Abstract The microbial safety of cosmetic products is an important consideration. The results of preservation challenge test are required for the safety assessment. Different methods are commonly available and used in the cosmetic industry. These methods are different in the type of microorganisms used, the inoculation of samples, the criteria of acceptance, and the consideration of preservative stability. They will be compared by applying them on a Q 10 containing body lotion. Using borderline concentrations of the preservative system shows how different criteria of acceptance lead to different assessments. However, in order to fix the period after opening (PAO) additional data beside the microbiological challenge test have to be considered.

INTRODUCTION

Cosmetic products should be safe under normal or reasonably foreseeable conditions of use. While the regulation on cosmetic products (EC 1223/2009) (1) requires that the cosmetic product safety report demonstrates the results of preservation challenge test to prove the microbiological stability, it does not specify the test procedure for the challenge test. Alongside the test from the EU (2) and US pharmacopoeia (3), the Personal Care Products Council (4), the ASEAN Cosmetics Association (5), in-house test protocols (6, 7) have been established for many years. A new method, the first edition of ISO 11930 was published in April 2012 under the title “Cosmetics - Microbiology - Evaluation of the antimicrobial protection of a cosmetic product” (8). This paper will examine the comparability of these methods.

REGULATIONS

Demands for challenge testing are specified only in the SCCS notes of guidance . In paragraph 4.4 “Guidelines on Microbiological of the Finished Cosmetic Product” in the 8th revision (December 2012) (9) of the SCCS publication “Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation” is an obligation for carrying out a preservation test with all cosmetic products which could be contaminated under normal storage and usage conditions or if an infection risk for the consumer exists.

The use of Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans from official collection strains from any state within the EU is obligatory. Standardized cultures and inoculum preparation is necessary to achieve a sufficient reproducibility (10, 11). In addition, the use of specific germs, which are known to lead to spoilage of cosmetic products, is recommended by the SCC.

METHODS APPLIED TO EVALUATE THE ANTIMICROBIAL PROTECTION OF COSMETICS

In vitro challenge testing of cosmetics has a good correlation to possible contaminations during its use (12). Accelerated test methods have been published (13), but according to our knowledge they are rarely used. For special applications like wet wipes particular methods are necessary (14). Similar tests are used for technical applications like water-born paints (15).

The following methods are commonly available and used in the cosmetic industry:

1. Pharmacopoeia methods:
   a. Ph. Eur. 7 - 5.1.3 “Efficacy of Antimicrobial Preservation” (2)
   b. USP 35 Chapter 51 “Antimicrobial Effectiveness Testing” (3)
2. CTFA Microbiology Guidelines:
   a. CTFA M-3 “A Method for Preservation Testing of Water-Miscible Personal Care Products” (4)
   b. CTFA M-4 “Method for Preservation Testing of Eye Area Cosmetics” (4)
   c. CTFA M-5 Methods for Preservation Testing of Nonwoven Substrate Personal Care Products (4)
   d. CTFA M-6 A Method for Preservation Testing of Atypical Personal Care Products (4)
   e. CTFA M-7 A Rapid Method for Preservation Testing of Water-Miscible Personal Care Products (4)

3. ASEAN Cosmetic Harmonized Testing Method
   Association of Southeast Asian Nations, Preservative efficacy test for cosmetic product, Document N° ACM MAL 08 (5):

4. In-house test protocols:
   a. schülke KoKo test “Determination of the Preserving Effect of Chemical Preservatives in Cosmetic Formulations” (6, 7)
   b. schülke FeuTuKo test “Method to Determine the Preserving Effect of Chemical Preservatives in Wet Tissues” (16)

5. ISO standard:
   a. ISO 11930 “Evaluation of the antimicrobial protection of a cosmetic product” (8)

For water soluble or water miscible products such as shampoos, lotions, creams etc. usually the pharmacopoeia methods, the KoKo test, the CTFA M-3, CTFA M-4 or the ASEAN test are used to prove the antimicrobial protection. As an ISO standard a new method was published in April 2012, the ISO 11930. These methods are compared in the following paragraphs.

Compared to other methods ISO 11930 is an overall standard to evaluate the antimicrobial stabilisation of a cosmetic product. With the decision diagram in Annex A also formulations intrinsically hostile to microbial growth, specific manufacturing conditions, packaging and/or conditions for use and the reference to ISO 29621 (Guidelines for the risk assessment and indemnification of microbiologically low risk products) (17) are covered.

