conditions and of inoculum preparation improves the reproducibility of the test.

NOTE 2 Changes in susceptibility of strains stored by freezing may be observed (due to repeated heat shocks) when multidose containers are used (for example, containers with several beads brought out of the freezer to take one bead, then replaced in the freezer).

5.4.2 Preparation of bacterial and *Candida albicans* suspensions

5.4.2.1 To prepare the working culture of the test micro-organism, prepare a subculture from the stock culture by streaking slant tubes or plates (TSA for bacteria, SDA for *C.albicans*) in order to obtain a confluent culture. Incubate at (32,5 ± 2,5) °C for 18 h to 24 h.

Prepare in the same way a second subculture, starting from the first, and incubate at (32,5 ± 2,5) °C for 18 h to 24 h. A third subculture can be grown in the same way, starting from the second. The second culture and the third one (if it was carried out) form the working culture.

If the second subculture cannot be carried out in a timely manner, then the first subculture can be kept for up to 48 h in the incubator (32,5 ± 2,5) °C and used to prepare the second subculture. In this case, prepare the third 18 h to 24 h subculture and use this in the test.

It is recommended that a fourth subculture not be prepared from the initial stock culture.

5.4.2.2 Take 10 ml of diluent (5.2.2.2) and place in a suitable sterile container with approximately 5 g of sterile glass beads. Transfer loopfuls of the cells harvested from the agar medium into the diluent; the cells should be suspended in the diluent by rubbing the loop in a small amount of the diluent against the side of the container to dislodge the cells.

5.4.2.3 Shake the container manually or mechanically, for a maximum of 3 min, to homogenize the suspension. Aspirate the upper part of the suspension (avoiding any contact with the glass beads) and transfer the obtained suspension to a sterile container.

5.4.2.4 Adjust the number of cells in the suspension to 1×10^7 cfu/ml to 1×10^8 cfu/ml (bacteria) or 1×10^6 cfu/ml to 1×10^7 cfu/ml (*C. albicans*) using the diluent (5.2.2.2) and in accordance with calibration data produced in the laboratory (e.g. using a spectrophotometer, see ISO 21148:2005, Annex C).

Use this calibrated suspension within 2 h.

5.4.2.5 At the time of the test, check the initial capacity of the suspension, N. Make successive tenfold dilutions of the calibrated suspension in the diluent (5.2.2.2). Perform the enumeration by duplicating 1 ml of the suitable dilutions (see 5.6.2) into TSA for bacteria and into SDA for *C.albicans*. Incubate the dishes at (32,5 ± 2,5) °C for 24 h to 48 h.

5.4.3 Preparation of *A. brasiliensis* (previously *A. niger)* spore suspension

5.4.3.1 To obtain the working culture of the test micro-organism, use a stock culture (on PDA) aged not more than 2 months and prepare a suspension in the diluent (5.2.2.2). Inoculate by flooding the surface of PDA, poured in a Roux flask (or use an appropriate number of Petri dishes), so as to obtain a confluent culture. Incubate at (22,5 ± 2,5) °C for 7 days to 11 days.

5.4.3.2 After incubation, transfer 10 ml of the polysorbate solution (5.2.2.2.3) to the Roux flask. Gently detach the spores from the culture surface, for example using a spatula or glass beads.

Transfer the suspension to an appropriate flask and stir gently for about 1 min in the presence of glass beads. Filter the suspension through a sintered glass filter of porosity 2 (i.e. 40 µm to 100 µm).

5.4.3.3 Carry out a microscopic examination (magnification ×400) to detect the presence of germinated spores or mycelium fragments.

— If germinated spores are present, the suspension shall be discarded.

— If mycelium is present in more than one field out of ten, wash the filtered suspension by centrifuging at 2 000 g for 20 min. Wash the spores at least twice by re-suspending them in the polysorbate solution (5.2.2.2.3) and centrifuging.

5.4.3.4 Adjust the number of spores in the suspension to a value of about 1×10^6 spores/ml to 1×10^7 spores/ml using the diluent (5.2.2.2) and any appropriate means.

NOTE The use of a cell enumeration device (e.g. a haemocytometer) is recommended to adjust the number of spores. If an appropriate cell count chamber is used, follow the instructions accurately.

