L-Ascorbic acid (Vitamin C) has been used in pharmaceutical and cosmetic preparations for a long time on the basis of its many favourable effects on the skin (1). It is an important antioxidant that protects the skin by scavenging and destroying free radicals and reactive oxygen-derived species (2). It could improve the morphogenesis of dermal epidermal junction, and is also known for its skin lightening properties (3, 4). L-Ascorbic acid is also used topically because of its ability to reduce wrinkles by promoting collagen synthesis and its skin-depigmenting activity (5). However, its low stability is a serious limitation. It is easily oxidized, especially under aerobic conditions and light exposure, being degraded first in a reversible step to dehydroascorbic acid and second to oxalic acid in an irreversible fashion (6). Chemical modification of ascorbic acid has led to more stable derivatives such as ascorbyl esters with C6 to C18 fatty acids or ascorbyl phosphate salts. Among the lipophilic derivatives, ascorbyl palmitate is often used in topical preparations against oxidative changes of biological components of the skin, and as an anti-oxidant to protect lipophilic ingredients in formulations (7). Ascorbyl palmitate has been studied in the food, pharmaceutical, and cosmetic industry due to its stability and antioxidant activity (8). In this research, it was selected as the active material. Ascorbyl palmitate has been proposed as an oxygen scavenger to remove headspace oxygen (9). This derivative of vitamin C is beneficial for its skin penetration: emulsions of ascorbyl palmitate have been prepared with a view to apply them for topical use (10). Given the low stability of ascorbic acid dissolved in aqueous vehicles, these new forms may be considered of great potential value. Well the technology of production of these alternative forms of vitamin C can raise the cost of the final product.

Accordingly, in this study the present study was undertaken to investigate the physical, chemical and microbiological stability of O/W emulsions containing ascorbic acid and ascorbyl palmitate in order to make a comparative assessment of these active principles.

INTRODUCTION

L-Ascorbic acid (Vitamin C) has been used in pharmaceutical and cosmetic preparations for a long time on the basis of its many favourable effects on the skin (1). It is an important antioxidant that protects the skin by scavenging and destroying free radicals and reactive oxygen-derived species (2). It could improve the morphogenesis of dermal epidermal junction, and is also known for its skin lightening properties (3, 4). L-Ascorbic acid is also used topically because of its ability to reduce wrinkles by promoting collagen synthesis and its skin-depigmenting activity (5). However, its low stability is a serious limitation. It is easily oxidized, especially under aerobic conditions and light exposure, being degraded first in a reversible step to dehydroascorbic acid and second to oxalic acid in an irreversible fashion (6). Chemical modification of ascorbic acid has led to more stable derivatives such as ascorbyl esters with C6 to C18 fatty acids or ascorbyl phosphate salts. Among the lipophilic derivatives, ascorbyl palmitate is often used in topical preparations against oxidative changes of biological components of the skin, and as an anti-oxidant to protect lipophilic ingredients in formulations (7). Ascorbyl palmitate has been studied in the food, pharmaceutical, and cosmetic industry due to its stability and antioxidant activity (8). In this research, it was selected as the active material. Ascorbyl palmitate has been proposed as an oxygen scavenger to remove headspace oxygen (9). This derivative of vitamin C is beneficial for its skin penetration: emulsions of ascorbyl palmitate have been prepared with a view to apply them for topical use (10). Given the low stability of ascorbic acid dissolved in aqueous vehicles, these new forms may be considered of great potential value. Well the technology of production of these alternative forms of vitamin C can raise the cost of the final product. Accordingly, in this study the present study was undertaken to investigate the physical, chemical and microbiological stability of O/W emulsions containing ascorbic acid and ascorbyl palmitate in order to make a comparative assessment of these active principles.

MATERIALS AND METHODS

Formulations

The components and concentrations of the formulations used in the study are shown in Table 1. Glycerol monostearate, ceteareth-25, isopropyl palmitate, cetearyl alcohol, ethyl paraben, polyethylene glycol, cetyl lactate, laureth 7 and disodium EDTA were added or not of 2% l-ascorbic acid and ascorbyl palmitate at pH 5.5, the active principle optimal pH. The O/W formulations were prepared and mixed in a Heidolph RZR 2021 shaker at 600 rpm.

