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Radical Skin/Sun Protection Factor RSF – Protection against UV-induced Free Radicals in Skin

Keywords: free radicals, UV-filter, ES

Abstract

Natural ageing of the skin is enhanced by environmental factors. Solar UV exposure is one of the main exogenous factors that is participating in that process and that can lead to photoageing, immunosuppression, and photocarcinogenesis. Using a new RSF method to determine UV-induced free radical injuries in the skin, it is possible to quantify the protective effect of sunscreens and UV-filters. The method is based on Electron Spin Resonance (ESR) spectroscopy measurements. Induction of free radicals in skin depends on the wavelength and dose of UV radiation. UVA penetrates into the deeper layers of the skin and induces more ROS as compared with UVB radiation. Therefore, UV filters that absorb or scatter UVA radiation give a stronger protection against free radical damage. In this study different sunscreen formulations have been tested in order to quantify their protection against free radicals.

Introduction

The sun emits a wide spectrum of electromagnetic waves of which ultraviolet light (UV) is the most aggressive towards cellular compounds. Large amounts of UVB and UVC are screened out by ozone, the major photoprotective agent formed in earth's atmosphere. Hence, solar UV radiation that reaches the earth as well as our skin, is composed of 5-10% highly energetic UVB (290-320 nm) and 90-95 % UVA (320-400 nm) which is less energetic, but penetrates deeper into the skin **(Fig. 1)** due to its longer wavelength.

Both UVA and UVB irradiation are very damaging to the skin. Depending on the wavelength, UV damage occurs via different mechanisms. UVA mainly produces free radicals (FR)/reactive oxygen species (ROS) though interaction with endogenous photosensitizers. These ROS will cause indirect damage to DNA, proteins and membranes. ROS is believed to be involved in photodamage of dermal connective tissue cells and proteins. On the contrary, DNA with its aromatic, heterocyclic bases is a strongly absorbing chromophore for UVB (absorption maximum at 260-265nm). Direct absorption of the UVB photons leads to disruption of DNA,

