	Title	Revision n°	date	Document n°
	PRESERVATIVE EFFICACY TEST FOR COSMETIC PRODUCT	0	**2/12/05**	ACM MAL 08

1 SCOPE AND FIELD OF APPLICATION

To determine the efficacy of the antimicrobial activity of preservatives used in cosmetic products.
The method covers the determination of the suitability of preservation of cosmetics products. It sets minimal requirements for preservation performance in the products.

Note: some cosmetic ingredients and formulae show intrinsic antimicrobial activity.

2 PRINCIPLE

The test consists in challenging a non contaminated product with a prescribed inoculum of suitable microorganisms and storing the inoculated product at a prescribed temperature. Using serial dilutions and plate counts, the number of organisms surviving in the test products are determined at specified interval of times. Products meeting the specified criteria will be considered adequately preserved for manufacture and consumers use. Products not meeting criteria will be considered inadequately preserved.

3 APPARATUS

3.1 Biohazard cabinet / Laminar air flow cabinet
3.2 Autoclave
3.3 Hot air oven
3.4 Incubator: 35 ± 2˚C and 25 ± 2˚C
3.5 Vortex mixer
3.6 Glass beads
3.7 Pipettes
3.8 Petri dishes
3.9 Haemocytometer & Phase-contrast Microscope (if available)
3.10 Spectrophotometer / Colorimeter (if available)
3.11 Colony counter
3.12 Media bottles
3.13 Universal bottles / Test tubes
3.14 Inoculating loops / spreader
3.15 Bunsen burner (or Bacticinerator)
3.16 Waterbath
3.17 Top pan balance
3.18 pH meter

4 MEDIA AND REAGENTS

For convenience, dehydrated media of any brand equivalent in function may be used.
Media should be tested for sterility and growth promotion using suitable organisms. Refer to standard Pharmacopeia.

4.1 Nutrient Agar (or other suitable equivalent media)
4.2 Letheen Agar (or other suitable equivalent media e.g. TSA with 1% Tween 80, "TSA t ")
4.3 Mycophil Agar, pH 4.7 (or other suitable equivalent media e.g. "PDA a" / "SDA a" , SDA with 1% Tween 80 "SDA t")
4.4 Letheen Broth (or peptone saline with 1% Tween 80)
4.5 Chloride buffer (or similar buffer)
4.6 Diluent 1 – Sterile solution containing 0.9% sodium chloride and 0.1% peptone
4.7 Diluent 2 – Sterile solution containing 0.9% sodium chloride and 0.05% Tween 80

Note : a = with antibiotic

5 TEST ORGANISMS

5.1 *Pseudomonas aeruginosa* ATCC 9027, CIP 82.118, or equivalent
5.2 *Staphylococcus aureus* ATCC 6538 (NCIMB 9518, CIP 4.83, NCTC 10788)
5.3 *Candida albicans* ATCC 10231 (NCPF 3179, IP 48.72)
5.4 *Enterobacter aerogenes* ATCC 13048
5.5 *Aspergillus niger* ATCC 16404 (IMI 149007, IP 1431.83)

6 MAINTENANCE OF MICRO-ORGANISMS

The micro-organisms listed shall be maintained accordingly :
6.1 Bacteria – Nutrient agar (TSA or other suitable equivalent media)
6.2 Yeasts and Fungi – Mycophil agar pH 4.7 (or other suitable equivalent media eg PDA / SDA with antibiotic)

7 PROCEDURES

7.1 Preparation of Challenge Micro-organisms

7.1.1 The viable micro-organisms used in the test must not be more than five passages removed from the original ATCC (or equivalent) culture.

7.1.2 Streak *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterobacter aerogenes* stock cultures onto TSA slants and incubate at 35 ± 2˚C for 18-24 hours.

7.1.3 Streak *Candida albicans* stock culture onto SDAa slant and incubate at 25 ± 2˚C for 48 hours.
7.1.4 Streak *Aspergillus niger* culture onto SDAa slant and incubate at 25 ± 2˚C for 7 – 14 days or until full sporulation is achieved.

7.2 Harvesting Microorganisms Cultures

7.2.1 Wash each slant of bacteria and yeast culture with 2 x 2.5 m of diluent 1; and fungi culture with 2 x 2.5 m of diluent 2, loosening the culture from the agar surface with the help of sterile glass beads. Transfer the suspension into sterile universal bottle and mix by using mechanical mixer to disperse evenly.

7.2.2 Adjust each wash with the same diluent to yield 108 cfu/m of bacterial suspension and 107 cfu/m of yeast and fungi suspensions either by using McFarland $BaSO_4$ standard No.2, direct microscopic count, turbidimetry, absorbance, or other method correlated to an aerobic plate count (APC as describe in paragraphs 3 and 4).

7.3 Determination of Challenge Bacterial Level, APC method – Surface Spread Technique

7.3.1 Pour about 15 – 20 m of melted TSA in sterile Petri dishes and let it solidify.

7.3.2 Fill up each of 10 sterile universal bottles/test tubes with 9 m diluent 1.

7.3.3 Inoculate 1 m of each bacterial suspension (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterobacter aerogenes*) into the first bottle/tube containing 9 m diluent 1 (label as 10-1). Repeat the 10-fold dilution process until 10-10. Mix by using mechanical mixer to ensure homogenous distribution.

7.3.4 Pipette in duplicate 0.5 m inoculum from 10-4 to 10-10 dilution and spread over TSA surface.

7.3.5 After inoculum is absorbed by the medium, invert the plates, and incubate at 35 ± 2 °C for 24 - 48 hours.

7.3.6 Record growth between 30 – 300 colonies, add the colony counts obtained from two plates and multiply the total colonies by the appropriate dilution factor.