The suspension should be used during the same working day. It can be used on the following day if stored between 2 °C and 8 °C, but, at the time of the test, the absence of germinated spores shall be checked.

5.4.3.5 At the time of the test, check the initial capacity of the suspension, N. Make successive tenfold dilutions of the calibrated suspension in the diluent (5.2.2.2). Perform the enumeration by duplicating 1 ml of the suitable dilutions (see 5.6.2) into PDA plates (using an appropriate number of Petri dishes). Incubate the dishes at (22,5 ± 2,5) °C for 3 days to 5 days.

5.5 Demonstration of the neutralizer efficacy

5.5.1 Principle

Verification, in the presence of each micro-organism, of the ability of the neutralizer to neutralize the antimicrobial activity of the tested formulation without inhibiting the test micro-organisms.

A calibrated suspension of micro-organisms (about 10^3 cfu/ml) is inoculated in the neutralizer in the presence (test) and in the absence (control) of the formulation. The neutralizer efficacy is demonstrated if the counts performed on the inoculum, N_v, and on the control, N_{vn} (mixture of the neutralizer and diluent), are equivalent and if the count in the test, N_{vf} (mixture of the neutralizer and the formulation), is at least 50 % of N_{vn} (see 5.5.4).

5.5.2 Procedure

Run the test separately for each strain.

a) Prepare a dilution of the calibrated suspension of micro-organisms [N is between 1×10^7 cfu/ml and 1×10^8 cfu/ml for bacteria, and between 1×10^6 cfu/ml and 1×10^7 cfu/ml for *C. albicans* and *A. brasiliensis* (see 5.4.2 and 5.4.3)] in order to obtain a suspension containing about 10^3 cfu/ml (inoculum).

b) Transfer 1 g or 1 ml of the formulation to be tested into 9 ml of neutralizer (5.2.2.3). Shake to disperse the formulation. If necessary (i.e. if the neutralizer is found to be ineffective), make a second tenfold dilution in the neutralizer.

Other test conditions are acceptable provided that at least 1 g or 1 ml of formulation is used and a minimal tenfold dilution is performed.

c) Leave the "test" tubes for (30 ± 15) min at room temperature. Run a control in parallel with the same neutralizer, replacing the tested formulation with 1 ml of diluent (5.2.2.2).

d) Inoculate the "test" tubes [tenfold and, if necessary, the additional dilution in 5.5.2 b)] and "control" tubes with 1 ml of inoculum [5.5.2 a)] (the final volume is 11 ml). Mix.

e) Prepare the "inoculum control". Add 1 ml of the inoculum [5.5.2 a)] to 10 ml of diluent (the final volume is 11 ml). Mix.

f) Enumerate in duplicate by inclusion of 1 ml of each mixture ("test", "control" and "inoculum control") into appropriate agar medium (TSA for bacteria, SDA for *C. albicans* and PDA for *A. brasiliensis*).

NOTE The use of a 1 ml volume of the calibrated suspension is recommended to improve the precision of the counts ("test", "control" and "inoculum control" mixtures).

g) Incubate at (32,5 ± 2,5) °C for 48 h to 72 h for the bacteria and *C.albicans* and at (22,5 ± 2,5) °C for 3 days to 5 days for *A. brasiliensis*.

5.5.3 Calculations

Calculate the number, N_v, of micro-organisms, in colony-forming units per millilitre, present in the inoculum control [see 5.5.2 e)].

$\overline{N}$ is the mean number of colonies counted in duplicate over the plates in a 1 ml sample;

N_v shall be about 100.

Calculate the number of micro-organisms, in colony-forming units per millilitre, present in the "test" mixture with the neutralizer in the presence of the formulation, N_{vf}, and in the "control" mixture with the neutralizer in the absence of the formulation, N_{vn}.

N_{vf} or N_{vn} is the mean number of colonies counted in duplicate over the plates in a 1 ml sample of "test" or "control" mixture.

5.5.4 Interpretation of results and conclusion on neutralizer efficacy

The efficacy of the neutralizer is demonstrated if $N_{vf} \geq 0{,}5N_{vn}$ and if N_{vn} is close to N_v. If N_{vn} is not close to N_v, the neutralizer is considered toxic for micro-organisms.

The inherent variability in enumeration on agar plates shall be taken into account. Two counts are usually considered different only if their difference exceeds 50 %.

