

# Determination of antioxidant efficacy of cosmetic formulations by non-invasive measurements

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**Background/aims:** Antioxidants have been proposed, over the last decade, as functional ingredients for anti aging preparations and to prevent and modulate oxidative skin damages. Up to date, beside the photo-induced oxidative skin damages model, none *in vivo* protocols have shown sufficient reproducibility for the validation of the antioxidant claim for a cosmetic finished product. To this aim, we have recently anticipated a new *in vivo* protocol based on a micro-inflammatory model, driven by reactive oxygen species. In the present study our model was validated by comparison with four different instrumental methods.

**Methods:** The effects of a pre-treatment of two different formulations based on antioxidant functional ingredients, were investigated on forearm skin of 15 healthy volunteers, and compared to a cosmetic base and control area. The instruments considered in the study were Chromameter (CR-300 Minolta), Tewameter TM 210 (Courage-khazaka, Cologne, Germany), Laser Doppler Perfusion Imager (PIM1.0 Lisca Development AB, Sweden), in comparison to DermAnalyzer<sup>®</sup>, an easy to use software program developed by us, using the CIE L\*a\*b\* color space parameters.

**Results:** The comparative measurements showed that the antioxidant formulations tested were all able to reduce, in different but statistically significant extent, the intensity of skin redness, and of cutaneous blood flow, when compared to control area ( $P < 0.0001$ ).

**Conclusions:** The methyl nicotinate (MN) based microinflammatory model, in conjunction with objective measurements, resulted an effective tool for *in vivo* assessment of oxidative skin injuries. In view of the high level of repeatability, short time of answer and simplicity, the procedure by us developed, is proposed as a possible protocol for the evaluation of *in vivo* efficacy of antioxidant functional ingredients in cosmetic formulations.

**Key words:** antioxidants – skin aging – *in vivo* evaluation – cosmetic formulations – skin color

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SKIN IS a highly metabolic tissue which possess the largest surface area in the body, and is also a major candidate and target of damaging free radicals. It is well known that free radicals and reactive oxygen species (ROS) are involved in the mechanism leading to cutaneous damages, such as early aging, inflammatory disorders and skin cancers. Elaborate and diversified antioxidant mechanisms, of both enzymatic and non-enzymatic nature, protect skin from oxidative damage. Over the last decade antioxidants have been proposed as functional ingredients for anti-aging preparations, and to prevent and modulate oxidative skin damages. Although it has been

shown that ROS are directly related to skin aging, simple methods for the evaluation of oxidative damage in skin, and the potential efficacy of antioxidant-containing cosmetic products are not yet available. Up to date, beside the photo-induced oxidative skin damages model, any other *in vivo* protocols have shown sufficient reproducibility for the validation of the above stated claim in a cosmetic finished product. Taking this into account, we have developed an *in vivo* protocol, based on a microinflammatory model, driven by reactive oxygen species, that allows the evaluation of the protective role of antioxidant-containing cosmetic/pharmaceutical formulations against

oxidative stress, and indirectly, the anti-aging efficacy (1). In the present study, the protocol developed by us is described. Objective measurements of the skin damages induced were measured, by comparison, with four different instrumental methods: Chromameter (CR-300 Minolta), Tewameter TM 210 (Courage-khazaka, Cologne, Germany), Laser Doppler Perfusion Imager (PIM1.0 Lisca Development AB, Sweden), compared to DermAnalyzer<sup>®</sup>, a new software program, developed by us, using the CIE L\*a\*b\* color space parameters.

## Materials and Methods

### *Subject and experimental design*

Fifteen Caucasian healthy volunteers were included in the study (10 women and five men; mean age 35.3 years), after giving their written consent. The clinical investigator assessed that they were in good health and had no dermatological diseases. The study was approved by the Ethics Committee. The investigation was performed according to a double-blind, randomized fashion, and in accordance to Colipa guidelines for the evaluation of the efficacy of cosmetic products (2). Four randomized areas (12 cm<sup>2</sup>) were outlined on the internal side of the forearm of each volunteer. The measurements were carried out in a partly air-conditioned room at a temperature of 23 ± 2 °C and an average relative humidity of 33%. The study was performed in two phases: the first was the pre-treatment phase consisting of the application of two antioxidant cosmetic products and a base cream twice a day for 30 days. On the 30th day, the second phase previews, after detecting the baseline value of treated and control areas with the four instrumental methods, the induction of a circular erythema spot in each area, by the application of an aqueous solution of methyl nicotinate (MN) for 3 min (0.5%, m/v; 100 mL; Merck). The volunteers rested during the examination in the laboratory area, with the test sites exposed to room temperature and measurements were performed at baseline and at regular times over 90 min. Non-invasive methods were used to evaluate the *in vivo* efficacy of antioxidant ingredients in cosmetic formulations: Chromameter (CR-300 Minolta), Tewameter TM 210 (Courage-khazaka, Cologne, Germany) Laser Doppler Perfusion Imager (PIM1.0 Lisca Development AB, Sweden), compared to DermAnalyzer<sup>®</sup>.