7.3.7 Use the suspension containing 108 cfu/m of bacteria for the test.

7.3.8 Use the organisms immediately or refrigerate them at 2 - 8˚C for no more than 72 hours.

7.4 Determination of Challenge Yeast and Fungi Level APC Method – Pour Plate Technique

7.4.1 Melt SDAa and maintain it at 45 - 50°C in a waterbath.

7.4.2 Fill up each of 10 sterile universal bottles/test tubes with 9 m of diluent (diluent 1 for *Candida albicans* and diluent 2 for *Aspergillus niger*).

7.4.3 Inoculate 1 m each of *Candida albicans* and *Aspergillus niger* suspension into the first bottle/tube containing 9 m diluent 1 and diluent 2 respectively. Repeat the 10-fold dilution process until 10-10. Mix by using mechanical mixer to ensure homogenous distribution.

7.4.4 Pipette in duplicate 1 m inoculum from 10-4 to 10-10 and transfer onto sterile Petri dishes. Pour about 15 – 20 m of melted SDAa (about 45°C) into each dish, cover and swirl gently to mix and let it solidify.

7.4.5 Invert plates and incubate at 25 ± 2 oC for 48 hours (yeast) and 72 hours (fungi).

7.4.6 Record growth between 30 – 300 colonies, average the colony counts obtained from two plates and multiply the total colonies by the appropriate dilution factor.

7.4.7 Use the suspension containing 107 cfu/m each of yeast and fungi for the test.

7.4.8 Use the organisms immediately or refrigerate them at 2 - 8˚C **for not more than 72 hours.**

7.5 Sample Preparation

7.5.1 Prepare 5 x 100 g of sample in five suitable glass containers. Label appropriately.

7.5.2 Liquid Cosmetics. Use product as it is for the test.

7.5.3 Water based cream and lotion. Dilute with the same weight of chloride buffer. Warm at 40 - 45˚C and with the help of the glass beads, mix by using mechanical mixer.

7.5.4 Solid and powders. Disperse product with the same weight of chloride buffer. Warm at 40 - 45˚C and with the help of the glass beads, mix by using mechanical mixer .

7.5.5 Oil based, waxy/ fatty products. Mix 100 g sample with 20 g mineral oil to form a paste. Add 80 m of chloride buffer. Warm at 40 - 45˚C and with the help of the glass beads, mix by using mechanical mixer.

7.5.6 Aerosol. Refrigerate the whole container (2 - 8˚C) for 30 minutes. Transfer 50 g of aerosol into suitable container containing 50 m of chloride buffer. With the help of the glass beads, mix by using mechanical mixer.

7.5.7 Proceed with the test as in paragraph 6.

7.6 Test Procedure

7.6.1 The sample must be verified for the absence of growth of bacteria, yeast and fungi, using test method "ACM THA 06". This is to be done before proceeding further on.

7.6.2 Inoculate each sample container with 1 m suspension of one of the test organisms to give an inoculum of 10^6 (for bacteria) or to 10^5 cfu/g (for yeast and fungi). **[The volume of the inoculum suspension should not exceed 1% of the sample taken].** Mix thoroughly using mechanical mixer.

1. Inoculate 100 g sample without preservative or 100 g peptone saline containing 1% Tween 80 with 1 m suspension of one of the test organisms to give an inoculum of 10^6 (for bacteria) or to 10^5 cfu/g (for yeast and fungi) as a control.

2. Remove 1 m of sample from each container immediately (0 day) and at intervals of 7, 14, 21 and 28 days. Proceed with a 10-fold serial dilutions using peptone saline containing 1% polysorbate 80.

3. Note: Sample is to be kept at ambient temperature (20 - 25˚C) for the whole duration of the test.

4. Determine the number of viable microorganisms in duplicate by surface spread technique on TSAt for bacteria, and pour plate technique using SDAt for yeast and fungi.

5. Incubate bacteria at 35 ± 2 °C for 24 to 48 hours, yeast and fungi at 25 ± 2 °C for 3 to 5 days. Count and calculate the number of surviving microorganisms per g or m of sample.

6. Verify the identity of the microorganisms by Gram staining where appropriate.

For validation purpose, the control test must be performed concurrently with the sample.

7.7 Interpretation of Data

The preservative shall show the following activity against test microorganisms :

1. Bacteria and yeasts should show at least a 99.9 % decrease within 7 days following each challenge and no increase thereafter for the remainder of the test within normal variation of data.

2. Fungi should decrease by at least 90 % within 28 days and show no increase during the test period within normal variation of data.

Controls or samples with no preservative (with the exception of self preserved products) should fail this test.

7.8 Precision and Bias

Precision and bias of this method have not been determined. Replicate samples are recommended.

8 REFERENCES

1. The American Society for Testing and Materials, Designation: E 640 – 78 (Reapproved 1998), Standard Test Method for Preservatives in Water-Containing Cosmetics, pp. 141 -142

2. AOAC Official Methods of Analysis (2000), Chapter 15, Efficacy of Preservation of Non-Eye Area Water-Miscible Cosmetic and Toiletry Formulations, pp 3 – 5

3. British Pharmacopoeia 2003 Version 7, Volume 3, Appendix XVI C, Efficacy of Antimicrobial Preservation, pp 136.

Harmonised method:

- **Issued by the chemical analysis group at the harmonization workshop in Kuala-Lumpur, on September 13th to 17th, 2004**
- **Approved by the harmonization workshop delegates workshop in Kuala-Lumpur, on September 13th to 17th, 2004,**
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