A preservation efficacy test is according to ISO 11930 not required for those cosmetic products for which the microbiological risk has been determined to be low in accordance with ISO 29621. Some examples of low risk products are shown in table 1.

### TEST MICROORGANISMS

The recommended microorganisms for the different test methods are summarised in table 2. The ISO 11930, the Ph. Eur. 7, the USP 35 and the ASEAN tests use only pathogenic microorganisms; they only partly fulfil the recommendation of the SCCS to use additional specific germs, which are known to lead to spoilage of cosmetic products. The Ph. Eur. 7 recommends an optional use of "strains or species that may represent likely contaminants to the preparation".

The test methods from the CTFA Microbiology Guidelines differentiate between testing of eye area cosmetics and other water miscible cosmetic products. Eye cosmetics are recommended to be tested with more species. Nevertheless, the CTFA methods have the biggest variability, only the use of different types of microorganisms is specified. The CTFA includes in their selection lists typical product spoiling microorganisms; they can be tested instead or in addition to pathogenic species.

The schülke KoKo test includes these microorganisms, selected using decades of experience from schülke’s support services to cosmetic producers through the scope of MGM (Microbiological Quality Management). Only the schülke KoKo test completely fulfill recommendations of the SCCS Notes of Guidance.

### CULTIVATION OF TEST MICROORGANISMS

The cultivation of the test germs is comparable for all methods. The surface of a suitable solid agar medium is inoculated separately with the specified microorganisms. Typical culture conditions are:

- Bacteria: 30-35°C / 18-24 h
- Yeasts: 20-25°C / 44-52 h
- Moulds: 20-25°C / 6-10 days

<table>
<thead>
<tr>
<th>Physico-chemical factor</th>
<th>Limit</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>≥5.0</td>
<td>Skin peels (glycolic acid)</td>
</tr>
<tr>
<td>pH</td>
<td>≥10.0</td>
<td>Hair relaxer</td>
</tr>
<tr>
<td>Ethanol or other alcohol</td>
<td>≥2%</td>
<td>Hair sprays, tonics, perfumes</td>
</tr>
<tr>
<td>Filling temperature</td>
<td>≥65°C</td>
<td>Lip balms, lotions, cream blushes</td>
</tr>
<tr>
<td>Water activities</td>
<td>≥0.75</td>
<td>Shampoo, based products, nail enamels</td>
</tr>
<tr>
<td>Ocuting products</td>
<td></td>
<td>Hair dry</td>
</tr>
<tr>
<td>Aluminium chlorhydrate</td>
<td>≥2%</td>
<td>Anti-perspirants</td>
</tr>
</tbody>
</table>

Table 1. Product characteristics and their combinations may be considered as examples of low-risk products.

Table 2. Microorganisms used for challenge testing.

<table>
<thead>
<tr>
<th>SCCS</th>
<th>Ph. Eur</th>
<th>USP &lt;51&gt;</th>
<th>CTFA M-3</th>
<th>CTFA M-4</th>
<th>ASEAN</th>
<th>KoKo</th>
<th>ISO 11930</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td></td>
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<tr>
<td>Actinomyces naeslundii</td>
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<tr>
<td>Bacillus subtilis</td>
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<tr>
<td>Kluverobacter caprae</td>
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<td></td>
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<tr>
<td>Enterococcus faecalis</td>
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<tr>
<td>Enterobacter aerogenes</td>
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<tr>
<td>Enterobacter cloacae</td>
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<tr>
<td>Flavobacterium species</td>
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<tr>
<td>Klebsiella pneumoniae</td>
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<tr>
<td>Kocuria rhizophila</td>
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<tr>
<td>Proteus vulgaris</td>
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<tr>
<td>Proteus mirabilis</td>
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<td></td>
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<tr>
<td>Pseudomonas aeruginosa</td>
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<tr>
<td>Pseudomonas putida</td>
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<tr>
<td>Pseudomonas stutzeri</td>
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<tr>
<td>Staphylococcus aureus</td>
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<tr>
<td>Staphylococcus epidermidis</td>
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<tr>
<td>Yeasts</td>
<td></td>
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<td></td>
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<tr>
<td>Candida albicans</td>
<td></td>
<td></td>
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<tr>
<td>Candida parapsilosis</td>
<td></td>
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<td></td>
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<tr>
<td>Moulds</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Aspergillus brasiliensis</td>
<td></td>
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<tr>
<td>Penicillium brevicompactum</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Penicillium simplicissimum</td>
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<td></td>
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<tr>
<td>Zygosporella matildae</td>
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</tr>
</tbody>
</table>

Legend:
- obligatory to be used
- at least one of this group
- at least two of this group

Table 2. Microorganisms used for challenge testing.
All tests have similar bacteria loads in the inoculated sample. The mould load is slightly higher in the pharmaceutical tests.

