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Review Article

The Radical Status Factor (RSF): a novel metric to characterize skin products

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Synopsis

A scale of skin treatments is analysed, which bases on the detection of UV-generated free radicals in pig skin. Physiological and anatomical similarities between man and pig made this animal a good model for man in many research areas. The determination of the Radical Status (free radical response) offers the possibility to see in an early stage the effect of exterior and interior influences. The detection of the skin's response after a normalized radical generation by defined UV dose combined with the application of external and internal influences enables a comprehensive and easy classification of skin products and therapies. The effect of substances, especially, applied topically on the skin, like it is usual in cosmetics and pharmacy, can be classified. The relevance of the RSF method is demonstrated with the application of numerous different treatments on the skin.

Introduction

The skin as the biggest human organ is permanently treated by various intrinsic and extrinsic influences (Fig. 1), and various types of free radicals like shown in Table I [1] are generated. It is the meeting point of the organs' internal and external radical stress [2–5]. This radical stress characterizes the Radical Status of the skin. The RSF describes the totality of free radicals present in the skin. The free radical cascade begins with the primary free radicals that are generated directly by the reaction of photons with oxygen to the superoxide anion radical followed by the reaction of $O_2^{-}\cdot$ with H_2O_2 to $OH^{-}\cdot$. Both superoxide and hydroxyl radicals are the cause for the generation of lipid radicals (secondary free radicals). Other reactive compounds in the skin are the radicalized (oxidized) antioxidants like ascorbyl and tocopherooxyl radicals, the ROS singlet oxygen and nitric oxide. Semi-stable compounds like melanin and metal ions perform persistent radicals.

The reaction of nitroxides with these free radicals and reactive oxygen species results in the loss of their ESR signal, indicating the possibility of using nitroxides as a probe for free radical detection [6–8]. These intrinsic radical chain reactions cause cell damages up to inflammations and permanent skin alterations.

On the basis of the measurement of free radical reactions in pig skin, the effect of various reactive products from pharmacy to cosmetics on the Radical Status of the skin can be quantified. The RSF of the skin enables the *ex vivo* classification of all active ingredients that are directed against or catalyse the generation of free radicals provided that they penetrate into the skin. The RSF [9], at first introduced as radical sun protection factor, comparable to the sun protection factor SPF was extended to the Radical Status Factor, which regards chemical and physical influences having an effect on the skin. The relevant part of the sun radiation reaching the earth and influencing the human skin and hair expands from infrared to UV enclosing wavelength from 1000 nm (IR) to 280 nm (UVB). The most painful effects are generated by UVB and UVA. Corresponding to their penetration depth, UVB and UVA radiation generate primary free radicals/ROS followed by secondary daughter radicals like lipid radicals.

The near ultraviolet (UVB–UVA) from 280 to 400 nm is absorbed very strongly in the surface layer of the skin by electron transitions. As we go to higher energies (UVC–UVB) from 100 to 280 nm (Table II), the ionization energies for many molecules are reached and the more dangerous photoionization processes take place. Sunburn is primarily an effect of near UV. Ionization produces the direct risk of skin cancer.

The measurement of the RSF can be performed on pig skin biopsies. Physiological and anatomical similarities between man and pig made this animal a good model for man in many research areas [10].

Materials and methods

Chemicals

The nitroxide 2,2,5,5 tetrametylpyrrolidine-N-oxyl(PCA) was obtained from Sigma-Aldrich (Taufkirchen, Germany).

As UV filter, the following formulations were used:

2.5% and 7.5% UV B – filter (Octyl Triazone OT)

3% UV A – filter (Butyl Methoxydibenzoylmetane BMDBM)

2.3% and 5% UV AB – filter (Bis ethylhexyloxyphenol methoxyphenyl triazine BEMT) from Ciba Speciality Chemicals Inc. and BASF AG.

