Effects of three acidic preservatives on microbiological quality of cosmetic emulsion from virgin olive oil

**Abstract**

The present study aimed to review the challenge test, which is used to evaluate the efficiency of three acidic preservatives in cosmetic emulsion from virgin olive oil. These preservatives were tested during our study: sorbic acid, benzoic acid and salicylic acid. These conservatives were tested separately and combined between them. The challenge test involved inoculating product with Gram positive bacteria (Micrococcus luteus and Staphylococcus aureus), Gram negative bacteria (Escherichia coli, Salmonella enterica and Pseudomonas aeruginosa) and fungi (Aspergillus niger and Candida albicans). Inhibition growth of these microorganisms at each concentration was followed over a 28 days period. Interestingly, it was the combination (0.5% sorbic acid and 0.5% salicylic acid) which inhibited most the microbial growth of microorganisms while preserving the physicochemical properties of the product. As a result, the challenge test is used in security and stability tests during product development and it is the most suitable to analyse preservative effectiveness.

**INTRODUCTION**

Chemical preservation has become an increasingly important practice in modern cosmetic technology with the increase in production of processed and convenience cosmetic products. These preservatives are deliberately added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes and thus increasing its shelf life. Sorbic acid (trans, trans-2,4-hexadienoic acid) (SA) is an antimycotic agent used as preservative in many pharmaceutical formulations, especially in dermatological and cosmetic preparations. During the last decades (SA) and its potassium salt have been accepted as ‘generally recognized as safe’ substances (1) and have become the leading preservatives for food as well as for pharmaceutical and cosmetic preparations (2). (SA) are generally effective to control mold and inhibit yeast growth, and against a wide range of bacterial attack (3, 4, 5). The pKa of SA is 4.76 and the working pH range is from 3 to 6.5. The extended preservation capacity up to pH 6.5 makes SA superior over other commonly used preservatives such as propionates and benzoates (6). Benzoic acid (BA) is commonly used as preservatives to prevent the alteration and degradation of foods and cosmetic products by microorganisms, since they exhibit inhibitory activity against fungi, yeasts, molds, and bacteria (7, 8, 9). The most prominent representative in the class of hydroxyacids is salicylic acids (SAA). In the last two decades, SAA has been widely incorporated into a variety of cosmetic products for daily use over long periods of time (10). The antiaging effects of HAS using (SAA) have become a prominent factor in cosmetic dermatology, leading to proliferation of hydroxyacid-containing cosmetic products and skin care systems (11). (SAA) is added to cosmetic products at concentrations usually less than 3% (12). The Cosmetic Ingredient Review Expert Panel has similarly recommended that effects on the skin’s sensitivity to sunlight be considered in the formulation and use of products containing SAA and salicylates (12).

Assessment of preservative efficacy in cosmetics is usually performed using the challenge test (13). This test provides assurance regarding the microbiological quality of the product at the time the test is performed. In 2003, Russell suggested that challenge test should be undertaken at the beginning, during and at the end of the shelf life of the product (14). Challenging a product with appropriate organisms is a major concern in determining how effective a preservative must be, is based on inoculation of the cosmetic product with bacteria, followed by incubation and sampling for survivors during the storage period (14). Considering the promising properties of additives and the growing interest in the use of efficient, this study postulates that acidic preservatives (SA, BA and SAA), which are naturally abundant and available may represent efficient additives. These agents can replace the commonly used expensive preservatives and can improve the cost-effectiveness of the overall production process, hence, the final end product. Accordingly, this study focused on the added value of SA, BA and SAA to cosmetic emulsion from virgin olive oil.
Here, we studied the microbial challenge test of the effect of the three acidic preservatives on cosmetic emulsion using a broad range of concentrations.

**MATERIALS AND METHODS**

The oil phase comprised of paraffin oil, Simulsol®4000, and virgin olive oil [3%] heated up to 75°C. The aqueous phase [distillate water] was added to the oil phase drop by drop with constant stirring using a mechanical stirrer set at 2000 rpm for 15 min until complete aqueous phase was added. During the stirring period and when the temperature reached 60°C, only three drops of lemon oil were added to give fragrance to the formulation. After the complete addition of the aqueous phase, the speed of the stirrer was reduced to 1000 rpm for 5min to achieve homogenization, and then further reduced to 500 rpm for 5min to reach complete homogenization. Agitation was maintained until the emulsion was cooled to room temperature.