**CRITERIA OF ACCEPTANCE**

The criteria of acceptance are summarised in Table 3. In Figure 2 the differences are visualised as graphic representation.

**PRESERVATIVE STABILITY**

Preservative stability is an important factor to ensure the microbial quality. The SCCS requires [9]:

<table>
<thead>
<tr>
<th>Methods</th>
<th>Instructions related to preservative stability</th>
</tr>
</thead>
</table>
| **Ph. Eur.** | The Ph. Eur. stipulates to investigate the antimicrobial activity with stored samples:  
- The antimicrobial activity of the preparation in its final container is investigated over the period of stability to ensure that such activity has not been impaired by storage. |
| **USP <51>** | The USP 35 does not address the issue of stability; there is only the statement:  
- The tests and criteria for effectiveness apply to a product in the original, unopened container in which it was distributed by the manufacturer. |
| **CTFA M-3** | Under “Other Considerations” the storage stability is included:  
- It is important that challenge tests also be conducted on samples aged in the final container in order to determine the stability of the preservative system. |
| **CTFA M-4** | For eye area cosmetic, it is clearly stated in the guidelines:  
- Product should be evaluated for preservative stability in commercial packaging by testing after storage that simulates warehouse, shipping and shelf-life conditions. |
| **ASEAN** | The sixfold inoculation includes automatically a preservative stability check, the sixth inoculation is performed on a sample which is at least six weeks old. Practical experience has shown that this time scale is sufficient to ensure microbial quality. |

**Table 3. Criteria of acceptance.**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Species</th>
<th>Log Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ph. Eur.</strong></td>
<td><strong>Bacteria</strong></td>
<td>21d 7d 14d 21d 28d 35d 42d</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>- x2 x3 - - Nl - -</td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>- - - x2 x3 - Nl -</td>
<td></td>
</tr>
<tr>
<td><strong>USP &lt;51&gt;</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - x2 x3 - x2 -</td>
</tr>
<tr>
<td><strong>CTFA M-3</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - - - - - -</td>
</tr>
<tr>
<td><strong>CTFA M-4</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - - - - - -</td>
</tr>
<tr>
<td><strong>KoKo</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - - - - - -</td>
</tr>
<tr>
<td><strong>ASUREN</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - - - - - -</td>
</tr>
<tr>
<td><strong>ISO 11930 A</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - - - - - -</td>
</tr>
<tr>
<td><strong>ISO 11930 B</strong></td>
<td><strong>Bacteria</strong></td>
<td>- - - - - - -</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td>- - - - - - -</td>
<td></td>
</tr>
<tr>
<td><strong>Yeast</strong></td>
<td>- - - - - - -</td>
<td></td>
</tr>
<tr>
<td><strong>Mould</strong></td>
<td>- - - - - - -</td>
<td></td>
</tr>
</tbody>
</table>

**Legend**

- No test
- Decrease reduction
- Continued reduction
- Continued reduction, to less than detectable

**Table 4. Instructions related to preservative stability in the different challenge test methods.**
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CLR maintains an integrated management system that combines both quality
and environmental aspects in accordance with ISO 9001 and 14001 standards.
- The efficacy of the preservation of a cosmetic product under development has to be assessed experimentally in order to ensure microbial stability and preservation during storage and use.

To ensure a safe preservation over the shelf life of a cosmetic product a single inoculation of a fresh prepared sample is insufficient. Degradation or inactivation of actives may occur, e.g. methylchloroisothiazolinone or bronopol at alkaline pH. Table 4 shows the instructions related to preservative stability of the different test methods.

**ASSESSMENT OF THE PERIOD AFTER OPENING**

In the Cosmetic Regulation (EC) No 1223/2009 (1) article 19c is required:
- Indication of the date of minimum durability shall not be mandatory for cosmetic products with a minimum durability of more than 30 months. For such products, there shall be an indication of the period of time after opening for which the product is safe and can be used without any harm to the consumer.

This requirement was already described under article 6(1)(c) of the Cosmetics Directive (76/768/EEC) (18). This period after opening (PAO) cannot be fixed only on the basis of a challenge test. The life cycle of a product is shown in figure 3.