### *Measures*

TEWAMETER TM 210 (Courage-khazaka, Cologne, Germany): TEWL was measured with a Tewameter TM 210 to assess the barrier function of the stratum corneum; the measured values are expressed in g/m<sup>2</sup>/h. To minimize the disturbance of air convection or draft on TEWL, the measurements were performed in a room without windows, air conditioners, and during the test the only persons in the room were the technician and the volunteer.

Measurements were performed before MN exposure (T0) and at 15, 60, and 90 min after MN removal.

CHROMAMETER (CR-300 Minolta): Chromameter CR-300 is a new hand-held tristimulus reflectance meters. In this study it was used to assess skin color of all sites (including the untreated control site) before and after the irritation induced by MN. With this instrument the skin is illuminated by a pulsed xenon arc lamp. The light reflected perpendicular to the surface, is collected for a tristimulus color analysis at 360–700, using the L\*a\*b\* color system, as determined by CIE. We considered only the a\* parameter (grade of redness). It represents changes along a red–green axis with changes from +60 for a red surface to –60 for a green surface. The skin measuring area is 8 mm in diameter. The arms of the volunteers were placed horizontally for these measurements, and special care was taken with the perpendicular placement of the Chromameter probe on the forearm. The manipulation of the probe required special attention so as to prevent changes in skin color due to pressure of the head on the skin. A single Chromameter measurement (consisting of the mean value of three readings) is performed for skin color determination. Measurements were performed before MN exposure (T0) and at 5, 10, 15, 30, 60, and 90 min after MN removal.

LASER DOPPLER PERFUSION IMAGER (PIM1.0 Lisca Development AB, Sweden): The cutaneous microcirculation of individual test sites was mapped using a Laser Doppler Perfusion Imager (3). This apparatus is a non-invasive instrument used to measure tissue perfusion. The non-invasive nature of LDPI, in combination with the ease of use and rapid response, makes this technique well suited for laboratory as well as clinical investigations of skin perfusion. It scans an area of skin with a laser, records changes in the light after it has bounced off moving blood, and generates a map of blood flow in the skin. Based

on the laser Doppler principle, it collects data without touching the tissue and generates a color-coded image of the spatial distribution of tissue perfusion.

The complete system comprises a low power He–Ne or solid state laser, a scanner, a personal computer with color monitor and a plotter. Light from a low power He–Ne or solid state laser sequentially scans up to 4096 measurement points over the tissue under study, covering a maximal surface area of approximately  $12 \times 12 \text{ cm}^2$ . At each measurement site, backscattered Doppler shifted laser light is detected by a photodetector and processed to a signal scaled proportional to blood flow. A color-coded perfusion image, representing the spatial variations in tissue blood flow is presented on a monitor and stored to file for further data and image analysis. Laser Doppler makes a scan of the whole area of interest, and the technique therefore provides the opportunity to study the heterogeneity of different grades of irritant and to study the size of the skin area affected. In this study this technique was found to be an important method for characterization and grading of the inflammatory response induced by MN. Measurements were performed before MN exposure (T0) and at 15, 30, 60, and 90 min after MN removal.

**DERMANALYZER** – (a) Image recording: True color images of the observed skin area were taken using a digital photo camera (Nikon coolpix900). The images were digitized by a frame-grabber, which was installed in a standard personal computer. Each image consisted of picture elements. The size of the skin area under study was  $12 \text{ cm}^2$ , and was located on the volar forearm. The arm was fixed to avoid large movements.

The position of the camera was adjusted so that the examined areas were in the middle of the images. For illumination, a diffuse light (D-65) was used to reduce shadows on the border of the arm and to avoid reflections on the skin. All the images were taken under the same constant lightening conditions. For this, the measurements were carried out in a special examination room, inside which no person entered during the recording. To analyze the development of the erythema response, a series of true color images were taken in the time over 90 min. Measurements were performed before MN exposure (T0) and at 5, 10, 15, 30, 60, and 90 min after MN removal.

(b) Software: The images were downloaded from the digital camera and archived on the

computer hard disk and afterwards analyzed singularly with the DermAnalyzer<sup>®</sup>. The program lets to choose each treated area simply drawing a line around the zone to be analyzed. After this step the program algorithm, developed by us, automatically pulls apart the treated from the untreated skin, and measures the CIE  $a^*$  components of the red area, excluding the other color components such as  $b^*$  (green–yellow). The final result consists in the mean  $a^*$  value of the whole area selected. This feature is particularly useful in the case of side effects (i.e. oedema) that, normally, would impair the measurement.

### *Statistic*

For each product, the values of redness, perfusion and TEWL, measured by the respective instrumental methods and referred to baseline values, were compared within the context of repeated-measurement Anova models, and Dunnett's post test. Differences were accepted as statistically significant at  $P < 0.05$ .

### *Test material*

The following three products were tested:

#### **Product A:** Sunscreen oil

**Ingredients:** Tocopherol, Cocoglycerides, Olea europea, Hexyldecanol, Hexyldecyl laurate, Triticum vulgare, Glicine soja, parfum, Triethyl citrate, Bisabolol, Lecithin, Oryzanol, Cupressus sempervirens, Anthemis nobilis, Lavandula officinalis, Rosmarinus officinalis, Pelargonium odouratissimum, Ascorbyl palmitate, Hypericum perforatum, Citric acid, Eucalyptus globulus, Eugenia caryophyllata, Daucus carota, Beta-carotene.