Take note of the test conditions (neutralizer, volume, etc.) and in particular the dilution of the formulation (1/10, 1/100 or other) for which the efficacy of the neutralizer was demonstrated.

If the results do not comply with the requirements, it is necessary to:

— either modify the neutralizer (see Annex C) or make a further dilution of the sample,

— or carry out a membrane filtration, if possible.

If the results still do not comply with the requirements, it is unlikely that the formulation can be contaminated by the strain concerned. It is possible, even in this case, to issue a test report [see 5.7 and 5.8 f)].

5.6 Determination of the preservation efficacy of the formulation

5.6.1 Procedure

Run the test separately for each strain.

5.6.1.1 Unpacking and aliquoting of test product

For each strain, dispense 20 g or 20 ml of the test formulation into a sterile container (5.2.1.4).

5.6.1.2 Inoculation of test micro-organisms

Add to each container 0,2 ml of calibrated inoculum (see 5.4.2 and 5.4.3) to obtain between 1×10^5 cfu/ml and 1×10^6 cfu/ml or g for bacteria, and between 1×10^4 cfu/ml and 1×10^5 cfu/ml or g for *C. albicans* and *A. brasiliensis* in the formulation (final concentration). Mix thoroughly to ensure a homogeneous distribution of the inoculum.

The initial concentration of micro-organisms present in the inoculated product, N_0, is calculated using the results of the enumeration of the calibrated inoculum, N [see 5.6.3.2 b)].

5.6.1.3 Incubation of the inoculated formulation

Store the containers holding the inoculated formulation at (22,5 $\pm$ 2, 5) °C.

5.6.1.4 Sampling and enumeration

a) At each specified sampling interval, 7 days (T7), 14 days (T14) and 28 days (T28), according to the test strain (see Annex B), take 1 g or 1 ml of the inoculated formulation.

Transfer each 1 g or 1 ml sample to 9 ml of neutralizer (5.2.2.3). Mix until homogeneous.

If the efficacy of the neutralizer has been demonstrated with the hundredfold dilution [see 5.5.2 b)], perform a second tenfold dilution in the neutralizer.

NOTE For the second dilution, another dilution factor is acceptable provided that the neutralization is demonstrated (see 5.5.4) and it is taken into account when calculating N_x (see 5.6.3.3).

Leave in contact for (30 $\pm$ 15) min at room temperature.

b) Starting from the 1/10 dilution in the neutralizer (or from the 1/100, or another, dilution for products where neutralizer efficacy is only demonstrated at the 1/100, or another, dilution), make successive tenfold dilutions in the diluent (5.2.2.2) to perform the enumeration of surviving micro-organisms.

c) All dilutions in the neutralizer (1/10 and eventually 1/100, or other) and in the diluent shall be counted at time T7. At times T14 and T28, the number of counted dilutions may be lowered in accordance with the results obtained at time T7.

d) Carry out microbial enumeration using a suitable agar medium (TSA for bacteria, SDA for *C.albicans* or PDA for *A.brasiliensis*). Microbial enumeration shall be performed in duplicate.

In Petri dishes of 85 mm to 100 mm in diameter, place 1 ml of each dilution and pour 15 ml to 20 ml of melted agar medium 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 dilution with the medium, carefully rotating or tilting the plates sufficiently to disperse the micro-organisms. Allow the mixture to solidify with the Petri dishes standing on a horizontal surface at room temperature.

Other methods of counting (spreading and membrane filtration) can be used. They require suitable adaptation to respect the parameters indicated above.

The use of a 1 ml volume of the counted dilution is recommended to improve the precision in the counts.

e) Incubate at (32,5 ± 2,5) °C for 48 h to 72 h for the bacteria and *C.albicans* and at (22,5 ± 2,5) °C for 3 days to 5 days for *A. brasiliensis*.

5.6.2 Counting of colonies

After incubation, enumerate the colonies on the incubated dishes. For all the enumerations [see 5.4.2.5, 5.4.3.5 and 5.6.1.4 c)], retain the dishes containing between 30 and 300 colonies for bacteria and *C.albicans* and between 15 and 150 colonies for *A. brasiliensis*.