The challenge test, especially the KoKo test, can guaranty the safe production and filling including the storage in the closed package. The fixing of the PAO has to include additional data beside the microbiological challenge test for example (19):
- Analytical data (e.g. analyses of preservatives, preferably according to the methods of analysis published by the European Commission (20))
- Type of packaging
- Experience with similar recipes and product
- Complaint statistics
- Consumer habits and practices

Modern soft preservatives, e.g. the combination phenoxyethanol / ethylhexylglycerin are not suspect to degradation. A good stability can be expected, the possible migration in the oil phase is according to our experience completed during the six weeks testing time of the KoKo. For the acid based preservatives at a minimum the pH stability of the cosmetic formulation has to be checked over a longer storage period.

For the highly reactive preservatives such as isothiazolinones, bronopol, formaldehyde donors etc., analytical data for the long term stability in the cosmetic product may be necessary.

**Table 5. Selected preservative systems used in the challenge tests.**

- Unpreserved lotion
- Lotion preservative 1: 0.69% phenoxyethanol, 0.10% ethylhexylglycerin
- Lotion preservative 2: 0.69% phenoxyethanol, 0.11% ethylhexylglycerin
- Lotion preservative 3: 0.50% phenoxyethanol, 0.20% ethylhexylglycerin
- Lotion preservative 4: 0.60% phenoxyethanol, 0.19% ethylhexylglycerin, 45 ppm methylchloroisothiazolinone
- Lotion preservative 5: 0.31% phenoxyethanol, 0.31% benzyl alcohol, 0.19% potassium sorbate (pH adjusted to 5.3)
- Lotion preservative 6: 0.74% phenoxyethanol, 0.12% benzoic acid, 0.07% sodium metabisulfite, 0.62% ethylhexylglycerin, 0.01% polyaminopropyl biguanide (pH adjusted to 5.1)
EXPERIMENTAL DATA

Using an unpreserved body lotion containing Q10 (INCI: aqua, glycerin, ethylhexyl stearate, isopropyl palmitate, ceteryl isononanoate, glyceryl stearate citrate, ceteryl alcohol, persea gratissima oil, tocopheryl acetate, creatine, parfum, carborner, allantoin, sodium hydroxide, xanthan gum, ubiquinone), we ran the preservative efficacy tests according to ISO 11930 in comparison to Ph. Eur. 7 and the schüleko KoKo test. Formulations containing coenzyme Q-10 (ubiquinone) are known to be more susceptible to mould growth (21). Table 5 shows the preservative systems which have been added for challenge testing.

For the ISO 11930 and Ph. Eur. test reduction in microbial counts are expressed in log units \( R = \lg N_0 - \lg N_x \). Negative figures indicate an increase of the microbial count in the test sample. The results are summarised in figure 4.

For the KoKo test, the preserving effect is evaluated semi-quantitatively by the growth of different streaks. The results are summarised in table 6.

SUMMARY EXPERIMENTAL DATA

The criteria of acceptance for ISO 11930 appear to be the easiest to fulfil. It is questionable if the demands of protection for a cosmetic product should not be stricter than for pharmaceutical products. The test for efficacy of antimicrobial preservation according to EU Pharmacopoeia and the criteria of acceptance are designed for the production of drugs under the strong requirements of the pharmaceutical GMP (Good Manufacturing Practice). Additionally the useable lifespan after opening of cosmetic products are generally longer than for pharmaceutical products.

The schüleko KoKo test is specifically verified for the assessment of cosmetic products. From more than 25 years of experience (6,7) with this test method, we can state a 30 month microbiological stability for a product passing criteria A, which is recommended for cosmetic products.

For all methods the period after opening has to be assessed separately.

The lotion with the preservative system 1 passed only ISO 11930 and Ph. Eur. criteria B. Nevertheless, even 28 days after the inoculation with Aspergillus brasiliensis it was still detectable.

Within 7 days the decrease of moulds is insufficient, the KoKo failed.

The lotion with the preservative system 2 and 3 passed ISO 11930 and Ph. Eur.: KoKo showed only slight growth in the last cycles, it fulfills criteria B.

PRECISION AND REPRODUCIBILITY OF PRESERVATIVE EFFICACY TESTS

To assess the differences in the results of the different test methods, it is important to realise the variation in microbiological test results. The GÖCH has published the results of a round robin test for Ph. Eur. - 5.1.3 “Efficacy of Antimicrobial Preservation” (22). The microbial counts differed widely between the laboratories. Figure 2 shows an overview of the results including expanded uncertainty.
are below those for ISO 11930. It is advisable to perform a test according to one of the methods designed for cosmetics.

REFERENCES AND NOTES

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