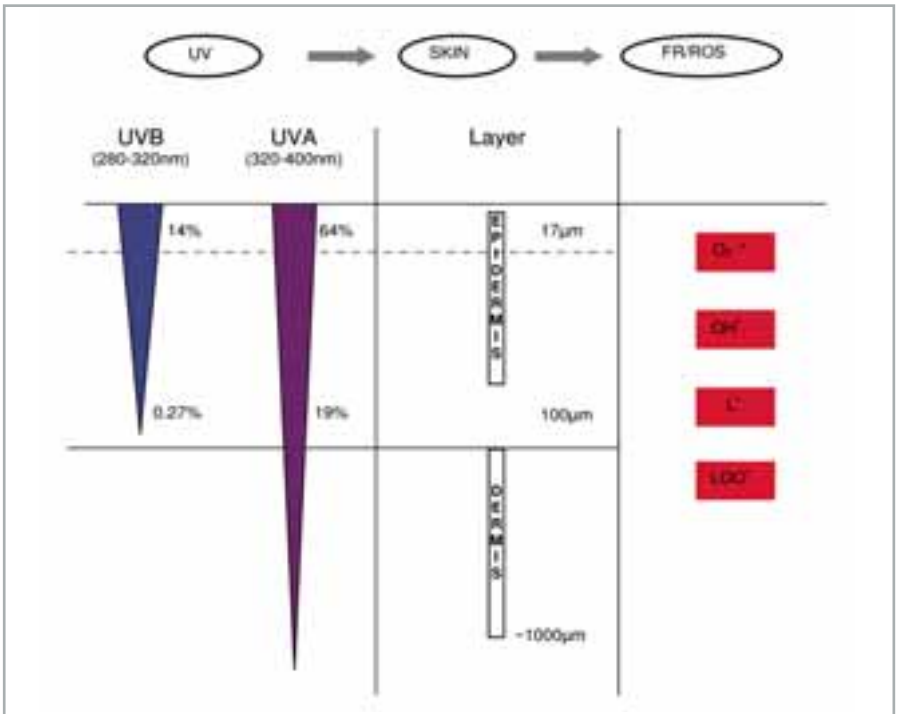

Fig. 1 Penetration of UVA and UVB in the skin and FR/ROS generated

with cyclobutane pyrimidine dimers (CPD) and pyrimidine pyrimidone photoproducts formation as a result.
Accumulation of DNA-damage may lead to mutations in genes which play a central role in tissue homeostasis and genome integrity, such as p53 tumor suppressor gene. P53 mutations are very commonly found in squamous cell carcinomas, actinic keratosis and even normal skin following UV exposure.
Hence, both UVA and UVB play a role in the pathogenesis of photosensitive diseases such as polymorphic light eruption (PLE), sunburn, immunosuppression, photoaging and even photocarcinogenesis.
Furthermore, free radicals can cause dermal connective tissue damage as well through the activation of transcription factors, matrix metalloproteinases, decrease in procollagen I and fibrillin-1 synthesis. These processes are increased by mitochondrial DNA mutations, protein oxidation, or apoptosis induction. Therefore antioxidant molecules and enzymes neutralizing free radicals have an important role in the cell defense mechanisms towards free radicals. For the therapy of photoageing local retinoids lived up to expectations, but the clinical effectiveness of antioxidant vitamins was lower than expected.
Free radical reactions in the skin are one of the most interesting subjects in skin research because they are involved in various skin diseases, including skin tumors, skin wrinkling and skin aging (1). Following UV-exposure, free radicals (FR) and reactive oxygen species (ROS) play a major role in producing lipid radicals ($L^{\cdot}$) that seem to be responsible for the destruction of the cell membrane and ultimately the cell (2,3).
UVA-UVB generated free radicals in skin can be measured by ESR spectroscopy (4) and imaging (5). The short lifetime **(Table 1)** of the generated free radicals (FR)/reactive oxygen species (ROS) like hydroxyl radical ($^{\cdot}OH$); superoxide anion radical ($O_2^{\cdot-}$) and lipid radical ($L^{\cdot}$) demands the application of radical traps for scavenging and accumulating FR/ROS to get sufficient signal-to-noise ratio (6).
Multiple lines of defense have evolved, aimed to protect the skin from oxidative stress, including prevention, interception, and repair. Primary defence mechanisms should prevent oxidative damage to occur by protecting the skin with UV filters against aggressive solar radiation. Secondary defence mechanisms interfere with processes elicited by reactive oxygen species, such as lipid peroxidation. Apart from using chemical and/or physical sunscreens to diminish the intensity of UV-radiation reaching the skin, supplementation of the skin with antioxidants, and thereby strengthening its antioxidative potential, is an emerging approach in limiting reactive oxygen species induced skin damage caused by UV-radiation (7,8).
Using ESR measurements, the protective effect of UV-filters (sunscreens) and antioxidants in the skin can be clearly assessed. The technique appears to be an attractive and effective approach to study processes in the skin as well. During the last years some reports have been published regarding the detection of UV generated free radicals/ROS in skin cells and skin biopsies by ESR spectroscopy.
With the RSF method presented herein the UV-induced ROS injury in skin can be quantified and the protective effect of UV filters, sunscreens and antioxidants can be determined.

Materials and Methods

Skin samples

For assessing the free radical protection of sunscreen and UV filter-containing formulations we used the pig skin model. Numerous reports suggest anatomical, physiological and biochemical similarities between man and pig skin. Pig skin, a waste product of the meat industry, can cheaply be obtained in large amounts and give reproducible results when studying photochemical and photo-toxicological processes (9-12). Pig skin was obtained from the local butcher. Skin strips (1x1cm) were placed in petri dishes (epidermal side up, in immediate contact with air) on filter paper soaked in PBS solution containing a nitroxyl probe as the free radical trap. UV filter formulations were then applied on the horny layer of the skin at a dose of

Reactive Species	Half-live	Half-live way
Hydroxyl radical ($^{\cdot}OH$)	0.3 ns	1.8 nm
Lipid alkyl radical ($L^{\cdot}$)	10 ns	60 nm
Lipid alkoxyl radical ($LO^{\cdot}$)	1 µs	6 µm
Lipid-peroxyl radical ($LOO^{\cdot}$)	1 – 10 s	
Superoxide anion radical ($^{\cdot}O_2^{-}$)	0,4 µs - 1ms	55 nm – 3 µm
Singlet oxygen ($^1O_2$)	ns - ms	
Nitric oxide (NO)	seconds	
Ascorbyl radical	seconds	
Tocopheroxyl radical	seconds	
Melanin	persistent	
Metal ions	persistent	

Table 1 ESR active species FR/ROS generated in skin by UV

2 mg/cm². Skin samples were treated with UV filter formulations for 20 minutes and kept in the dark before pursuing the experiment. A punch biopsy (Ø 4 mm) was then taken and exposed to UV radiations. ESR measurements were then performed as described underneath.