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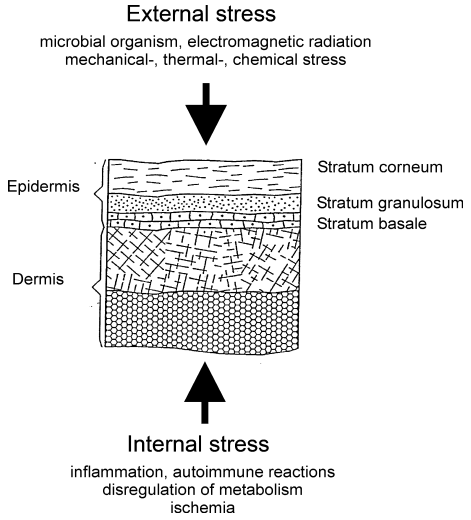

Figure 1 External and internal stress factors of skin.

Table I Half-lives and half-life ways of biologically relevant, free radicals and reactive oxygen species

Reactive species	Half-life	Half-life way
Hydroxyl radical (· OH)	0.3 ns	1.8 nm
Lipid alkyl radical (L·)	10 ns	60 nm
Lipid alkoxyl radical (LO·)	1 μs	6 μm
Lipid-peroxyl radical (LOO·)	1–10 s	
Superoxide anion radical (O_2^{-}·)	0.4 μs–1 ms	55 nm–3 μm
Singlet oxygen ($^1O_2$)	μs–ms	
Nitric oxide (NO)	Seconds	
Ascorbyl radical	Seconds	
Tocopheroxyl radical	Seconds	
Melanin	Persistent	
Metal ions	Persistent	

Data according to Pryor (1986).

Table II Spectra of different parts of sun radiation and its photon energy ε

Spectrum		Wave length λ	Energy ε
UV	C	100–280 nm	12.3–9.8 eV
	B	280–320 nm	9.8–9.2 eV
	A	320–400 nm	9.2–8.1 eV
IR		760 nm–1 mm	1.6–10^{-3} eV

Antioxidants caffeic acid, grape seed extract and rooibos extract were supplied from Sigma-Aldrich.
Dihydroxyacetone (DHA) was given by Sigma – Aldrich.

Skin

Skin biopsies from pig were used in all experiments. Pig skin has the greatest similarities to human skin and has the main advantage of a high structural and functional homogeneity. The ears of 6-month-old pigs from local slaughter were washed, the cartilage and the subdermal fat were removed, and the skin was cut into 1×1 cm^2 pieces and stored in phosphate-buffered saline (PBS) with a pH = 7.4 at 77°K until used. Skin biopsies (Ø 4 mm) were taken from the prepared skin flap. All samples (UV filter, antioxidants and DHA) were applied on the epidermal side of the pig ear.

Free radical indicator

Oxygen- and carbon-centred free radicals generated in skin during UV irradiation were detected using a radical trap on the basis of nitroxyl compounds (2,2,5,5 tetrametylpyrrolidine-N-oxyl - PCA).

UV irradiation

The UV irradiation was performed with xenon arc lamp Solar Simulator from Newport-ORIEL Product Line 81260 (US, Newport Solar Simulators – product specifications) equipped with a 300 W xenon lamp supplying an irradiance in the plane of the sample of 16.5 mW cm^{-2} for UVA (330–400 nm) and 5.0 mW cm^{-2} for UVB (290–330 nm). The 81 260 has a UVB/UVA dichroic mirror as a standard device. It passes 280–400 nm and greatly reduces the VIS and IR output of the lamp. The measurements were performed with an UV-Meter-BASIC (hönle UV technology, Gräfelfing, Germany). The UV solar simulator emits a continuous spectrum with no gaps or extreme peaks of emission in the UV region. The output from the solar simulator is stable, uniform across the whole output beam and suitably filtered to create a spectral quality that complies with the required acceptance limits. The RCE% values are complied [11, 12].

ESR Spectrometer

A X-band ESR spectrometer Miniscope 200 Magnettech, Germany, was used for *ex vivo* radical detection. A special tissue cell (GZ 170P) from Magnettech for skin measurements was also applied.