#### **Product B:** Body cream

**Ingredients:** Aqua, Dicaprylyl ether, Glycerin, Cocoglycerides, Cetearyl alcohol, Prunus dulcis, Palmitoyl proline, Dimethicone, Behenyl alcohol, Santalum album, Cetearyl glucoside, Potassium sorbate, Citrus dulcis, Citrus amara, Triethyl citrate, Hydrolyzed glycosaminoglicans, Sodium Hydroxymethylglycinate, Urea, Lactic acid, Arachidyl glucoside, Sodium palmitoyl sarcosinate, Magnesium palmitoyl glutamate, Isopropylbenzylsalicylate, Sodium PCA, Citrus limonum, Sclerotium Gum, Cera alba, Sorbitol, Fructose, Glicine, Sodium glutamate, Hydrolyzed wheat protein, Glucose, Bisabolol, Echinacea pallida, Citrus grandis, Escin, Rutin, Isodecylsalicylate,

Centella asiatica, Sodium Hyaluronate, Prunus amara, Citric acid, Glycolic acid, Tartaric acid, Malic acid, Lysine, Lecithin, Sericin, Ascorbyl palmitate.

**Product C:** Cosmetic base cream:

**Ingredients:** Aqua, Glyceryl stearate, Caprylic/Capric Triglyceride, Diethylexylcyclohexane, Dimethicone, Glycerin, Cetearyl Alcohol, Cetear-eth-20, Cetear-eth-12, Imidazolidinyl Urea, Methylparaben, Propylparaben, Disodium Edta.

They were compared to control area (treated with MN only).

## Results

### Chromameter

Mean values and the standard error of measurements (SEM) for instrumental recordings  $a^*$  are shown in Table 1.

Quantification of the erythema produced by the typical vasodilator methyl nicotinate could be easily demonstrated by means of the  $a^*$  Chromameter parameter. As it can be observed (Fig. 1) the pre-treatment of the sites with the examined cosmetic products A and B, is significantly effective in the inhibition of MN-induced skin erythema compared to control area and the cosmetic base ( $P < 0.0001$ ). In fact, the cosmetic base was less effective than the finished formulations. Differences between individual test sites compared to control area were calculated with Dunnett's multiple comparison test, and approached significance both for A and B ( $P < 0.05$ ), but, as one may expect, not for the base. After 30 min from exposure to MN, the increase in redness with respect to baseline values for the control area was

36.4% and 38.9% compared to A and B, respectively (Table 2). On the other hand, after 15 min, the increase of  $a^*$  for the control area was only 7.2% and 4.6% compared to the pre-treated areas. In fact, as shown in Fig. 1, between 10 and 15 min, there was an initial decrease in the control area in the  $a^*$  value, that has been attributed to an initial vasocompression due to tissue oedema. After recovery of oedema the grade of redness reached its maximum, than subsided during the following 90 min.

Furthermore, the treated areas showed a quite complete recovery of the baseline values, the control area showed a consistent delay in the recovery

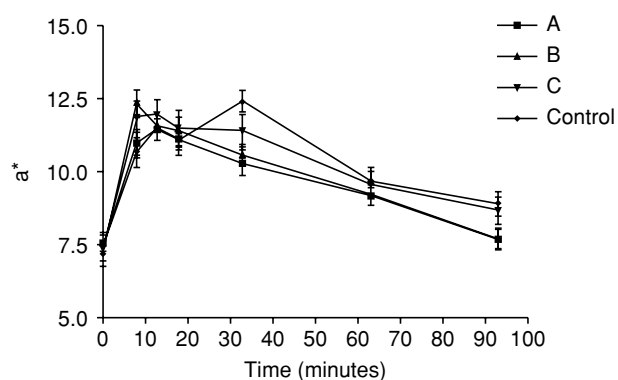


Fig. 1. Mean values ( $n = 15$ ) are given for  $a^*$  measured with the Chromameter CR-300 for the three formulations and the control area. Higher values reflect increase of redness. A, sunscreen oil; B, body cream; C, base cream.

TABLE 2. Percentage of relative difference [(value after – value before)/value before  $\times 100$ ] from the baseline value (T0) of each examined area, after 15 min and 30 min from exposition to MN

Time	A	B	C	Control
15 min	47.2	49.8	55.2	54.4
30 min	36.4	38.9	54	73.3

TABLE 1. Means  $\pm$  SEM ( $n = 15$ ) are given for  $a^*$  measured with the Chromameter CR-300 Minolta, for the three tested areas pre-treated with the different formulations and the control area (no pre-treated and irritated with MN). The values are given as arbitrary units. Higher values reflect increase of redness. A, sunscreen oil; B, body cream; C, base cream. MN: methyl nicotinate. T = minutes