Test samples

Six UV filter-containing formulations and one marketed sunscreen product (Daylong extrême SPF 50+) were manufactured and supplied by Spirig Pharma AG, Switzerland. These formulations differ in the amount and proportion in various UVA and UVB filters. UV filters used in this study are depicted in **Table 2** .

UV irradiation

UV irradiation of the skin samples was performed using a solar simulator SOL 2 (Hönle AG, Germany). Irradiance as integrated value over the spectral ranges was E (UVB=280-320) = 2.4 mW/cm² and E (UVA = 320-400nm) = 28.9 mW/cm². To test the effect of different UV doses the irradiation time was varied accordingly.

Instrumentation

ESR measurements on skin biopsies were performed using a commercial high sensitive X-band bench top Electron Spin Resonance Spectrometer MiniScope MS200 (Magnettech GmbH Berlin, Germany). Skin biopsies were supported in a special tissue cell.

Free radicals/ROS detection method

Radical trapping experiments have the potential to allow the identification of the generated free radical species and were employed successfully in the detection of oxygen and carbon centered free radicals and singlet oxygen generated in skin exposed to UV radiation. Nitroxyl probes as traps for the detection of free radicals/ROS have therefore been used in this study. The nitroxyl probe is suitable to monitor the biological redox reaction, particularly when a nitroxyl probe is localized in an area of interest. Nitroxyl probes are susceptible to oxygen concentration, reactive oxygen species (ROS), and biological redox systems, and are widely used in ESR measurements. The reaction of nitroxyl probes with ROS results in the loss of their ESR signal, indicating their potential as tool for ROS detection. With the application of nitroxyl probes we measured the influence of UV irradiation (280-400 nm) on the formation of free radicals and reactive oxygen species (ROS) in skin samples treated or not with sunscreens. While UVB (280-320 nm) rays penetrate only the upper layer of the epidermis, UVA rays penetrate into the deep layers of the dermis as well. The common nitroxyl probe which was tested for its application to detect free radicals/ROS was purchased from Sigma (München, Germany).
Other reagents used were of the highest grade of purity commercially available.

Filter	Absorbtion wavelength (nm)	Specific Extinction E 1%/1cm	Solubility
1. Isoamyl p-Methoxycinnamate	260 – 340	> 980 (307 nm)	lipophilic
2. Ethylhexyl Triazone	260 - 325	>1500 (314 nm)	lipophilic
3. Butyl Methoxy-dibenzoylmethane	315-390	> 1100 (355 nm)	lipophilic
4. Methylene Bis-Benzotriazoyl Tetramethylbutylphenol (Tinosorb M)	290-390	> 600 (357 nm)	Microparticle suspension, Ø 200 nm
5. Bis Ethylhexyloxyphenol Methoxyphenyl Triazine (Tinosorb S)	290-390	> 819 (340 nm)	lipophilic

Table 2 UV filter used in the different sunscreen formulation

Results

Effect of sunscreens

Sunscreens are the first line of defence which should prevent the generation of primary free radicals ($^{\cdot}OH$, $O_2^{\cdot-}$) following UVA and UVB irradiation which indirectly damage the skin. UVB alone can directly damage the cells. Sunscreen formulations which were tested contained different concentrations of UVB, UVA and broadband UVA/B filters. Their protective effect against UV radiation when applied on the skin is listed in **Table 3** and depicted in **Fig. 2**. These are the results of 3 independent experiments in triplicate for each condition. Both representations show the calculated Radical Skin/Sun protection Factor RSF of the tested sunscreens/UV filter formulations.

$$RSF = \frac{N(\text{free radicals})_{\text{unprotected}}}{N(\text{free radicals})_{\text{protected}}}$$