Experimental process

The test products (2 mg cm^{-2}, as defined by the COLIPA standard) were applied one time on a 1×1 cm^2 skin biopsy (epidermal side). The treated skin samples were allowed to remain for 15 min in the dark room at room temperature in a normal humidity atmosphere. After that, they were placed on a paper (filter discs grade 389, 84 g m^{-2} from Munktell&Filtrak GmbH, Bärenstein, Germany) imbued with a 1 mM solution of the radical indicator (PCA) for 5 min. Then, a skin biopsy of 4 mm diameter was taken with a biopsy punch, the skin was fixed on the tissue cell, and the ESR spectrum was recorded. The skin in the tissue cell was UV-irradiated with different UV doses (corresponding to irradiation times between 0.5 and 5 min), and different irradiance corresponding to the optical transmission was given by the density filters. After that process, the ESR spectra of the skin biopsy were measured and the data were analysed corresponding to a given numerical algorithm [13].

Determination of the radical skin status factor the calibration curve

Principle. The determination of the RSF starts with the generation of free radicals by a defined number of photons characterized by irradiance and irradiation time followed by the measurement of the radical response. The skin is treated with the radical indicator, the

Table III Irradiation time t (s) and the corresponding UV dose D (J cm^{-2}) for an irradiance of 5.1 mW cm^{-2} (UVB). The SED data are valid for the assumption that 1 SED = 0.01 J cm^{-2}

Time t (s)	Dose D (J cm^{-2})	SED
0	0	0
30	0.153	15.3
60	0.306	30.6
120	0.612	61.2
180	0.918	91.8
300	1.530	153.0

nitroxides 2,2,5,5 tetrametylpyrrolidine-N-oxyl(PCA). Different free radicals ($O_2^{-}\cdot$, $\cdot OH^{-}$, L·, LO·, LOO·) were generated by UV irradiation and react with PCA [8] decreasing its amplitude. Corresponding to a numerical algorithm [14] over a time interval of 5 min, six data points (0′, 30″, 1′, 2′, 3′, 5′) were measured. The different time interval corresponds to different UV doses (see Table III). The measurements are expressed in SED (standard erythema dose), where 1 SED = 100 J m^{-2} normalized to 298 nm [15]. The k-factor (reaction velocity) of the reduction curve was estimated. The number of free radicals generated in the skin is directly proportional to the k-factor. Figure 2 shows the calibration curves for three different density filters (PF = 1, PF = 10 and PF = 50). The density filters were supplied from ORIEL Instruments (Stratford, CT, U.S.A.). An optical density filter is a thin quartz plate provided with a corresponding vacuum deposited metal alloy resulting in an attenuation of the transmittance. PF = 10 means an attenuation to 1/10 of the incident light. For 6 (n = 6) different optical density filters (PF1, PF2, PF 5; PF 10, PF 20 and PF 50), calibration curves were measured and normalized to the full light intensity (non-irradiated case). A second calibration curve $k_1/k_n = RSF_n$ of skin is determined. This curve correlates with the curve of decreased irradiance I (see Fig. 3).

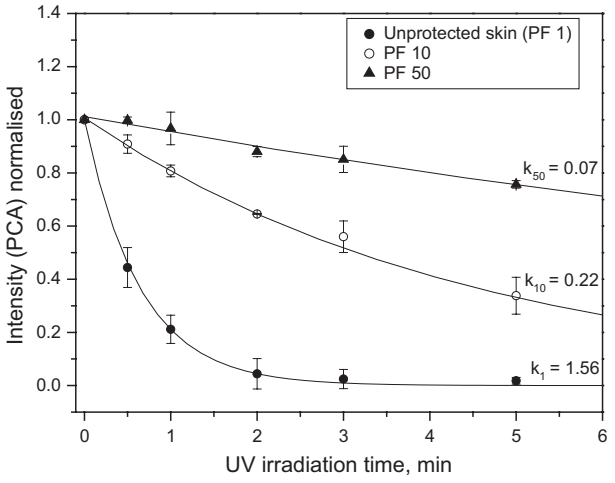

Figure 2 Calibration curves and determination of the rate constant k for the optical density filters PF 1, PF 10 and PF 50 as a function of UV irradiation time.