When counts of surviving micro-organisms obtained in 5.6.1.4 c) are out of these limits [more than 300 (150 for *A. brasiliensis*) or less than 30 (15 for *A. brasiliensis)*], note the result as >300 (>150 for *A. brasiliensis) or <30 (<15 for A. brasiliensis).*

Determine the number of micro-organisms present in the sample at time t_0 ($N_0 = N/100$) in accordance with 5.6.3.2, and the number of survivors at each sampling time, N_x, in accordance with 5.6.3.3.

5.6.3 Calculations

5.6.3.1 General

Check that the neutralizer efficacy is verified (see 5.5.4) and that the experimental data are in accordance with the rules indicated in 5.6.2.

5.6.3.2 Determination of the initial numbers of micro-organisms, N and N_0

a) Calculate N, the number of micro-organisms present in the calibrated suspensions (see 5.4.2 and 5.4.3), in colony-forming units per millilitre, using Equation (1):

$$N = \bar{C} / (V \times d) \quad (1)$$

where

$\bar{C}$ is the mean number of colonies (see 5.6.2) counted in duplicate over the plates;

V is the volume of inoculum applied to each dish, in millilitres (1 ml, in accordance with 5.4.2 and 5.4.3);

d is the dilution factor of the counted dilution;

N shall be between 1×10^7 cfu/ml and 1×10^8 cfu/ml for bacteria, and between 1×10^6 cfu/ml and 1×10^7 cfu/ml for *C. albicans* and *A. brasiliensis*.

b) Determine N_0, the number of micro-organisms inoculated in the formulation at time t_0 using Equation (2):

$$N_0 = N/100 \quad (2)$$

N_0 shall be between 1×10^5 cfu/ml and 1×10^6 cfu/ml or g for the bacteria, and between 1×10^4 cfu/ml and 1×10^5 cfu/ml or g for *C. albicans* and *A. brasiliensis*.

5.6.3.3 Enumeration of the micro-organisms at each sampling time, N_x

Calculate N_x the number of surviving micro-organisms in the contaminated formulation, in colony-forming units per millilitre or grams, at each sampling time, t_x, (T7, T14 or T28) using Equation (3):

$$N_x = C / (V \times d) \quad (3)$$

where

C is the mean number of colonies (see 5.6.2) counted in duplicate over the plates;

V is the volume of inoculum applied to each dish, in millilitres [1 ml, in accordance with 5.6.1.4 a)];

d is the dilution factor corresponding to the retained and counted dilution [see 5.6.1.4 c), taking into account the second tenfold dilution in the neutralizer as in 5.6.1.4 a)].

5.6.3.4 Reduction in microbial counts

Calculate the reduction values, R_x, expressed in log units, obtained at each sampling time using Equation (4):

$$R_x = \lg N_0 - \lg N_x \quad (4)$$

where

N_0 is the number of micro-organisms inoculated at time t_0 [see 5.6.3.2 b)];

N_x is the number of surviving micro-organisms at each sampling time, t_x (see 5.6.3.3).

There may be no reduction, and there may even be an increase in the micro-organism count.

5.7 Interpretation of test results and conclusions

5.7.1 Criteria

The obtained log reduction values, R_x, (see 5.6.3.4) are compared to the minimum values required for evaluation criterion A or B reported in Annex B.

The criteria representing the protection capacities of a cosmetic formulation are:

— criterion A, whereby the formulation is protected against microbial proliferation that may present a potential risk for the user and no additional factors are considered [see 6.2 a)];

— criterion B, whereby the level of protection is acceptable if the risk analysis demonstrates the existence of control factors not related to the formulation indicating that the microbiological risk is tolerable for the cosmetic product [see 6.2 b)].

The criteria are expressed either by a minimum log reduction value or by “NI” when the requirement is that there be no increase in the microbial population.

The inherent variability in microbial counts that are used to determine R_x values shall be taken into consideration when comparing the obtained R_x values and the preset criteria A or B. In this International Standard, a deviation of 0,5 log units from the preset criteria is considered acceptable.