The RSF is a factor characterizing the protection of a sunscreen against the generation of free radicals and is the ratio of the number N of generated free radicals in unprotected and protected skin samples assuming the same applied UV dose (constant irradiance, variable irradiation time) in both cases. It is also a measure for the increase in time one could stay in the sun by using UV filter protection assuming the generation of the same amount N of free radical/ROS like for the unprotected skin.
Table 3 and **Fig. 2** show clear differences between the RSF values of the individual UV filter formulations; these are due to the absorption spectrum of the UV filter used in the formulations and their applied concentration. A normalization of

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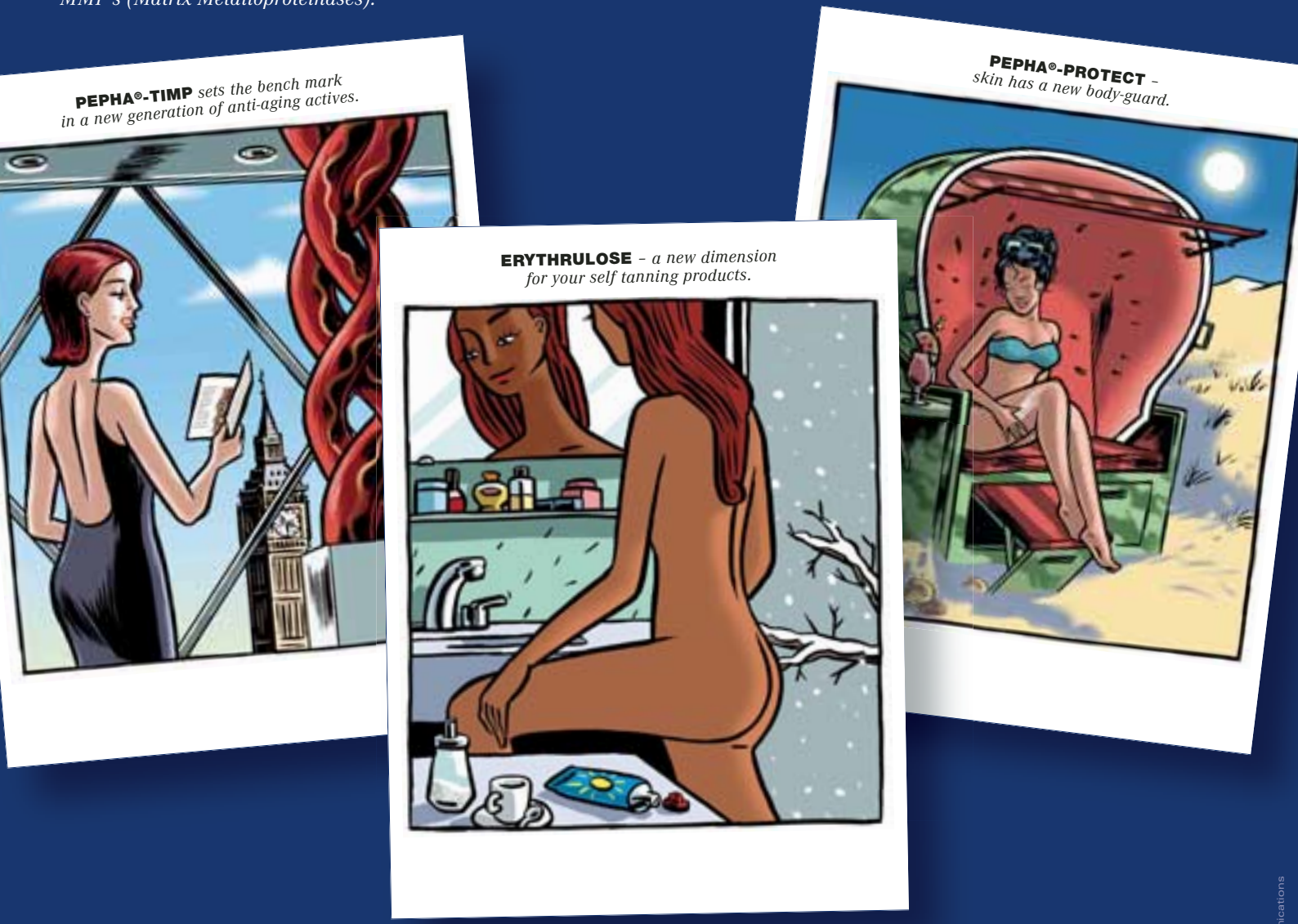

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the results with regards to the applied UV filter concentration represents the radical protection quality of the different UV filter systems as shown in **Fig. 3**. These representations show a slightly different ranking than that of **Fig. 2**. The excellent UVA protection is reflected by the highest radical protection. In contrast little or no UVA protection results in low radical protection.