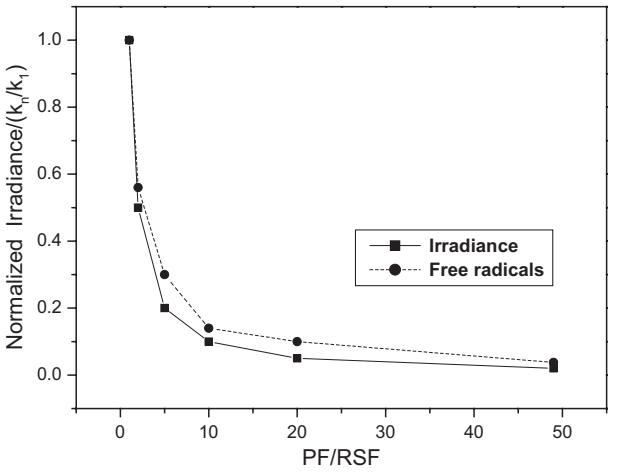

Figure 3 Correlation between the normalized number of generated free radicals and the corresponding irradiance as a function of the applied density filters (PF ≥ 1).

$$\frac{N(\text{free radicals in untreated skin})}{N(\text{free radicals in treated skin})} = \text{RSF} \qquad (1)$$

$$N(\text{R}\cdot \text{intreated skin}) = N(\text{R}\cdot \text{inuntreated skin})/\text{RSF} \qquad (2)$$

It is the basis for the determination of the RSF of an unknown sunscreen, which is measured as a comparison with this calibration curve.

On the basis of this observation, it is possible to present the relation between the RSF and the number of corresponded free radicals. The so-called second calibration curve documents this correlation. For radical protection, the curve corresponds to a 1/x-function, which is also applied to radical promotion.

Radical protector vs. radical promoter

Processes or substances that increase the number of generated free radicals are characterized by RSF < 1 and that decrease the number of generated free radicals are characterized by RSF > 1. The normal untreated skin is characterized by a RSF = 1, and the number of measured free radicals corresponds to a concentration of 100%. All products that can characterized by RSF > 1 are radical protectors. Products that labelled by RSF < 1 are radical promoters (Fig. 4).

Results

Protection very high increase RSF ≫ 1

UV filters can avoid the generation of free radicals by absorbing or scattering the ultraviolet radiation. They perform the first defence line against external stress caused by UV irradiation. The RSF values of arbitrarily chosen sunscreens containing different UV filter with different concentrations presented in Fig. 5. All filters are stirred in the same formulation. The formulation contains aqua,

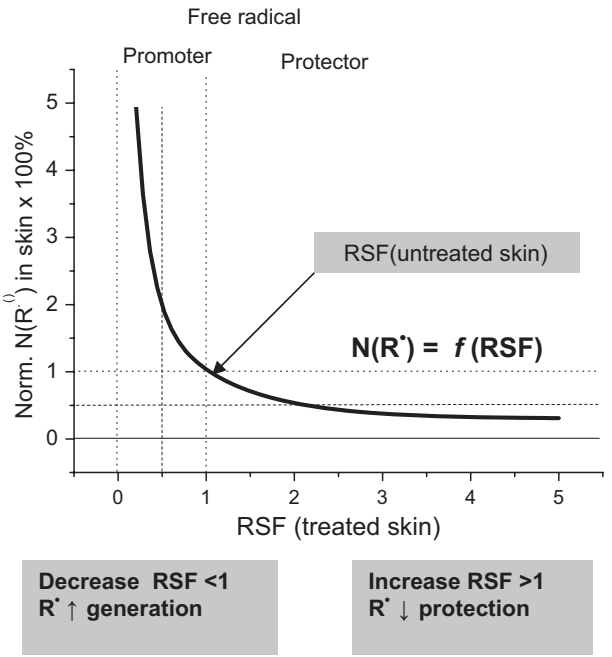

Figure 4 The normalized number N(R·) of generated free radicals in skin as a function of RSF > 0.