Physical stability

The obtained emulsion was submitted to a set of organoleptic (colour, thickness, look, feel) and physical (creaming and phase separation) analyses.
Stability Tests
Stability tests were performed at different conditions for emulsions to investigate the effect of these conditions on the storage of emulsions. These tests were performed on samples kept at 8°C ± 2°C (in refrigerator), 25°C ± 2°C (in incubator) and 40°C ± 2°C (in incubator). The physical, i.e. colour, and the organoleptic, i.e. liquefaction and phase separation, characteristics of emulsions were observed at various intervals for 28 days [11, 12].

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 [11, 12].

Skin Measurement (Measurement of Transepidermal Water Loss)
TEWL is determined using skin biophysical technique, Tewameter® CM 210 from Courage Khazaka (Cologne, Germany). TEWL, which is related to skin barrier function, was expressed as g/m² h [13].

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 [14].

Chemical stability
All samples were stored in well-closed 25 ml glass flasks. During storage samples were kept at room temperature (22±1 °C) in the dark, except for those used for studying the influence of light. The amount of non-degraded active ingredient in samples was determined quantitatively at the beginning of storage and subsequently on the 1st, 2nd, 3rd, 7th, 14th and 28th day.

Chromatographic conditions
The HPLC apparatus consisted of JASCO HPLC, a sample injector with a 20 µl sample loop and a wavelength detector. For ascorbyl palmitate, the stationary phase was 125 mm x 4 mm column packed with 5 µm LiChrospher RP-18 and the mobile phase was methanol : acetonitrile : 0.02 M phosphate buffer pH 2.5 (75 : 10 : 15). The flow rate was set at 1.5 mL/min and the determination by ultraviolet (UV) detection at 254 nm.

Sample preparation for stability testing
A standard stock solution of ascorbyl palmitate (0.4 mg/mL) was prepared by dissolving appropriate amount in methanol. The standard solution was obtained by diluting the standard stock solution with methanol to yield a solution containing 0.04 mg/mL. Approximately 0.4 g of cream was exactly weighed, placed into a 50 mL volumetric flask, taken to volume with methanol and shaken for about 5 min for ascorbyl palmitate analysis. The solutions were passed through a 0.45-µm membrane filter before injection. The stability of ascorbyl phosphate was determined by HPLC in samples kept at room temperature (22±1 °C) in the dark for 4 weeks.

Microbiological stability
In order to assess the degree of contamination, 1g of material was dispersed in a 4-ml sterile Ringer solution containing 0.25% tween 80. Appropriate dilutions were made in the same dispersing vehicle, and 0.1 ml was plated out on the appropriate solid medium using the surface viable method. Emergent colonies were counted after the necessary incubation. All operations were carried out in duplicates [15].

Aerobic plate count
Aerobic plate counts were determined by inoculating 0.1 mL of the homogenate sample onto triplicate sterile plates of prepared and dried Standard Methods Agar using the surface spread technique. The plates were then incubated for 48 h at 35°C [16]. The Standard Methods Agar is a standardized medium for the enumeration of microorganisms from materials of sanitary importance. Duplicates of each dilution (1 mL) of neutralized and non-neutralized samples were pour-plated using Standard Methods Agar (Oxoid, Basingstoke, Hampshire, England) and incubated at 30 ± 1°C for 48 ± 3 h. Plates containing 25–250 colonies were selected and counted, and the average number of CFU/mL was calculated.

Pseudomonas aeruginosa count
Pseudomonas aeruginosa were enumerated on Pseudomonas Agar Base (CM 559, Oxoid) supplemented with cetrimide, fucidin, and cephaloridine, providing a selective isolation medium for Pseudomonas aeruginosa. Colonies were counted after 2 days of incubation at 25°C [17].

Staphylococcus aureus
Surviving population of Staphylococcus aureus was determined by standard plating methods [18]. At each sampling time, colonies of Staphylococcus were selected, Gram-stained, and observed for catalase and oxidase reactions to confirm the presence of Staphylococcus aureus. Microbiological data were transformed into logarithms of the number of colony-forming units (CFU/g).

Yeast and mould counts
The method involved enumeration of colonies on Sabouraud dextrose chloramphenicol agar medium. Enumeration was carried out as a pour plate, surface spread, or membrane filtration method [15]. Microbiological tests were repeated for fresh and formulations at 25°C after 7, 14, 21 and 28 days of preparation.