Discussion and Conclusion

ESR spectroscopy measurements (measurements at X-band frequency [9.5 GHz]) were performed on skin samples using a modified and adapted experimental setup supplied by Magnettech GmbH (Germany). The amount of free radicals induced by UV radiation in skin was characterized by a new RSF (radical sun protection) Factor.

Different UV filter-containing formulations show very different RSF values. The main protection against UV-induced free radicals is provided by UVA filters closely followed by broadband filters. UVB filters contribute only marginally to the radical protection. Daylong extrême sunscreen with a RSF of nearly 40 is an excellent sunscreen formulation in terms of radical protection, mainly due to the presence of the broadband filters Tinosorb S and M as well as the UVA filter Butyl Methoxydibenzoylmethane. A RSF value of 40 means that only 2.5% of the total UVA/B radiation penetrates into the skin. In order to compare different UV filters, the measured RSF values have been normalised to a 1% (w/w) filter concentration. Again it results that UVA filters are more effective in reducing free radical injury in skin than UVB filters.

In conclusion, UVA/B filters drastically reduce the total number of UV-generated free radicals **(see Fig. 2** and **Table 3)**. On the other hand UVB filters protect only against free radicals and damages generated in the epidermis and have no influence on UVA-generated free radicals in the dermis. As a consequence only UVA filters can effectively protect the dermis against those damages. Damages generated by UVB can be caused indirectly through the generation of free radicals or directly through absorption of UVB rays by cell components such as membranes or DNA. The possible declaration of the RSF for a sunscreen product would give an information about its common radical protection of the skin.

Antioxidants present in the dermis and epidermis scavenge mainly the secondary radicals. Their role is to avoid cell damages caused by free radicals. Thus antioxidants give only an indirect protection. They »repair« damages caused by the primary radicals (ROS) resulting in secondary radicals ($L^{\cdot}$, $LO^{\cdot}$, $LOO^{\cdot}$) or break free radicals chain reactions. The evaluation of the protection capacity of sunscreen and antioxidant formulations using ESR measurements is a new promising method for characterizing the »real« sun protection of commercial products over the entire range of the solar spectrum (UVA and UVB).

Although sun avoidance is obviously the most efficient way of photoprotection, it

Product	RSF Norm.	RSF (1%)
No product	1.00 ± 0.05	
Daylong extreme	40.2 ± 2.01	
base with 2.5 % Butyl Methoxydibenzoylmethane	22.97 ± 1.14	9.19
base with 5 % Tinosorb S	39.4 ± 2.05	7.88
base with 5 % Ethylhexyl Triazone	4.31 ± 0.42	0.86
base with 7.5 % Isoamyl p- Methoxycinnamate	12.14 ± 0.92	1.62
base with 3 % Tinosorb M	10.14 ± 1.51	3.38