	T0 (before MN irritation)	T5	T10	T15	T30	T60	T90
Test site 1 (A + MN)	7.53 ( $\pm 0.39$ )	10.96 ( $\pm 0.48$ )	11.43 ( $\pm 0.37$ )	11.10 ( $\pm 0.38$ )	10.28 ( $\pm 0.43$ )	9.14 ( $\pm 0.30$ )	7.68 ( $\pm 0.36$ )
Test site 2 (B + MN)	7.58 ( $\pm 0.32$ )	10.65 ( $\pm 0.49$ )	11.55 ( $\pm 0.50$ )	11.37 ( $\pm 0.47$ )	10.54 ( $\pm 0.36$ )	9.19 ( $\pm 0.37$ )	7.68 ( $\pm 0.33$ )
Test site 3 (C + MN)	7.38 ( $\pm 0.44$ )	11.87 ( $\pm 0.55$ )	11.93 ( $\pm 0.53$ )	11.46 ( $\pm 0.62$ )	11.37 ( $\pm 0.56$ )	9.53 ( $\pm 0.45$ )	8.66 ( $\pm 0.46$ )
Test site 4 (Control area (MN))	7.15 ( $\pm 0.42$ )	12.33 ( $\pm 0.44$ )	11.54 ( $\pm 0.47$ )	11.05 ( $\pm 0.50$ )	12.41 ( $\pm 0.39$ )	9.67 ( $\pm 0.48$ )	8.87 ( $\pm 0.42$ )

of initial skin color; indeed, the  $a^*$  value at T90 was 23.9% higher than the baseline value (T0).

*DermAnalyzer*<sup>®</sup>

Mean values and the standard error of measurements (SEM) for instrumental recordings  $a^*$  are shown in Table 3.

The data obtained with this technique confirm the above discussed results of Chromameter. In fact, as shown in Fig. 2, the antioxidant formulations tested were able to reduce, in statistically significant extent, the intensity of skin redness induced by MN ( $P < 0.0001$ ), compared to the control area. The differences between individual test sites compared to control area were calculated with Dunnett's multiple comparison test, and approached significance either for A and B ( $P < 0.01$ ). As expected, also in this case cosmetic base resulted less effective as compared to the finished antioxidant formulations A and B ( $P < 0.01$ ). After exposure to MN, the increase in redness for

the control area, compared to baseline value (T0), was 26.2% after 30 min, whereas the increase for A and B was 21.8% and 20.4%, respectively (Table 4). Finally, *DermAnalyzer*<sup>®</sup>, different from Chromameter, is able to consider only the red irritated area, excluding the oedema, it is thus possible to achieve curves with a more linear progression (Fig. 2).

*Comparison between Chromameter and DermAnalyzer*<sup>®</sup>

As described above, the redness values of skin treated area was measured with two different instrumental methods, such as a new hand-held tristimulus reflectance meter (Chromameter CR-300) and a new software program *DermAnalyzer*<sup>®</sup> using both the CIE  $L^*a^*b^*$  space color system. Because *DermAnalyzer*<sup>®</sup> has been recently proposed by us as an alternative technique to traditional colorimetry (1), we considered useful to compare the  $a^*$  parameter values, obtained with the two different instruments, both *in vitro* and *in vivo*. Furthermore, we carried out the correlation values by linear analysis regression *in vitro* (using standardization color charts) and *in vivo* (using the values assessed over the whole range of measurements of the induced color changes).

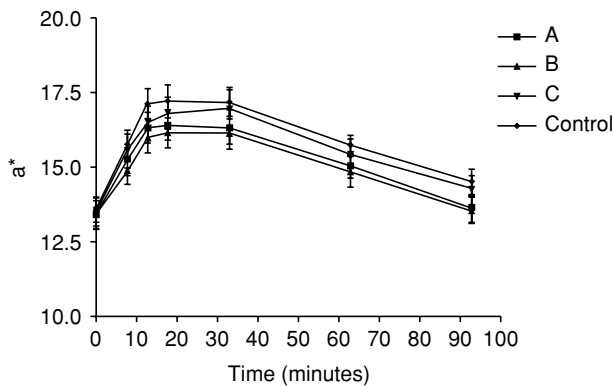


Fig. 2. Mean values (n = 15) are given for  $a^*$  measured with the *DermAnalyzer*<sup>®</sup> for the three formulations and the control area. Higher values reflect increase of redness. A, sunscreen oil; B, body cream; C, base cream.

TABLE 4. Percentage of relative difference [(value after – value before)/value before × 100] from the baseline value (T0) of each examined area, after 15 min and 30 min from exposition to MN

Time	A	B	C	Control
15 min	22.3	20.3	24.4	26.6
30 min	21.8	20.4	25.6	26.2

TABLE 3. Means ± SEM (n = 15) are given for  $a^*$  measured with the *DermAnalyzer*<sup>®</sup> for the three tested areas pre-treated with the different formulations and the control area (no pre-treated and irritated with MN). The values are given as arbitrary units. Higher values reflect increase of redness. A, sunscreen oil; B, body cream; C, base cream. MN: methyl nicotinate. T = minutes