**Preservatives**

In our study, three acidic preservatives have been tested: (SA), trans, trans-2,4-hexadienoic acid, is a natural organic compound. It has the chemical formula C₆H₈O₂. It is a colourless solid that is slightly soluble in water. BA is a colourless crystalline solid. It has the chemical formula C₆H₄COOH and the SAA has the formula C₆H₄(OH)COOH, a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. It is a colourless crystalline. Firstly, these preservatives are added separately to the cosmetic emulsion: 1 % SA; 1% BA and 1% SAA. Thereafter, preservatives are used in combination according to the following concentrations: 0.5 % SA + 0.5% BA; 0.5 % SA+ 0.5% SAA and 0.5 % BA + 0.5% SAA. Sample without any additives was used as the control.

**Microbial challenge test**

Each of the tested formulations was inoculated with each of the following microorganisms: Micrococcus luteus LB 14110, Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 8739, Salmonella enterica ATCC 43972, Pseudomonas aeruginosa ATCC 9027, Aspergillus niger ATCC16404 and Candida albicans ATCC 10231, using the test procedure modified from United States Pharmacopeia (15).

Microorganisms were grown in aerobic conditions. Salmonella enterica ATCC 43972, Pseudomonas aeruginosa ATCC 9027 and Micrococcus luteus LB 14110 were grown 24 h at 30°C in Luria Bertani medium LB: 10 g/l peptone; 5 g/l NaCl; 5 g/l yeast extract; pH 7.2 while Escherichia coli ATCC 8739 and Staphylococcus aureus ATCC 6538 were grown at 37°C on the same medium [LB]. Yeast and fungal strains were cultivated on Sabouraud dextrose agar (Difco Laboratories) at 25°C for 48 h and 1 week, respectively. All microorganisms were harvested by washing the cells from the slants with sterile saline. The growth colony in terms of colony forming units per milliliter (CFU/ml) of each organism was determined. Twenty ml of the samples were placed into sterile glass bottles, one for each organism and inoculated with the tested microorganism (10⁵-10⁶ colonies or spores/ml sample). The containers were incubated at room temperature (35°C) for a total of 28 days with periodic examination. Examination was made initially and after 3, 7, 14, 21 and 28 days of inoculation. Plate counts were performed using media corresponding to those in primary cultivation.

**Stability test at ambient condition**

**Centrifugation tests**

Centrifugal tests were performed for emulsions immediately after preparation. Those tests were repeated for emulsions after 24 hours, 7 days, 14 days, 21 days, and 28 days of preparation. They were performed at 5000 rpm and 25°C for 10 minutes by placing 10g of the sample in centrifugal tubes.

**pH determination**

The pH value of freshly prepared emulsions and emulsions kept at different conditions were determined using a digital pH-Meter. The pH tests were repeated for emulsions after 24 hours, 3 days, 7 days, 14 days, 21 days, and 28 days of preparation.

**Stability studies**

Cosmetic emulsions were formulated for stability test. The samples in glass containers were stored at room temperature (35°C) for three months. After the storage, the preservative efficacy was tested. The thermal stability of formulations was studied by placing the samples in glass tubes in a humidity chamber at 45°C and 75% relative humidity. Their appearance and physical stability were inspected each month for a period of three months (16).

**Statistical analysis**

All measurements were carried out in triplicates, and all microbial counts were converted into base-10 logarithms of colony forming units per ml of thigh samples. Data were subjected to analysis of variance (ANOVA) using the General Linear Models procedure of the Statistical Analysis System software of SAS Institute (17). Differences among the mean values of the various treatments and storage periods were determined by the least significant difference (LSD) test, and the significance was defined at P < 0.05. The differences which are equal to or more than the identified LSD values are considered statistically significant.
contamination, addition of system preservatives is needed according to microbial sensibility of the cosmetic product and its use by consumers.

Microbiological evaluation
To evaluate the microbiological effect of additives on the cosmetic emulsion from virgin olive oil, SA, BA and SAA combinations were prepared at different concentrations [Table 1]. Thus, we studied their capacities to inhibit Gram positive bacteria ([Micrococcus luteus LB 14110 and Staphylococcus aureus]), Gram negative bacteria (Escherichia coli ATCC 8739, Salmonella enterica ATCC 43972, Pseudomonas aeruginosa ATCC 9027 and moulds (Candida albicans ATCC 10231 and Aspergillus niger ATCC 16404).