Table 3 RSF and norm. RSF (1%) of UV-filters and sunscreen formulation

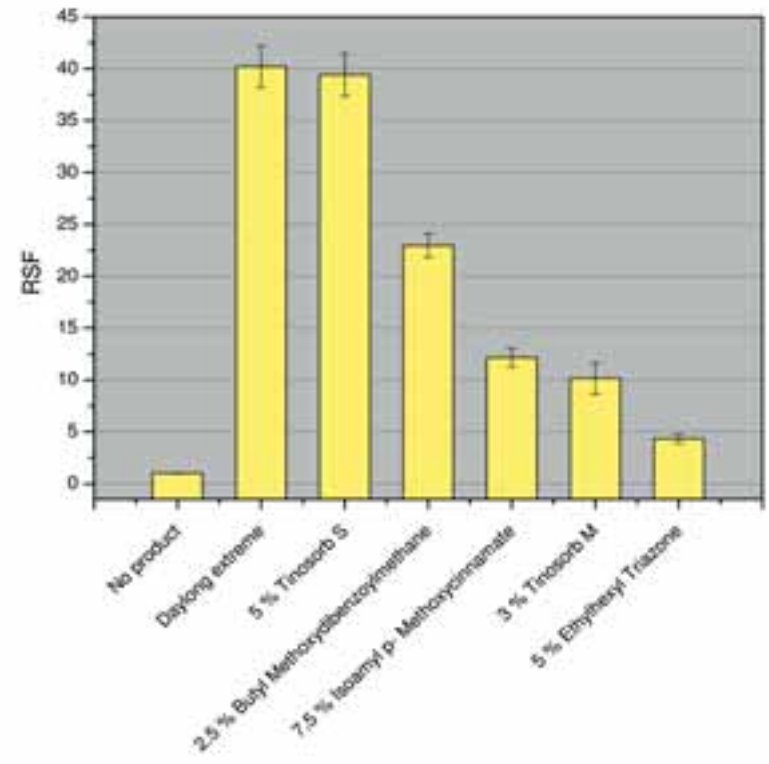

Fig. 2 RSF values of the tested sunscreen formulations

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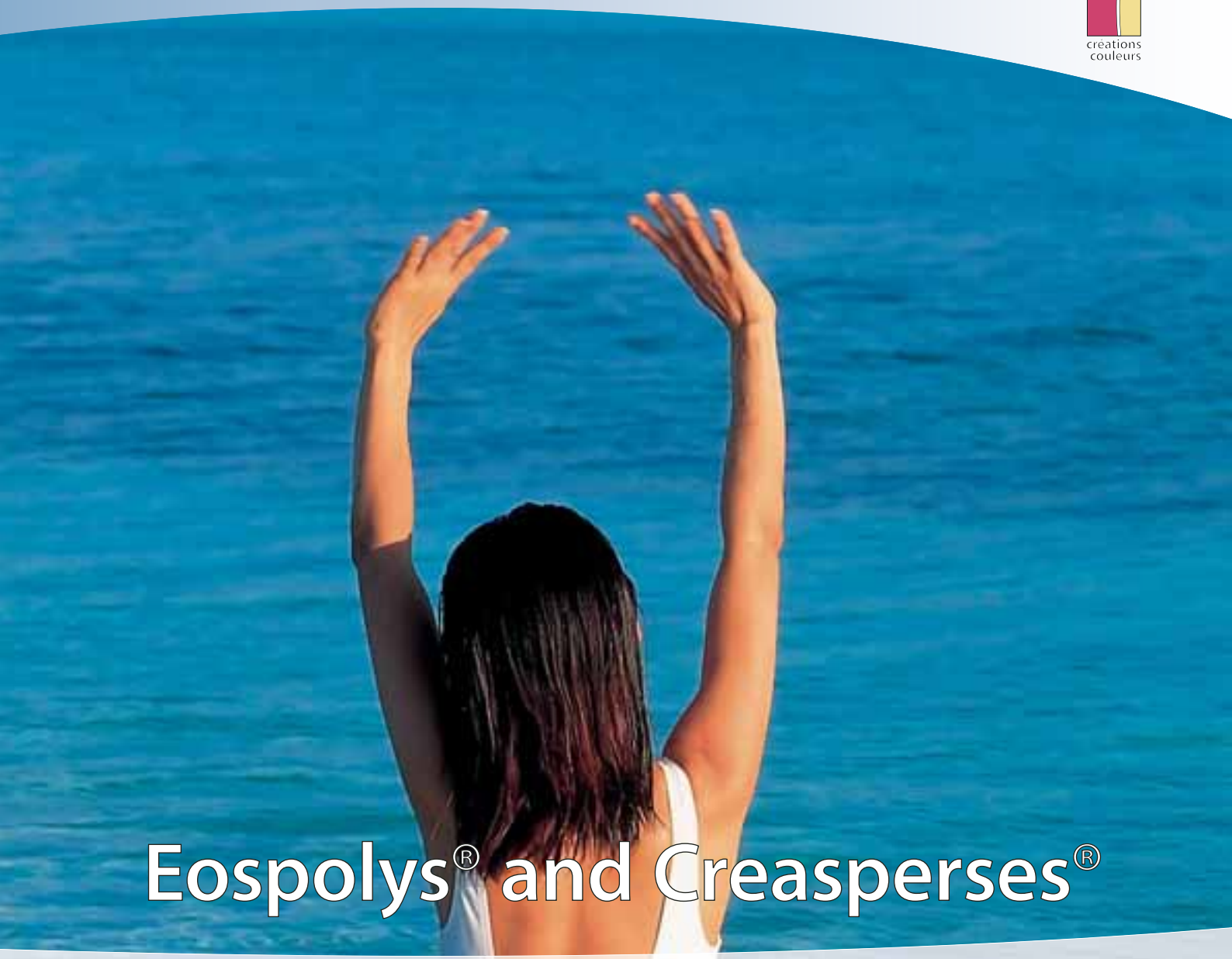