	T0 (before MN irritation)	T5	T10	T15	T30	T60	T90
Test site 1 (A + MN)	13.41 (±0.46)	15.25 (±0.53)	16.33 (±0.50)	16.40 (±0.51)	16.34 (±0.57)	15.04 (±0.41)	13.61 (±0.44)
Test site 2 (B + MN)	13.42 (±0.52)	14.89 (±0.46)	16.02 (±0.52)	16.16 (±0.50)	16.17 (±0.55)	14.86 (±0.51)	13.54 (±0.44)
Test site 3 (C + MN)	13.50 (±0.47)	15.56 (±0.57)	16.48 (±0.55)	16.81 (±0.54)	16.97 (±0.63)	15.44 (±0.49)	14.24 (±0.48)
Test site 4 (Control area (MN))	13.58 (±0.43)	15.76 (±0.47)	17.13 (±0.52)	17.21 (±0.54)	17.16 (±0.53)	15.72 (±0.37)	14.49 (±0.42)

**In vitro**

To assess the *in vitro* range of measurements, standardized color charts were used, and for each color parameter the lowest and highest experimental values were noticed. Parameter  $a^*$  assessed by Chromameter varies from  $-50.79$  to  $+59.48$ . The corresponding  $a^*$  values for the Derm Analyzer<sup>®</sup> were from  $-46$  to  $+61$ .

Correlations were calculated between related color parameters obtained with the different instruments. Figure 3 shows the scattergram related to the correlation between the respective  $a^*$  values data measured, during the *in vitro* experiments, by Derm Analyzer<sup>®</sup> and Chromameter CR-300. The correlation value was very high ( $R = 0.99$ ).

**In vivo**

The inter-individual range of measurements of  $a^*$  assessed by Chromameter varied from 5 to 16.2.

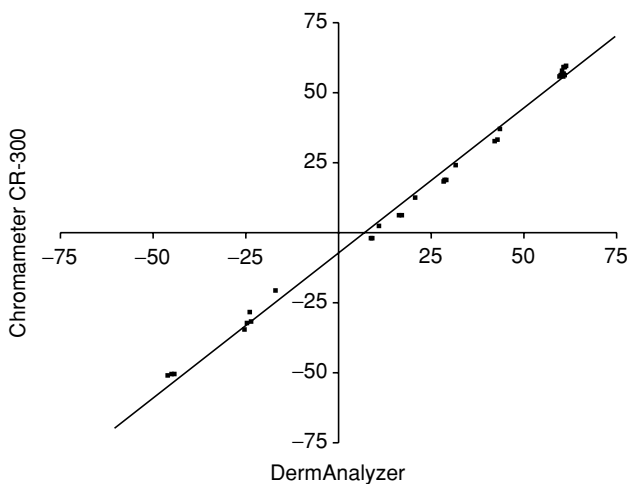


Fig. 3. Relationships between the  $a^*$  values, measured during the *in vitro* experiments, by Derm Analyzer<sup>®</sup> and Chromameter CR-300 Minolta. The correlation between  $a^*$  values was very high ( $R = 0.99$ ).

The corresponding  $a^*$  values, calculated with Derm Analyzer<sup>®</sup>, varied from 10.8 to 23.

Figure 4 shows the scattergram related to correlation between  $a^*$  parameter measured, during the *in vivo* experiments, by Derm Analyzer<sup>®</sup> and Chromameter CR-300, respectively. The mean correlation value was good ( $R = 0.86$ ).

**Laser Doppler**

Mean values and the standard error of measurements (SEM) for instrumental recordings  $a^*$  are shown in Table 5.

Figure 5 shows the time course of cutaneous blood flow, for each cosmetic formulation tested compared to control, after exposure to an irritant agent. A statistically significant difference in the increase of flow values, between control and pre-treated areas, was found ( $P < 0.0001$ ). The differences between individual test sites compared to control area, approached significance either for product A ( $P < 0.05$ ) and B ( $P < 0.01$ ), but not for the base. After 30 min from exposure to MN, the

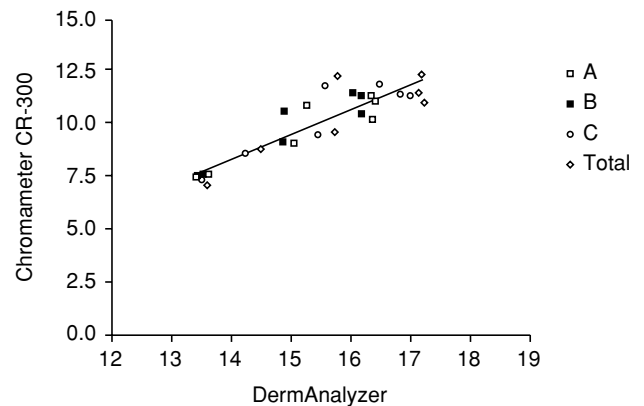


Fig. 4. Relationships between the  $a^*$  values, measured during the *in vivo* experiments, by Derm Analyzer<sup>®</sup> and Chromameter CR-300 Minolta. The correlation between  $a^*$  values was good ( $R = 0.86$ ).

