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) 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 29261 (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 MQM (Microbiological Quality Management). Only the schülke KoKo test completely fulfil 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>Microorganisms</th>
<th>Limit</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>±0.9</td>
<td>Skin peels (glycolic acid)</td>
</tr>
<tr>
<td>pH</td>
<td>±0.5</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, lipsticks, cream blushes</td>
</tr>
<tr>
<td>Water activities</td>
<td>8-9,7</td>
<td>Shower-based products hair-removals</td>
</tr>
<tr>
<td>Oozing products</td>
<td>94%</td>
<td>Hair-dyes</td>
</tr>
<tr>
<td>Moisture</td>
<td>±2%</td>
<td>Anti-pariements</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Ph. Eur</th>
<th>USP</th>
<th>CTFA M-3</th>
<th>CTFA M-4</th>
<th>ASEAN</th>
<th>KoKo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces candidatus</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcaligenes eutropha</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter gergonii</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter hartmanni</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacterium species</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kocuria rhizophila</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>2±1</td>
<td></td>
<td>2±1</td>
<td>2±1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Yeasts</th>
<th>KoKo</th>
<th>ISO 11930</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>2±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>2±1</td>
<td>2±1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Moulds</th>
<th>KoKo</th>
<th>ISO 11930</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus brasiliensis</td>
<td>2±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Penicillium lilacinum</td>
<td>2±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Penicillium glaucum</td>
<td>2±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Penicillium expansum</td>
<td>2±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Zygosporella capitata</td>
<td>2±1</td>
<td>2±1</td>
</tr>
</tbody>
</table>

Table 2. Microorganisms used for challenge testing.
PRODUCTION OF INOCULUM

For the production of an inoculum, microorganisms are washed off from the nutrient media plates and adjusted to the required starting germ count by dilution. The inoculum is then directly used to inoculate the test samples according to ISO 11930, Ph. Eur., 7and ASEAN. The CTFA tests accept the use of either as well pure or mixed culture suspensions, the schülke KoKo test uses mixed culture suspensions (i.e., single cultivated microorganisms brought together into a mixed suspension). To guarantee a constant composition of the mixed inoculum the mixed suspension is stored for maximum of three days in a refrigerator. A new inoculation suspension is prepared for each inoculation cycle.

INOCULATION OF SAMPLES

Each container is inoculated with a measured amount of inoculum and thoroughly mixed to ensure homogeneous distribution of inoculum. The quantity of inoculum is typically 1% of the test sample. The initial concentration of microorganisms present in the inoculated product with regard to the different test methods are demonstrated in the figure 1.

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.

For Ph. Eur.; KoKo and ISO 11930 challenge testing A and B criteria are defined. The differentiation for Ph. Eur. test is related to the risk. Criteria B is acceptable if a preservation fulfilling criteria A, for example leads to an increased risk of adverse reactions. For ISO 11930 and KoKo test the microbiological risk analysis is the possible differentiation. The existence of control factors not related to the formulation; for example, a protective package such as a pump providing a higher level of protection than a jar and/or following strong demands on good manufacturing practice has to be demonstrated to accept criteria B.

PRESERVATIVE STABILITY

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

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Species</th>
<th>Log Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph. Eur.</td>
<td>Bacteria</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>USP &lt;51&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CTFA M-3</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CTFA M-4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>KoKo</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td>ASEAN</td>
<td>-</td>
<td>x3</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>x3</td>
</tr>
<tr>
<td>ISO 11930 A</td>
<td>Yeast</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td>ISO 11930 B</td>
<td>Mould</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Criteria of acceptance.

Figure 1. Microorganism counts and the quantity of inoculum used for challenge testing.

Figure 2. Criteria of acceptance - graphical representation.

Table 4. Instructions related to preservative stability in the different challenge test methods.
Tumbling market.
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How?

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- Relieves itching

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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 guarantee 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 4 shows the instructions related to preservative stability of the different test methods.

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

<table>
<thead>
<tr>
<th>Preservative System</th>
<th>Unpreserved lotion</th>
<th>Lotion preservative 1</th>
<th>Lotion preservative 2</th>
<th>Lotion preservative 3</th>
<th>Lotion preservative 4</th>
<th>Lotion preservative 5</th>
<th>Lotion preservative 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.69% phenoxethanol</td>
<td>0.09% phenoxethanol</td>
<td>0.59% phenoxethanol</td>
<td>0.09% phenoxethanol</td>
<td>0.31% phenoxethanol</td>
<td>0.74% phenoxethanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10% ethylhexylglycerin</td>
<td>0.99% phenoxethanol</td>
<td>0.20% ethylhexylglycerin</td>
<td>0.11% ethylhexylglycerin</td>
<td>0.31% benzyl alcohol</td>
<td>0.12% benzoic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07% diethylamine acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.02% ethylhexylglycerin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01% polyamino propyl biguanide (pH adjusted to 5.1)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Life cycle of a cosmetic product.

Figure 4. Comparison of the test results Ph. Eur. and ISO 11930.
EXPERIMENTAL DATA

Using an unpreserved body lotion containing Q10 (INCI: aqua, glycerin, ethylhexyl stearate, isopropyl palmitate, cetaryl isononanoate, glyceryl stearate citrate, cetareth alcohol, persea gratissima oil, tocopheryl acetate, creatine, parfum, carbomer, allantoin, sodium hydroxide, xanthan gum, ubiquinone), we ran the preservative efficacy tests according to ISO 11930 in comparison to Ph. Eur. 7 and the schülke 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 \( R = \log N_0 - \log N_x \). 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 useful lifespan after opening of cosmetic products are generally longer than for pharmaceutical products.

The schülke 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.

CONCLUSIONS

The ISO 11930 as new standard to evaluate the antimicrobial protection of a cosmetic product includes a reference method to evaluate the preservation, as well as a decision diagram to evaluate the microbiological risk. The recommended preservation efficacy test is less strict than the Ph. Eur. and the KoKo test (see 9. Experimental data). The microbial strains did not include typical product spoiling microorganisms along side the pathogenic germs as required by the SCCS (9). The stability of a preservative is not taken into account by ISO 11930.

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

For the other methods this data is not available, probably however it can be assumed that it is in the same range.

Borderline results with low reduction in microbial counts might fail in a replicate. Uncertain contaminations during production combined with an insufficient speed of killing may lead to detection of contamination in the market place. The worst case is the inclusion in the RAPEX notifications (23).

Figure 5. GÖCH results of round robin test 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.

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