5.7.2 General case (the efficacy of the neutralizer is demonstrated for all strains)

For each micro-organism, compare the values of R_x to criterion A or B (Annex B and 5.7).

a) If all the reduction values comply with criterion A, the formulation satisfies requirements A of the preservation efficacy test and, in accordance with 6.2 a), meets the requirements of this International Standard.

b) If all the reduction values comply only with criterion B, the formulation satisfies requirement B of the preservation efficacy test. Additional justification should be provided to show that the product satisfies the requirements of this International Standard [see 6.2 b)].

c) If one or more of the reduction values do not comply with criterion A or B, then the formulation does not satisfy the requirements of the preservation efficacy test. The status of the product shall be evaluated solely according to the microbiological risk assessment [see 6.2 c)].

5.7.3 Case of formulations for which the efficacy of the neutralizer is not demonstrated for some strains

If the efficacy of the neutralizer has not been demonstrated for some strains despite additional tests (see 5.5.4), the formulation can be considered not susceptible to contamination from these microbial strains. Note the result as "not susceptible to contamination". This result is deemed equivalent to R_X given by the preset criterion of logarithmic reduction given in Annex B.

Interpret the results for the strains for which the efficacy of the neutralizer is demonstrated and compare the values of R_X to the preset criterion A or B (see Annex B and 5.7).

If all the reduction values comply with criterion A (or B), the formulation satisfies by extrapolation the requirements A (or B) of the preservation efficacy test.

If one or more of the reduction values do not comply with criterion A (or B), the formulation does not satisfy the requirements of the preservation efficacy test.

5.8 Test report

The test report shall contain the following information:

a) a reference of this International Standard, i.e. ISO 11930;

b) the identification of the testing laboratory;

c) the identification of the cosmetic product (or formulation):

   1) name of product,

   2) batch number or lot date code,

   3) name of the entity responsible for marketing the product and name of the manufacturer, if known,

   4) date of reception at the laboratory,

   5) conditions of storage at the laboratory;

d) the enumeration method used;

e) the experimental conditions:

   1) analysis period,

   2) conditions of incubation of the inoculated formulation,

   3) composition of the neutralizer,

   4) incubation temperature of Petri dishes,

   5) culture media used,

   6) test strains (origin and modes of storage),

7) modes of contamination of the formulation with the test micro-organisms (mass/volume of product formulation, volume of calibrated suspension);

f) the test results:

1) quantity of the initial numbers of micro-organisms, N and N_0 [see 5.6.3.2 a) and 5.6.3.2 b)],

2) results of the demonstration of the neutralizer efficacy for each test strain and concentration of the formulation for which the neutralization is demonstrated (in the case of non-demonstration for some strains, indicate the test results obtained and the tests carried out to achieve neutralization),

3) test results (enumerations and logarithmic reductions) for each of the test strains and each sampling time (see 5.6.3.3 and 5.6.3.4);

g) conclusions.

6 Overall evaluation of the antimicrobial protection of the cosmetic product

6.1 General

Antimicrobial protection is based on a combination of formulation characteristics, production conditions and final packaging. The overall evaluation takes into account the microbiological risk assessment together with the preservation efficacy test results, if relevant, as set out in the decision diagram (see Annex A). It is the manufacturer's responsibility to provide information to demonstrate that safety has been satisfactorily demonstrated and that the level of risk is tolerable.

6.2 Case 1 — The preservation efficacy test has been performed on the formulation

a) If the formulation meets criterion A, the microbiological risk is considered to be tolerable (the cosmetic product is protected against microbial proliferation that may present a potential risk for the user) and the cosmetic product is deemed to meet the requirements of this International Standard without additional rationale.

b) If the formulation meets criterion B, the microbiological risk analysis shall demonstrate the existence of control factors not related to the formulation; for example, a protective package such as a pump provides a higher level of protection than a jar (see Annex D). This would be considered a protective device for risk reduction**.**

Hence, if the risk analysis demonstrates the existence of control factors, the cosmetic product is deemed to meet the requirements of this International Standard on the basis of criterion B plus additional characteristics indicating that the microbiological risk is tolerable.

c) If the formulation does not meet the requirements of either criterion A or B, then the status of the product shall be evaluated solely according to the microbiological risk assessment. For example, a product in single-dose units can be considered a tolerable microbiological risk, even if the formulation does not meet the requirements of either criterion A or B, provided that the microbiological quality of the finished product is assured at the time of release.

NOTE Other data such as consumer in-use data can be used to substantiate the microbiological risk of a product.