TABLE 5. Means  $\pm$  SEM ( $n = 15$ ) are given for perfusion units measured with the Laser Doppler for the three tested areas pre-treated with the different formulations and the control area (no pre-treated and irritated with MN). The values are given as arbitrary units. Higher values reflect increase of cutaneous perfusions. A, sunscreen oil; B, body cream; C, base cream. MN: methyl nicotinate. T = minutes

	T0 (before MN irritation)	T10	T30	T60	T90
Test site 1 (A + MN)	0.83 ( $\pm 0.03$ )	1.55 ( $\pm 0.11$ )	1.97 ( $\pm 0.10$ )	0.89 ( $\pm 0.05$ )	0.77 ( $\pm 0.02$ )
Test site 2 (B + MN)	0.76 ( $\pm 0.03$ )	1.54 ( $\pm 0.14$ )	1.94 ( $\pm 0.13$ )	0.85 ( $\pm 0.05$ )	0.73 ( $\pm 0.02$ )
Test site 3 (C + MN)	0.74 ( $\pm 0.03$ )	1.73 ( $\pm 0.13$ )	2.1 ( $\pm 0.12$ )	0.97 ( $\pm 0.07$ )	0.75 ( $\pm 0.02$ )
Test site 4 Control area (MN)	0.79 ( $\pm 0.02$ )	1.8 ( $\pm 0.13$ )	2.12 ( $\pm 0.14$ )	1 ( $\pm 0.07$ )	0.79 ( $\pm 0.02$ )

increase in skin perfusion for the control area, with respect to baseline values, was 40.9% and 24.9% as compared to A and B, respectively (Table 6). Whereas the  $a^*$  values, collected by the previous techniques examined, showed a consistent delay in the recovery of initial skin color in the control area even after 90 min, the perfusion value of control area, collected by laser Doppler, showed a recovery of the baseline value as early as after 60 min (Table 5).

Figure 6 shows the variations in cutaneous blood flow after exposure to methyl nicotinate.

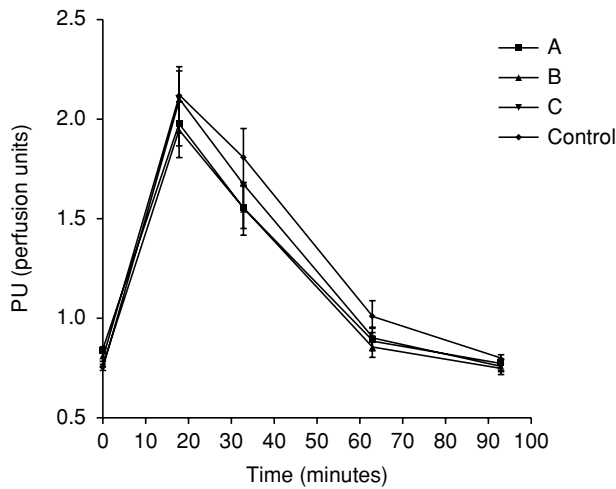
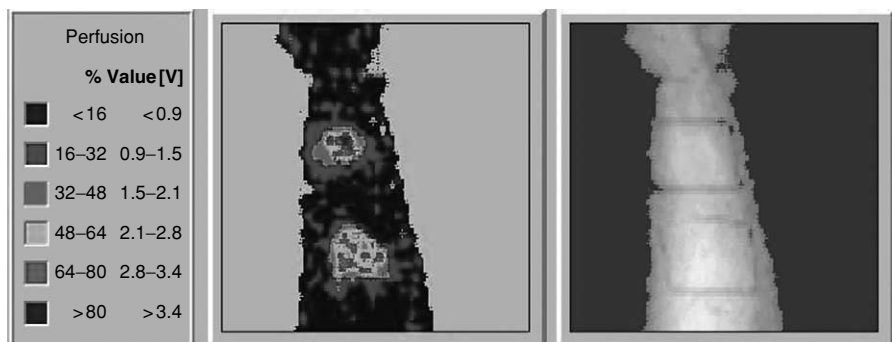


Fig. 5. Mean values ( $n = 15$ ) are given for perfusion arbitrary units measured with the Laser Doppler Perfusion Imager (PIM1.0 Lisca Development AB) for the three formulations and the control area. Higher values reflect increase of cutaneous perfusion. A, sunscreen oil; B, body cream; C, base cream.

TABLE 6. Percentage of relative difference  $[(value\ after - value\ before) / value\ before \times 100]$  from the PU baseline value ( $T_0$ ) of each examined area, after 15 min and 30 min from exposition to MN

Time	A	B	C	Control
15 min	135.6	154.1	182.9	166.6
30 min	85.8	101.8	124.7	126.7

Fig. 6. Variations in cutaneous blood flow on volar forearm, after 15 min from the exposure to MN.



The control area is shown as compared to the site pre-treated with B. The increase in blood flow was detectable after 5 min from the stimulus; a maximum was reached after 15 min, and decreased during the following 90 min. The highest peak in blood flow was observed in the control site.

### Tewameter

The results collected by Tewameter did not consent to obtain useful information about the efficacy of the two cosmetic formulations tested. Whereas exposure of the skin to irritant agents generally results in an increase of TEWL (4), in our study (Fig. 7) an inverse relationship was found. In fact, after exposure to MN, in each sites tested there a decrease of TEWL was observed, as compared to the baseline values. However, as expected, at baseline (before irritation), the pre-treatment of the sites with the examined cosmetic products was effective in reducing TEWL with respect to the control area.