Gram positive Bacteria
The initial populations of Micrococcus luteus LB 14110 in unpreserved samples is $6 \log_{10} \text{CFU/ml}$ (Table 2). This population was slightly increased until the seventh day reaching $6.7 \log_{10} \text{CFU/ml}$ and then it was decreased to $4.0 \log_{10} \text{CFU/ml}$ at the end of storage time in control (sample 1). For all treated samples (2, 3, 4, 5, 6 and 7), a significant reduction ($P < 0.05$) in Micrococcus luteus population was observed in comparison with sample 1. A 99.99% reduction in bacterial cell number ($P < 0.05$) was observed after 28 days in the tested cosmetic product according to the challenge test (Table 2) except samples treated with BA (3) at 1%. On the 21th day of storage, SA / SAA combination reduced the number of Micrococcus luteus to 99.99%. In fact a logarithmic reduction of 6 ($P < 0.05$) by the 21th day was noted in the sample treated with SA / SAA combination (6) (Table 2).

Staphylococcus aureus ATCC 6538 is a common skin organism (18). Most preservative challenge test methods use it to challenge frequently used cosmetic products because it is the most common contaminant that may pose threats to consumers (19). It represents Gram-positive cocci in many tests. Since its nutrient needs are comparatively demanding, it does not always seem to be a logical choice as a challenge inoculum.

### RESULTS AND DISCUSSION

Microbial contamination of cosmetic products is of great importance for the suppliers and the consumers. The microbial growth is at the origin of organoleptic and formulation alterations. To prevent microbial contamination, addition of system preservatives is needed according to microbial sensibility of the cosmetic product and its use by consumers.

<table>
<thead>
<tr>
<th>Preservatives</th>
<th>Concentration used in formula No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>SA</td>
<td>1.0</td>
</tr>
<tr>
<td>BA</td>
<td>-</td>
</tr>
<tr>
<td>SAA</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1.** Different percentages of preservatives used in formulations

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Microorganisms (Gram + Bacteria)</th>
<th>Initial Microbial load (log_{10} CFU/ml)</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E. coli ATCC 8739</td>
<td>$5.7 \pm 0.08$</td>
<td>$5.34 \pm 0.09$</td>
<td>$5.23 \pm 0.01$</td>
<td>$4.73 \pm 0.08$</td>
<td>$3.13 \pm 0.04$</td>
</tr>
<tr>
<td>2</td>
<td>S. enterica ATCC 43972</td>
<td>$5.0 \pm 0.03$</td>
<td>$5.44 \pm 0.05$</td>
<td>$4.78 \pm 0.07$</td>
<td>$3.13 \pm 0.08$</td>
<td>$2.37 \pm 0.04$</td>
</tr>
<tr>
<td>3</td>
<td>P. aeruginosa ATCC 9027</td>
<td>$5.0 \pm 0.04$</td>
<td>$4.49 \pm 0.04$</td>
<td>$3.85 \pm 0.06$</td>
<td>$2.90 \pm 0.02$</td>
<td>$1.65 \pm 0.03$</td>
</tr>
<tr>
<td>4</td>
<td>E. coli ATCC 8739</td>
<td>$5.0 \pm 0.05$</td>
<td>$5.75 \pm 0.12$</td>
<td>$3.55 \pm 0.07$</td>
<td>$2.12 \pm 0.06$</td>
<td>$1.10 \pm 0.02$</td>
</tr>
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<td>$1.43 \pm 0.03$</td>
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<td>$3.85 \pm 0.06$</td>
<td>$2.77 \pm 0.04$</td>
<td>$1.43 \pm 0.03$</td>
</tr>
</tbody>
</table>

**Table 2.** Effect of preservatives on the microbial load of Gram + Bacteria: Micrococcus luteus LB 14110 and Staphylococcus aureus ATCC 6538

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<tr>
<th>Sample No.</th>
<th>Microorganisms (Gram + Bacteria)</th>
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<td>S. enterica ATCC 43972</td>
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<td>$1.43 \pm 0.03$</td>
</tr>
</tbody>
</table>

**Table 3.** Effect of preservatives on the microbial load of Gram – Bacteria: Escherichia coli ATCC 8739; Salmonella enterica ATCC 43972 and Pseudomonas aeruginosa ATCC 9027