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is not always convenient and sometimes not possible. In the last decades however, there has been an enormous increase in sun exposure which can – at least partially- be attributed to the popularity of »sun bathing holidays«. This attitude has led to an increased incidence of melanoma as well as non-melanoma skin cancers (BCC and SCC).

In future we may have to take the influence of climate changes on skin cancer risk into account. As a consequence of ozone depletion, a greater amount of UV could cross the atmosphere which results in a more hazardous terrestrial UV-spectrum. On the other hand, global warming might influence people's behaviour and the time they spend outdoor, resulting in an increased exposure to sun light. Therefore it is, now more than ever, important to protect our skin against the damaging effects of UV rays. A general approach to reduce the unwanted effects of UV irradiation is therefore to either restrict UV exposure time or use effective sunscreen products with broadband UV protection and clearly defined declaration of the UV protection.

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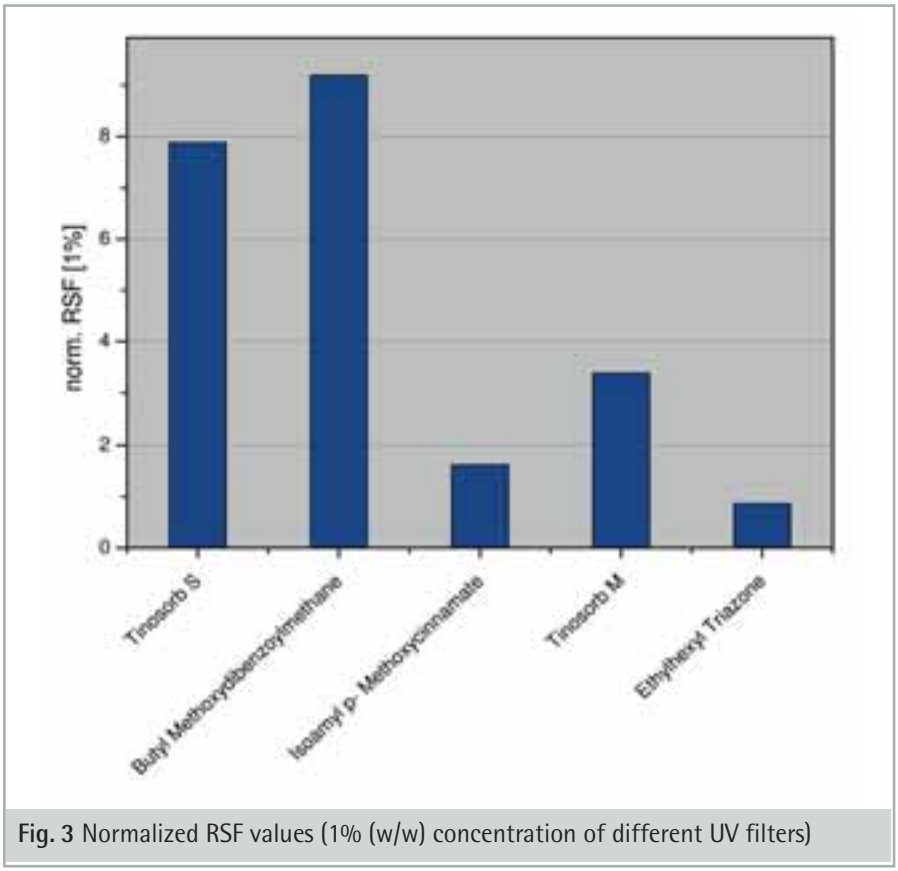

Fig. 3 Normalized RSF values (1% (w/w) concentration of different UV filters)

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