Hence, if the risk analysis demonstrates the existence of strengthened control factors (risk reduction), then the cosmetic product is deemed to meet the requirements of this International Standard.

d) If the cosmetic product does not comply with any of the three previous situations, it does not satisfy the requirements of this International Standard.

6.3 Case 2 — The preservation efficacy test has not been performed on the formulation

On the basis of ISO 29621, the entity responsible for the production and/or the marketing of the cosmetic product shall identify those characteristics of the formulation or the finished cosmetic product, and the control factors, that ensure that the microbiological risk is low.

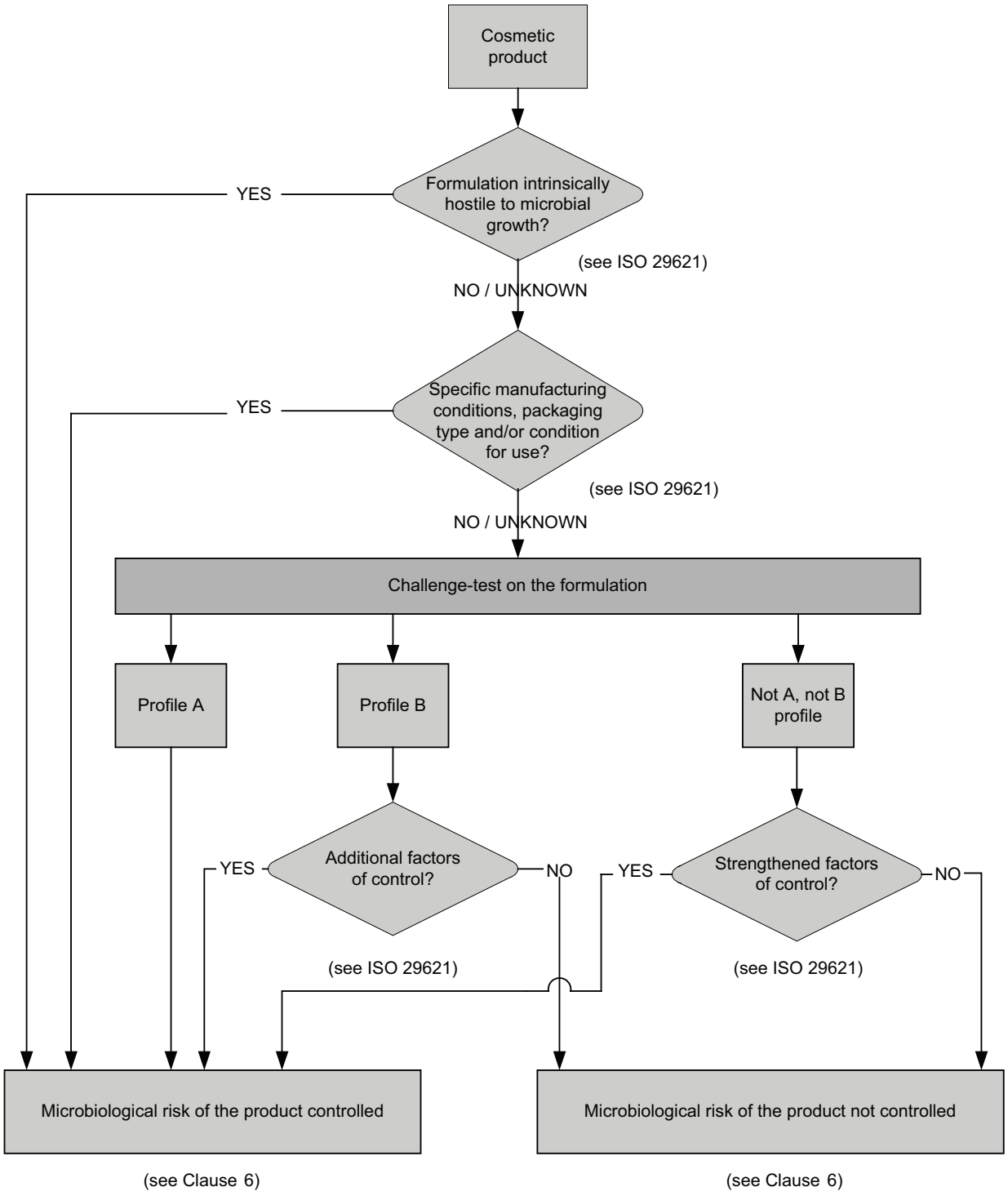
Those characteristics, and any supporting documentation, that demonstrates that the microbiological risk is tolerable, shall be reported.