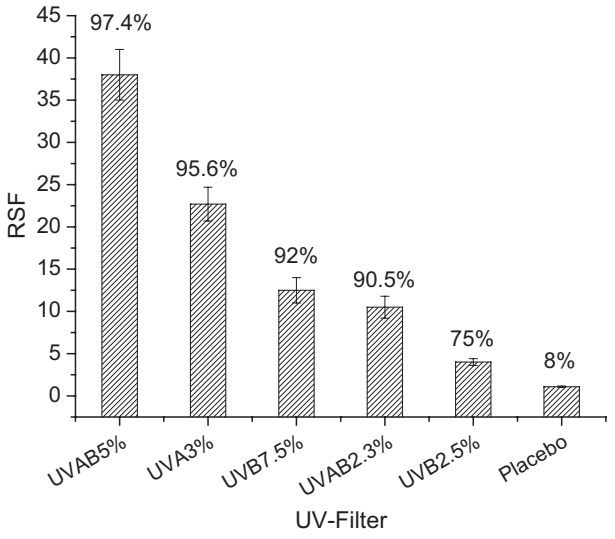

Figure 5 RSF inclusive their radical prevention(given in %)of formulations containing different concentrations of UV-filters.

pentylene glycol, tetramethylbutylphenol, cocoglyceride, dicaprylyl carbonate, C12-15 alkyl-benzoate, dimethione, cetearyl alcohol, polymethyl methacrylate, glycerin, xanthan gum, alkyl acrylate crosspolymer and sodium hydroxide. A formulation that contains 5% UVB filter (ethylhexyl triazone) results in 75% radical protection (RSF = 4 means 25% remaining free radicals). For 3% UVA/B filter (Tinosorb M), a RSF = 10.5 is reached, which corresponds to a radical protection of 90.5%. 7.5% UVB filter (isoamyl p-methoxycinnamate) gives a radical protection of 92% (RSF = 12.5). With 3% UVA filter (butyl methoxydibenzoylmethane), a RSF = 22.7 or a power of 95.6% radical protection can be reached. A 97.5% radical protection (RSF = 38) for 5% UVAB filter (Bemotrizinol, Tinosorb S) content is possible.

Moderate RSF > 1

Antioxidants are a heterogeneous class of molecules, which can neutralize free radicals and can stop radical chain reactions. The efficacy of these active ingredients is measured by the Antioxidative Power (AP) [16, 17], quantified by its capacity (represented by Antioxidative Unit [AU]) and qualified by its reaction time t_r (min). Three antioxidants (listed in Table IV) were tested for their influence on the radical status. They were applied with three different concentrations in the final formulation. Contrary to the application of formulations containing UV filter, the measured RSF values of antioxidant skin products are obviously smaller. With antioxidants, mostly RSF values < 2.0 (means 50% radical protection) are obtained.

These influences are time-dependent processes that cause a time-dependent function RSF = f(t_A) presented in Fig. 6. It describes the quality of reaction kinetics with RSF > 1 (Protector); RSF < 1 (Promoter). The factor k determines in this case the rate constant for different application times.

For the demonstration of the correctness of this approach, some examples were chosen and presented. So it can distinguish between different products corresponding to their RSF.

Table IV Three antioxidants ingredients in different concentrations in a formulation result in different RSF, and AP with the reaction times t_r

Antioxidant ingredient in the formulation	Concentration of antioxidant %	AP (AU)	t_r (min)	RSF	R·↓ (%)
Caffeic acid	0.18	2000.000	0.16	1.5	(33)
Grape seed extract	0.3	300.000	0.9	1.4	(29)
Rooibos extract	1.2	120.000	1.2	1.9	(47)

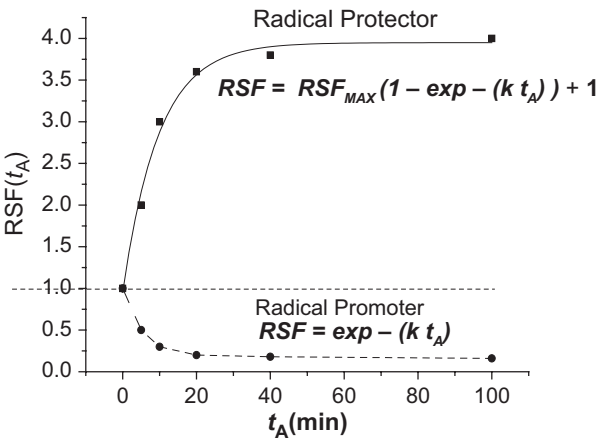

Figure 6 Diagram of RSF(t_A) as a function of application time t_A for products which act as radical protector or promotor.