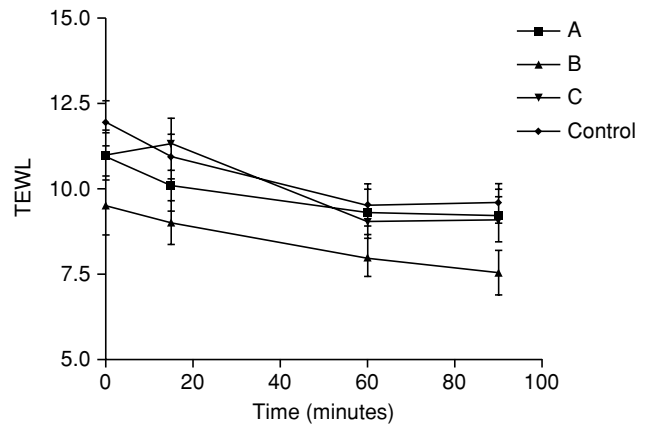


Fig. 7. Mean values ( $n = 15$ ) are given for  $a^*$  measured with the Tewameter (Courage-Khazaka) for the three formulations and the control area. Lower values reflect increase of barrier functions. A, sunscreen oil; B, body cream; C, base cream.

## Discussion

In skin, free radicals can be generated by UV-irradiation or chemical insults, and up to date, the most widely method to assess the antioxidant/radical scavenging activity of a compound is the photo-oxidative-induced skin erythema (5). Taking this into account, we have developed a protocol that directly allows the evaluation of the protective role of antioxidant-containing cosmetic/pharmaceutical formulations against oxidative stress, and indirectly, the anti-aging efficacy. In particular, the protective role of antioxidants have been investigated through 30 days pre-treatment, in order to integrate and reinforce the skin physiological defence, of selected areas with different antioxidant formulations, as compared to the cosmetic base and control areas. We believe the pre-treatment a key question in the application of antioxidants, because the oxidative skin damage is a rapid event, and antioxidants prevent this occurrence only, when present in adequate concentration at the site of action and when the oxidative damage take place (6). Thus, post-treatment with antioxidants is not effective as an intervention aimed to increase the antioxidant reservoir against possible oxidative damages. The present *in vivo* study takes advantage from the recently reported evidences of the generation of reactive oxygen species by MN induced micro inflammation; this in turn, stimulates cyclooxygenase and the prostaglandins synthesis (7). This cascade triggers the generation of endoperoxides thus leading to the amplification of the original stimulus by production of additional reactive oxygen species. The relationship between skin aging and inflammation is at the basis of the microinflammatory model of skin aging (8,9) that postulate peroxidation of skin cellular lipids, induced by endogenously or exogenously generated free radicals, as the promoter of a subclinical inflammatory state. ROS are believed to be involved in many inflammatory skin disorders, such as those constitutively produced in epidermal keratinocytes, and can be induced by chemical irritants (10). In this study we used cutaneous parameters (i.e. redness, cutaneous blood flow, and TEWL) modified by MN induced oxidative stress, to assess by non-invasive bioengineering methods, the efficacy of functional ingredients,

included in antioxidant/anti-aging cosmetic formulations. In order to measure objectively the skin redness, various color measuring devices have been developed (11) and in this paper we used DermAnalyzer<sup>®</sup>, an effective color analysis method recently developed by us (1). Data obtained with this method were compared with those collected by Chromameter, both using the L\*a\*b\* color system, but our method provide an objective, accurate, quantitative, and cost-effective way that overcome the drawbacks of traditional instrumental methods used to assess the skin color. The characteristics of this method are (a) the possibility to achieve a digitalized picture of the skin, suitable for successive re-evaluation, and (b) a quantitative evaluation, of the mean redness value of the whole area considered. The traditional Chromameter, instead, only consider a limited area (generally a circular surface of about 0.5 cm<sup>2</sup>) with problems in the repeatability of the measure. Moreover, in such cases when measures are complicated by the presence of side effects (i.e. oedema), these latter feature consents to achieve always a data suitable for statistic analysis. On the contrary, severe reactions may be difficult to measure by the Chromameter due to oedema formation.

The data obtained with both colorimetric techniques, demonstrated the *in vivo* efficacy of the pre-treatment with the antioxidant formulations examined, as compared to control and base cream, in the reduction of MN-induced skin redness. These results have been unequivocally confirmed measuring cutaneous blood flow variations by Laser Doppler, one of the other, non-invasive methods, considered in this study. The measurements performed confirm that in MN irritation, color changes are associated with a marked increase in dermal microperfusion (12). Thus, the a\* color factor and the blood perfusion are proved as adequate parameters for objective measurements of the grade of irritation induced by methyl nicotinate (13).

On the contrary, although tewametry has been demonstrated as a valuable technique, in other human skin inflammation models (14), for the determination of anti-inflammatory effects; we were not able to detect any coherent correlation. This occurrence is so far unexplained, may be this technique is not adequate to follow the skin changes induced by the MN model.