Hence, if the results of the microbiological risk analysis show that the formulation or the finished product would be considered low risk, then the cosmetic product is deemed to meet the requirements of this International Standard on the basis of a microbiological risk evaluation, provided that the cosmetic product is produced in compliance with good manufacturing practice.

If the results of the microbiological risk assessment show that the formula or the finished product would not be considered a tolerable risk, then the cosmetic product does not meet the requirements of this International Standard. Risk reduction shall be performed to bring the product into compliance.

Annex A
(normative)

Decision diagram



Annex B
(normative)

Evaluation criteria for the preservation efficacy test (see 5.7)

Table B.1 — Evaluation criteria

Log reduction values ($R_x = \lg N_0 - \lg N_x$) required[a]								
Micro-organisms	**Bacteria**			***C. albicans***			***A. brasiliensis***	
Sampling time	T7	T14	T28	T7	T14	T28	T14	T28
Criteria A	≥3	≥3 and NI[b]	≥3 and NI	≥1	≥1 and NI	≥1 and NI	≥0[c]	≥1
Criteria B	Not performed	≥3	≥3 and NI	Not performed	≥1	≥1 and NI	≥0	≥0 and NI

[a] In this test, an acceptable range of deviation of 0,5 log is accepted (see 5.7).

[b] NI: no increase in the count from the previous contact time.

[c] $R_x = 0$ when $\lg N_0 = \lg N_x$ (no increase from the initial count).

Annex C
(informative)

Examples of neutralizers for the antimicrobial activity of preservatives and washing liquids

Table C.1 — Examples of neutralizers for the antimicrobial activity of preservatives and washing liquids

Preservative	Chemical compounds able to neutralize the antimicrobial activity of preservatives	Suitable neutralizers and washing liquids (for membrane filtration methods)
Phenolic substances: Parabens, phenoxyethanol, phenylethanol, etc. Anilides	Lecithin Polysorbate 80 Fatty alcohol ethylene oxide condensate Non-ionic surfactants	Polysorbate 80, 30 g/l + lecithin, 3 g/l. Fatty alcohol ethylene oxide condensate, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l. D/E neutralizing broth[a]; SDCLP broth[b] Washing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Quaternary ammonium salts Cationic surfactants	Lecithin, saponin, polysorbate 80, sodium dodecylsulphate Fatty alcohol ethylene oxide condensate	Polysorbate 80, 30 g/l + sodium dodecylsulphate, 4 g/l + lecithin, 3 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. D/E neutralizing broth[a]; SDCLP broth[b]. Washing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Aldehydes Formaldehyde-generating 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[a]; SDCLP broth[b] Washing liquid: polysorbate 80, 3 g/l + L-histidine 0,5 g/l.
Oxidizing agents	Sodium thiosulphate	Sodium thiosulphate, 5 g/l. Washing liquid: sodium thiosulphate, 3 g/l.
Isothiazolinones Imidazoles	Lecithin, saponin Amines, sulphates, mercaptans, sodium bisulphite, sodium thioglycolate	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. Washing 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. Washing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Metal salts (Cu, Zn, Hg) Organo-mercurials	Sodium bisulphite, L-cysteine Sulphydryl compounds, thioglycolic acid	Sodium thioglycolate, 0,5 g/l or 5 g/l. L-cysteine, 0,8 g/l or 1,5 g/l. D/E neutralizing broth[a]; SDCLP broth[b] Washing liquid: sodium thioglycolate, 0,5 g/l.

[a] Dey/Engley neutralizing broth.

[b] Soybean-casein digest broth with lecithin and polysorbate 80.

Annex D
(informative)

Packaging characteristics

Consideration should be given to the configuration of the container. Package design plays a major role in the risk assessment scheme and in determining the overall antimicrobial protection of a cosmetic product.