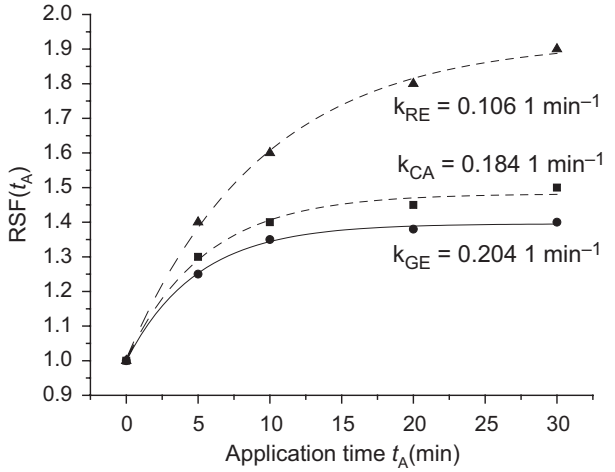

Figure 7 RSF(t_A) inclusive their rate constants k (1 min^{-1}) of three different antioxidants: Rooibos Extract, Caffeic Acid, Grape Seed Extract as a function of application time t_A.

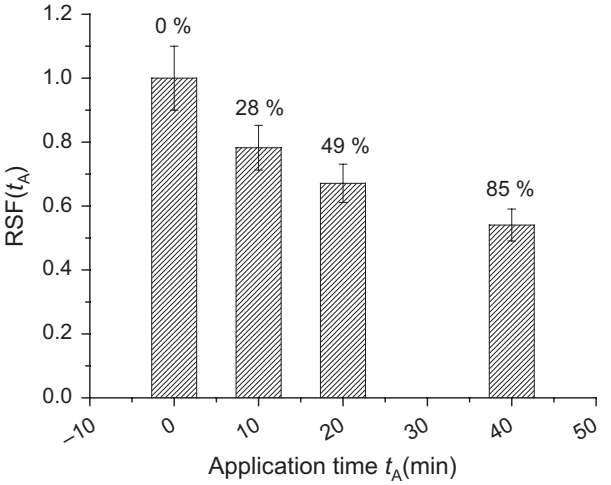

Figure 8 The treatment of skin with a 5% DHA (Dihydroxyacetone) agent solution over different time intervals results in different RSF 11 decrease from RSF = 0.78 (28% additional radical generation) for 10 min application time to RSF = 0.54 (85% additional radical generation) for 40 min application time.

The obtained RSF values are correlated with the corresponding free radical decrease(R·↓), which depends on the penetration properties of antioxidant ingredient in the skin. The changes in the RSF as a function of application time of formulation with antioxidant ingredient can give information on the application process itself. Their penetration behaviour can be characterized by their rate constant k. Figure 7 shows the rate constants of the three antioxidant formulations presented in Table. IV. Grape seed extract that has the highest rate constant with k_{GE} = 0.204 1 min^{-1} has reached its saturation point after 20 min followed by caffeic acid with k_{CA} = 0.184 1 min^{-1} reached the saturation after 30 min. Rooibos extract with k_{RE} = 0.106 1 min^{-1} does not reach its saturation point after 30 min. The maximum values (saturation points) are RSF_{MAX} = 1.9 for roibos extract, RSF_{MAX} = 1.5 for caffeic acid and RSF_{MAX} = 1.4 for grape seed extract. All the antioxidant ingredients were stirred in an Unguentum emulsificans aquosum (DAC) formulation.

Promotion RSF < 1

Substances containing active radical promoting ingredients show a decreased RSF during UV irradiation. Dihydroxyacetone (20%) that undergoes a Maillard reaction in the skin was tested [18] at three penetration times (Fig. 8). During UV radiation of the treated skin, the first reaction products and intermediates of the Maillard reaction are susceptible to UV and generate a huge amount of additional free radicals inside the skin. After 10-min treatment with 20% DHA (Dihydroxyacetone) solution, a RSF of 0.79 was found. That means that about 27% of additional free radicals are generated in the skin during UV exposure. For a treatment time of 20 min, 49% free radicals are generated, which corresponds to an RSF of 0.67. 40-min penetration time generates 85% additionally free radicals that corresponds to an RSF = 0.54 (Fig. 8).