### Table 1. Components and concentrations of the formulations

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F₁</th>
<th>F₂</th>
<th>F₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol (monostearate)</td>
<td>5%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>Caprool 2</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Isopropyl palmitate</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>3%</td>
<td>5%</td>
<td>2%</td>
</tr>
<tr>
<td>Ethyl paraben</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>6%</td>
<td>6%</td>
<td>6%</td>
</tr>
<tr>
<td>Carbomer 940</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Acryl palmitate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>81%</td>
<td>79%</td>
<td>79%</td>
</tr>
</tbody>
</table>
DEFENSIL®-PLUS
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MORE QUALITY OF LIFE
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ATOPIC ECZEMA

42%

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www.rahn-group.com
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 formulations (log10 CFU/g). Data were subjected to analysis of variance (ANOVA) using the General Linear Models procedure of the Statistical Analysis System software of SAS Institute (19). 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.

RESULTS AND DISCUSSION

Physical stability of formulated emulsions
In this study, formulations were placed in different storage conditions i.e. 8±2°C, 25±2°C and 40±2°C for a period of four weeks in stability chambers. The samples were observed for change in colour, liquefaction, and phase separation, as presented in Table 2.

Color
The findings revealed that the freshly prepared emulsions were white and yellowish white in colour for F1, F2, and F3. Little changes in color were observed for emulsions well the end of storage is marked. (Table2). For example, for F1, the change in colour appeared from the 21st day and persisted up to the 28th day of the analyses period. The change in colour at the end of the observation period was presumably due to the oily phase separation which was promoted at higher temperature. Interestingly, no change in colour was observed for F4 at the different storage conditions, i.e., 8 ±2°C, 25 ±2°C and 40 ±2°C, up to 28 days of observation.

Liquefaction
The viscosity of emulsion is often reported to play a vital role in its flow properties (20). Starting from the emulsion preparation, the time and temperature processes begin to affect its properties, leading to a decrease in viscosity which, in turn, results in liquefaction increase (19). In fact, liquefaction is a sign of instability, and might attribute to the passage of water from the internal phase to external phase as described in several reports in the literature (21). Slight liquefaction was observed in the samples kept at 40°C on 21st day for F1 [control] and F2 (l-ascorbic acid). No liquefaction was observed for the emulsions in any of the storage conditions under investigation, i.e., 8 ±2°C, 25 ±2°C and 40 ±2°C throughout the 28 days of observation for F3. The absence of liquefaction provided strong evidence for the stability of the emulsions under investigation.

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 solid and a liquid, and is a useful tool for assessing and predicting the shelf life of emulsions (22). No phase separation was observed after centrifugation in any of the samples kept at different storage conditions up to 21 days. A slight phase separation was, however, recorded on centrifugation from the 21st day and up to the 28th day of observation in the samples F1 and F2 kept at 40°C. No other phase separations were observed till the end of the experimental period. This was presumably due to the proper homogenization speed during emulsion formulation which might have prevented the breakage of the emulsions during testing (23).

pH testing
The most important parts of chemical stability are performances on accelerated testing and kinetics of pH profiles (14). As far as the effectiveness of the cosmetic formulations 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. The multiple emulsions prepared in this work had a pH value of 6.93, which is close to the neutral pH. Moreover, the pH of the various emulsion samples kept at ambient temperature were noted to undergo a continuous decrease up to the 28th day of observation, pH analysis was performed on three samples. The pH of the control (F1) was, for instance, noted to continuously decrease from the 1st day and up to the last day (28th day), on which the pH was recorded to attain 5.98. The pH of F2 was also noted to decrease continuously, reaching 6.32 on the 28th day of observation. Likewise, the pH values of the sample F3 showed continuous decreases, reaching 6.22 on the 28th day of observation, respectively (Table 2). So the addition of L-ascorbic acid and ascorbyl palmitate lightly decreases the pH during 28 days of storage.

<table>
<thead>
<tr>
<th>Liquefaction</th>
<th>Colour</th>
<th>Phase separation</th>
<th>Centrifugation</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>8°C</td>
<td>25°C</td>
<td>40°C</td>
<td>8°C</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24 h</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>3 day</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>7 day</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>14 day</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>21 day</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>W W</td>
<td>W</td>
<td>-</td>
</tr>
<tr>
<td>28 day</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: Physical characteristics of F1, F2 and F3, formulations kept at 8 ±2°C, 25 ±2°C and 40 ±2°C.