Results and Discussion

The initial populations of Staphylococcus aureus in unpreserved samples is $5.7 \log_{10} \text{CFU/ml}$ (Table 2). This value is decreased with the storage time reaching $4.0 \log_{10} \text{CFU/ml}$ in the 28th days of storage. However, for all the treated samples, the initial load of Staphylococcus aureus is decreased rapidly until the total elimination of...
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the inoculum. In fact, after 21 days of storage, absence of *Staphylococcus aureus* was observed in samples treated with BA, SAA and SA/SAA combination. During storage time, *Micrococcus luteus* and *Staphylococcus aureus* counts were increased in control but it was clear that all treatment resulted in a significant population reduction (P<0.05) in the cosmetic emulsion.

**Table 3.** Effect of preservatives on the microbial load of yeast and fungal: *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404. ± : Standard deviation of three replicates 

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Microorganisms (strain and fungus)</th>
<th>Initial (log10 CFU/ml)</th>
<th>Microbial load (log10 CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>3.9 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>4.2 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>4.6 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>4.8 ± 0.3</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>5.0 ± 0.2</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>5.2 ± 0.3</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td><em>C. albicans</em> ATCC 10231</td>
<td>5.4 ± 0.2</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

In accordance with our findings, BA alone is known as a non-specific antifungal agent with the wide spectrum of the activities against human pathogenic fungi with different minimum inhibitory concentration (MIC) values (25). Moreover, the preservative action of SA is based on the evidences that it can inhibit certain enzymes responsible for the growth of microorganisms (12). It has been investigated earlier that the microbial growth would be stopped by the inhibition of one or more sulphydryl enzymes by SA (12). SA has been found to be an effective inhibitor of fumarase, aspartase and succinic dehydrogenase in microorganisms (12).

**Stability test at ambient condition**

**Centrifugation test**

The centrifugation test is based on the principle of using centrifugal force to separate two or more substances of varied densities, such as two different liquids or a liquid and a solid, and is a useful tool for assessing and predicting the shelf life of emulsions (26). No phase separation was observed after centrifugation in any of the samples kept at ambient storage conditions up to 14 days.

**pH test**

The most important parts of chemical stability are performances on accelerated testing and kinetics of pH profiles (27). As far as the effectiveness of the cream is concerned, the pH is often regarded as a significant parameter. The pH of human skin normally range from 4.5 to 6.0.
and 5.5 is considered as an average pH of human skin. Therefore, in order for a formulation to possibly gain admission for industrial application, it should have a pH that is close to this range (28). The multiple emulsions prepared in this work had a pH value of 7.15, which is close to the neutral pH. Moreover, the pH of the various emulsion samples kept at ambient storage conditions were noted to undergo a continuous decrease up to the 28th day of observation. pH analysis was performed on four samples (1, 5, 6 and 7). The pH of the control (1) was, for instance, noted to continuously decrease from the 1st day and up to the last day (28 day), on which the pH was recorded to attain 5.2. The pH of the sample (0.5 % SA + 0.5 % BA) (5) was also noted to decrease continuously, reaching 4.0 on the 28th day of observation. Likewise, the pH values of the samples 0.5 % SA + 0.5 % SAA and 0.5 % BA + 0.5 % SAA (6 and 7) showed continued decreases, reaching 4.2 and 4.0 on the 28th day of observation, respectively (Figure 1). This decrease in pH was presumably due to the presence of fatty acids, such as palmitic acid, oleic acid, arachidic acid (saturated fatty acid), palmitoleic acid, oleic acid, linoleic acid, linolenic acid, and eicosenoic acid (unsaturated fatty acid) in virgin olive oil (29). So the addition of organic acids decreases the pH during 28 days of storage.

**Stability Study**

Stability and acceptability of organoleptic properties (odour and colour) of formulations during the storage period indicated that they are chemically and physically stable (data not shown). SA/SAA combination (7) inhibited most the microbial growth of microorganisms and preserving the physicochemical properties of the cosmetic emulsion from virgin olive oil.

**REFERENCES AND NOTES**


Figure 1. pH values of Control (♦), (0.5 % SA + 0.5% BA) (▲), 0.5 % SA + 0.5 % SAA (●), and 0.5 % BA + 0.5 % SAA (■).