The application time of DHA determines the intensity of the Maillard reaction [19] in the skin. The radical injury is a function of applied concentration and application time. The result is a radical burst associated with a decreasing RSF < 1. The penetration time of DHA in the skin is characterized by the used formulation and is given by the function RSF = f(t_A), which results in a rate constant k_{DHA} = 0.027 1 min^{-1} (Fig. 9).

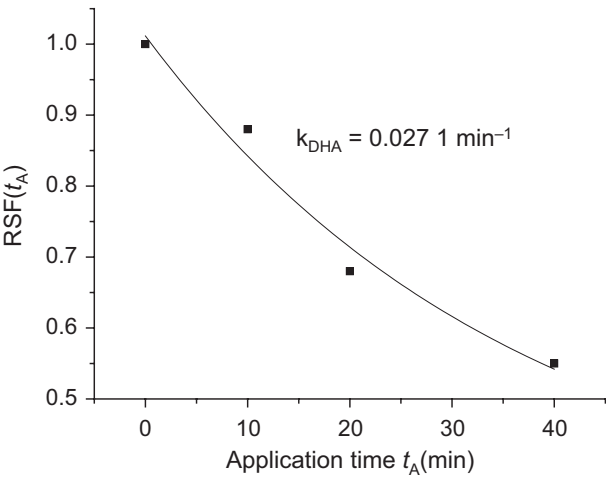

Figure 9 RSF(t_A) decrease as a function of application time of the DHA formulation on the skin resulting in rate constant k_{DHA} = 0.027 1 min^{-1}.

Discussion

The (RSF) describes the properties of a substance to protect against or to promote the generation of free radicals. The existence of free radicals is strongly correlated to all redox processes that take place in the skin. So it is in the case of sun exposure. The radical generation begins at 380 nm and goes in a steady state from 300 nm on [20]. The rate of rise is a linear process, which covers UVA and UVB. Because of the penetration depth of the electromagnetic waves, UVB is limited to the epidermis. Considering both UVA and

UVB radiation in the sun spectrum, more free radicals are generated in the dermis. Therefore, it is necessary to protect the whole skin with a product, which covers a range beginning with at least 380 nm and ended with the limit of terrestrial radiation (normally 280 nm). The question, which radical type is generated in this moment at a defined place, depends on the energy input of the photon (marked by the wave length λ) and the molecule itself. Of course, the radical process is a temporary process that begins with OH^-, O_2^- and ends mostly with the generation of lipid radicals, which have a good reaction with PCA. A uniform, wideband protection over UVA + UVB is of vital interest. It is not a question of the ratio of UVB/UVA protection.

Substances that have a good protection against free radicals are marked by factors which are RSF $\gg$ 1. First of all, UV filters are mentioned that have the highest RSF. Radical protection of RSF = 100 is the currently upper limit of radical protection. It is nearly a perfect protection. It allows only 1% radical generation. A moderate protection is reached by using antioxidants. They can act as a second defence line against sun damage. RSF > 2, meaning 50% radical protection, is hard to achieve. But antioxidants can penetrate in the dermis to protect the skin from the inner side contrary to UV filter, which are deposited on the surface only.

The normal untreated skin is characterized by RSF = 1, which means no protection against and no promotion of free radicals. One < RSF < 0 is valid for all substances that promote the generation of free radicals. Detergents or other surface-active materials belong to this group of products that show a drastic radical increase after UV irradiation [21]. A free radical generation of 300% corresponds to a RSF = 0.25.

The RSF provides the characterization of the redox – status of the skin by a simple factor. This factor considers physical, chemical and biological influences as well as topically applied substances affecting the skin. The RSF is not only a metric for sun protection products comparable to the SPF or the UVA-PF. It is a more comprehensive parameter to classify cosmetic and pharmaceutical products.

Conflict of interest

The authors state no conflict of interest.

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