The configuration of the packaging container, or use of a refillable container, may influence the choice of preservative system. The chances of microbial contamination by the consumer are increased when the container has a wide orifice and is subjected to direct consumer contact. Likewise, the chances of microbial contamination by the consumer are decreased when the container is a unit dose, ophthalmic tip tube, one-way valve or when direct consumer contact is prevented. Package size versus quantity used per application should be considered since use of a large package which contains more products and extends usage over a longer period of time increases the possibility of contamination. The presence of supplementary applicators such as brushes, pads or puffs will also affect the antimicrobial protection needs.

The following factors are among those taken into consideration when assessing product risk with regards to packaging:

— single- or multiple-use packaging;

— size of the package;

— mode of dispensing the product;

— predicted use-up rate;

— whether the package type allows for direct consumer contact;

— whether the package is pressurized.

Bibliography

[1] ISO/IEC Guide 51, *Safety aspects — Guidelines for their inclusion in standards*

[2] EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal, mycobactericidal, sporicidal and fungicidal activity*

[3] IBRAHIM Y.K.E., GEISS H.K., SONNAG H.-G., Alternatives to the traditional preservatives, SöFW, **118**, June 1192

[4] LÜCK E., JAGER M., *Antimicrobial food additives; characteristics, uses, effects*, Springer (ed), 1995, pp. 122-124

[5] *Application of Water Activity Determination to Nonsterile Pharmaceuticals Products*, USPC, Inc., **30**(5), 2006, in-process revision: <1112>

[6] CTFA Microbiology guidelines 2007

[7] RUSSELL N.J., GOULD G.W., *Food preservatives*, Blackie and Son Ltd., 1991, pp. 16-17

[8] DENYERS S., BAIRD R., *Guide to microbiological control in pharmaceuticals*, 1990, pp. 16-17 and p. 285

[9] RAHMAN M.S., *Handbook of food preservation*, Marcel Dekker, Inc., 1999, pp. 95-172 and p. 386

[10] MEIER M., FISHER F.X., KELLER M., HALFMANN H.-J., Influence of alternative propellants on microbial viability in comparison to chlorofluorocarbons, *Pharm. Ind.*, **58**, pp. 78-82, 1996

[11] DECLERCK J., CAIRE-MAURISIER F., GENOT P., LEVACHER E., MICHAUT A., SCHEIBER G., TARDIVET S., Les gaz propulseurs: les HFC (hydrofluorocarbones) alternatives aux CFC (chlorofluorocarbones) pour les préparations pharmaceutiques pressurisées, *SFSTP Pharmapratiques*, **16**(1), January/February 2006, pp.61-72

[12] BLOOMFIELD S.F., BAIRD R., LEAK R.E., LEECH R., Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries, *Ellis Horwood Series in Pharmaceutical Technology*, 1988, p. 78

[13] SAWYER E., GREEN B., COLTON H., Microorganisms survival in non-CFC propellant P134a and a combination of CFC propellants P11 and P12, *Pharmaceutical Technology*, March 2001, pp. 90-96

[14] BRANNAN D.K., Packaging's role in preservation, *Cosmetics and Toiletries*, **113**, Abilene Christian University, Abilene, TX, April 1998

[15] SHARPELL F. and MANOWITZ M., Preservation of cosmetics, *Disinfection, sterilization and preservation*, fourth edition, Seymour S. Block — Lea & Febiger, 1991

[16] IBRAHIM Y.K.E., SONNAG H.-G., Preservative potentials of some aerosol propellants, *Effectiveness in some pharmaceutical oils*, Drugs made in Germany, **2**, 1995

[17] KABARA J.J., ORTH D.S., Principles for product preservation, *Preservative-free and self-preserving cosmetics and drugs, principles and practice*, Marcel Dekker, Inc. (ed.), New York, 1997, pp. 1-14

[18] FRIEDEL R.R., CUNDELL A.M., The application of water activity measurement to the microbiological attributes testing of nonsterile over-the-counter drug products, *Pharmacopeial Forum*, **24**(2), March-April 1998

[19] BRANNAN D.K., Dille J.C., Type of closure prevents microbial contamination of cosmetics during consumer use, *Appl. Environ. Microbiol*., **56**, 1990, pp. 1476-1479

[20] ENIGL D.C., SORRELS K.M., Water activity and self-preserving formulas, *Preservative-free and self-preserving cosmetics and drugs, principles and practice*, Marcel Dekker, Inc. (ed.), New York, 1997, pp. 45-71

ICS 07.100.99;71.100.70

Price based on 20 pages