No Change; + = Slight Change; YW= Yellowish White; W= White.
is in excess. The also occur in larger extent in more dilute systems, indicating that the initial concentration of the active ingredient is an important factor concerning the extent of its degradation (27). When ascorbyl palmitate used as an antioxidant to stabilize formulation; if used as an active ingredient the concentrations are higher, usually 1–2%. In our study, ascorbyl palmitate was incorporated in emulsion at 2.00%. Fraction of nondegraded ascorbyl palmitate determined at different time intervals of storage in the dark is shown in Table 3.

After 28 days, 37% of ascorbyl palmitate remained fraction against only 8% of ascorbic acid. Compared to l-ascorbic acid, ascorbyl palmitate was significantly more stable. These results support the use of ascorbyl palmitate as an active ingredient in cosmetic and pharmaceutical preparations.

Microbiological stability of formulated emulsions

Aerobic plate count

The increase in storage time resulted in significant proliferations in Aerobic plate counts regardless of the type of treatment being applied (Table 4). The log mean count recorded for the Aerobic plate count of samples on day 0 was about $2 \log_{10}$ CFU/g. On day 28 of storage, the log mean count of Aerobic plate count reached 3.13, 2.93 and 2.35 for F1, F2 and F3, respectively, which did not approximate the maximum limit of $6.9 \log_{10}$ CFU/g for Aerobic plate count recommended by ISO NF-21149 (2006) in processed cosmetics (16).

Table 3. Percentages of non-degraded l-ascorbic acid (F2) and ascorbyl palmitate (F3) (n=3) in o/w emulsions at 2.00%.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>100</td>
<td>71.1±0.56</td>
<td>84±5.44</td>
<td>45.4±7.82</td>
<td>26.7±0.78</td>
<td>16.4±1.39</td>
<td>8.4±1.26</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>100</td>
<td>95.9±4.20</td>
<td>79±18.80</td>
<td>68.7±9.23</td>
<td>58.1±16.00</td>
<td>48.2±3.49</td>
<td>37±18.00</td>
<td></td>
</tr>
</tbody>
</table>

Evaluation of skin measurements

Only the formulation containing ascorbic acid (F2) led to an enhancement in TEWL values (Fig. 1) contributing alteration of skin barrier function. During the process of terminal differentiation, loss of water and several biochemicals changes and occur on the skin surface (24) and because ascorbic acid led to an increase in TEWL, we have an indication that it enhanced the epidermal cell renewal process on human skin. For F2 and F3, the values of TEWL remain constant during storage.

Chemical stability

Chromatographic analysis of ascorbyl palmitate (F3)

Several chromatographic analytical methods for quantifying the amount of ascorbyl palmitate are described in the literature. Ascorbyl palmitate has been determined using amino and cyanopropyl columns (6, 25, 26). The instability of ascorbyl palmitate is a result of its oxidative degradation. Generally, the kinetics of oxidative reactions are second order, but can usually be simplified to pseudo first order if oxygen...
**Pseudomonas aeruginosa and Staphylococcus aureus counts**
The results from the Pseudomonas and Staphylococcus aureus detection tests were negative, thus confirming that all the treated samples met the conventional standards specified with regards to fitness for human consumption (Table 4) [17, 18].

![Table 4. Microbial load of aerobic plate count, Pseudomonas spp, Staphylococcus aureus and Yeast and moulds count of F1, F2 and F3 during 28 days of storage at 25 ± 2°C ±: Standard deviation of three replicates CFU: Colony-forming units a–c: Averages for different microbial analyses with different letters in the same column are different (P<0.05).](image)

**CONCLUSIONS**
L-ascorbic acid and its derivative: ascorbyl palmitate seems to be very interesting since it preserved physicochemical properties of the product and was efficient against the proliferation of various spoilage microorganisms. In fact, their stability (physical, chemical and microbiological) was studied in o/w emulsions. In fact, ascorbyl palmitate is more stable than l-ascorbic acid at initial concentration used 2.00%. Therefore, ascorbyl palmitate can be used as an active ingredient in cosmetic and pharmaceutical preparations on the basis of its stability. The newly formulated cream of proved to exhibit a number of promising properties and attributes that might open new opportunities for the construction of more efficient, safe, and cost-effective skin-care, cosmetic, and pharmaceutical products.

**REFERENCES AND NOTES**
17. AFNOR, ISO NF- 22718 (2008)