## Conclusions

As stated above, one of the major concerns in the application of dermo-cosmetic preparations, based on the antioxidant claim, is the lack of reliable, fast and effective *in vivo* methods for claim substantiation. In this paper we have presented a new approach to assess the *in vivo* antioxidant efficacy of cosmetic formulations by non-invasive bioengineering methods. This was based on the capability of antioxidants to counteract the skin reaction following a MN-induced microinflammatory stimulus. Three different parameters were used: skin redness, cutaneous blood flow, and TEWL. The degree of skin redness was comparatively evaluated by two different techniques: Chromameter and DermAnalyzer<sup>®</sup>, a new software for skin color analysis recently developed by us, using the CIE L\*a\*b\*. Our results have clearly demonstrated that a microoxidative model of skin aging is a valuable approach to evaluate the efficacy of antioxidant cosmetic formulations. Moreover, the comparative study, also consented us to further validate DermAnalyzer, our recently developed technique for skin redness evaluation, endowed with good repeatability. Indeed, we have demonstrated the good relationships between a\* values parameter collected by the two instruments and that DermAnalyzer<sup>®</sup> also consent reliable measurement in case of severe reactions with oedema formation. While Laser Doppler fully confirmed the data obtained by the two latter techniques, we were not able to achieve reliable results by Tewameter. In conclusion, we believe our approach of value in the determination of the *in vivo* efficacy of antioxidant dermo-cosmetic formulations. Finally, an additional advantage consists in the concomitant application of the DermAnalyzer<sup>®</sup> program which, in comparison to the traditional color measuring devices, presents the following advantages: suitable for color determination of the whole treated area also in case of oedema formation, visualization and storage of the skin area considered for successive re-evaluations, low costs.

## References

1. Manfredini S, Vertuani S, Solaroli N, Ziosi P, Pavan B, Baratto G, Levratti A, Corazza M, Virgili A. Presented in part at the 22th IFSCC Congress, Edinburgh (UK) 23–26 September 2002. A comparative *in vivo* study of antioxidant efficacy of cosmetic formulations.

2. The European cosmetic toiletry and perfumery association (COLIPA). *Guidelines for the Evaluation of the Efficacy of Cosmetic Products*, 1997; 1–10.
3. Fullerton A, Stucker M, Wilhelm KP, Wardell K, Anderson C, Fischer T, Nilsson GE, Serup J. Guidelines for visualization of cutaneous blood flow by laser Doppler perfusion imaging. *Contact Dermatitis* 2002; 46: 129–140.
4. Fluhr JW, Kuss O, Diepgen T, Lazzerini S, Pelosi A, Gloor M, Berardesca E. Testing for irritation with a multifactorial approach: comparison of eight non-invasive measuring techniques on five different irritation types. *Br J Dermatol* 2001; 145: 696–703.
5. Aquino R, Morelli S, Tomaino A, Pellegrino M, Saija A, Grumetto L, Puglia C, Ventura D, Bonina F. Antioxidant and photoprotective activity of a crude extract of *Culciturium reflexum* H.B.K. leaves and their major flavonoids. *J Ethnopharmacol* 2002; 279: 183–191.
6. Dreher F, Denig N, Gabard B, Schwindt DA, Maibach HI. Effect of topical antioxidants on UV-induced erythema formation when administered after exposure. *Dermatology* 1999; 198: 52–55.
7. Hibatallah J, Carduner C, Poelman MC. In-vivo and in-vitro assessment of the free-radical-scavenger activity of Ginkgo flavone glycosides at high concentration. *J Pharm Pharmacol* 1999; 51: 1435–1440.
8. Khodr B, Khalil Z. Modulation of inflammation by reactive oxygen species: implications for aging and tissue repair. *Free Radic Biol Med* 2001; 30: 1–8.
9. Giacomoni PU, Declercq L, Hellemans L, Maes D. Aging of human skin: review of a mechanistic model and first experimental data. *IUBMB Life* 2000; 49: 259–263.
10. Fuchs J, Zollner TM, Kaufmann R, Podda M. Redox-modulated pathways in inflammatory skin diseases. *Free Radic Biol Med* 2001; 15: 337–353.
11. Clarys P, Alewaeters K, Lambrecht R, Barel AO. Skin colour measurements: comparison between three instruments: the Chromameter, the Deraspectrometer, and the Mexameter. *Skin Res Technol* 2000; 6: 230–238.
12. Issachar N, Gall Y, Borrel MT, Poelman MC. Correlation between percutaneous penetration of methyl nicotinate and sensitive skin, using laser Doppler imaging. *Contact Dermatitis* 1998; 39: 182–186.
13. Boelsma E, Anderson C, Karlsson AM, Ponc M. Microdialysis technique as a method to study the percutaneous penetration of methyl nicotinate through excised human skin, reconstructed epidermis, and human skin *in vivo*. *Pharm Res* 2000; 17: 141–147.
14. Fuchs M, Schliemann-Willers S, Heinemann C, Elsner P. Tacrolimus enhances irritation in a 5-day human irritancy *in vivo* model. *Contact Dermatitis* 2002; 46: 290–294.

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