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On the assessment of photostability of sunscreens exposed to UVA irradiation: From glass plates to pig/human skin, which is best?

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ABSTRACT

Photostability of suncare products is a great area of interest since several sunscreens on the market are photounstable, and this is primarily a problem concerning the UVA region (320–400 nm). Here we report a comparative study on the photostability assessment of two commercial sunscreens with same SPF, spread onto glass plates or onto full thickness pig ear skin or human/pig SCE membranes, and exposed to 183 kJ/m^2 UVA. Absorbance spectra and lipid peroxidation (measured by TBARS production) were determined. The results indicate: (a) sunscreen performance consequent to UVA exposure is independent of whether it is spread onto a non-biological and chemically inert substrate such as glass, or on biological substrates such as skin/SCE membranes; (b) despite the same SPF, sunscreen performance and photostability can be very different; (c) the data on human SCE membranes are similar to those on pig SCE membranes, indicating the suitability of the latter as a model for human skin. However, since the results obtained using skin membranes, akin to the more realistic conditions of use in vivo, do not substantially differ from those obtained on glass plates, the method proposed here using the latter may be applied for rapid, inexpensive, efficacy screening of photostability of sunscreens. Photostability testing should be a mandatory requirement for safer sunscreen protection products, since the results clearly show that some are still far from perfect.

1. Introduction

The use of sunscreens is the most popular, universal method for preventing skin damage caused by sun over-exposure, which manifests itself as sunburn/erythema in the short-term and photocarcinogenesis/photoageing in the long-term (Clydesdale et al., 2001; Trautinger, 2001; Ullrich, 2007). The active ingredients in sunscreens are a mixture of UV filters designed to absorb/reflect/scatter the UVB rays (290–320 nm), UVA rays (320–400 nm) or both, thus reducing the amount of UV light reaching the viable skin layers (Palm and O'Donoghue, 2007). Most UV filters are sufficiently, photochemically stable, i.e. their absorbance spectra remain relatively unchanged during UV exposure. However, it is well known that some common ones are not. Their absorbance spectra change following UV exposure and this leads to a loss in absorbance which ultimately translates into reduced photoprotection of the sunscreens containing them (Bonda, 2005; Dondi et al., 2006). In addition, in some cases chemical photoinstability is accompanied by the formation of photoproducts, free radicals, reactive oxygen species (ROS) which may not only interact with other co-formulated ingredients of sunscreen products, but also with skin constituents such as lipids, proteins, and nucleic acids (Allen et al., 1996; Butt and Christensen, 2000; Karlsson et al., 2009; Schwack and Rudolph, 1995). Hence, obtaining knowledge on the photostability of individual UV filters and, more importantly, of their photochemical behaviour when combined in a sunscreen, should be worthwhile pursuing for product safety and skin photoprotection. In fact, there is plentiful literature on the behaviour of individual UV filters but their performance may change when co-formulated with others in a sunscreen product (Damiani et al., 2007; Dondi et al., 2006; Maier et al., 2001; Roscher et al., 1994; Tarras-Wahlberg et al., 1999). Unfortunately, the majority of sunscreens on the market do not have a photostability label, since this is not a regulatory requirement for marketing, but only a SPF

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(sun protection factor) label which is an indicator only for protection against erythema, largely caused by UVB wavelengths (EC, 2007; Stanfield et al., 2010). No information is given on protection against UVA wavelengths which penetrate much deeper into the dermal skin layers than UVB ones. Furthermore, some studies have demonstrated that sunscreens' photoinstability is primarily a problem concerning the UVA region (Hojerova et al., 2011; Maier et al., 2001). High SPF value sunscreens imply that the UV filters present in them should remain photostable for the entire period of sunlight exposure providing long-lasting photoprotection. However, this may not be the case if the wrong combination of UV filters is present in the sunscreen and if they are prone to photoinstability.

At this regard, we recently developed a simple and effective method for assessing photostability and photoinduced ROS generation in sunscreens containing individual UV filters and their combination (Damiani et al., 2010). However, sunscreens were not applied to skin itself but on glass plates, hence the information obtained may not totally reflect the true behaviour of sunscreens when applied to skin. It is known that sunscreen performance is dependent on whether it is as a thin film or disrupted such as in real application to the irregular surface of the skin (Farr and Diffey, 1985; Haywood, 2006). Furthermore, the different pigmentation of skin (the UV and visible light reflected, scattered, absorbed and dissipated by chromophores in various layers of skin depending on the different skin types/tones) may affect the true behaviour of sunscreens. Based on our previous method, the present study takes one step further to gain more realistic information on the photostability of UV filters present in sunscreens. For this purpose, the behaviour of two commercial sunscreens with same SPF was investigated on human stratum corneum/epidermis (SCE) membranes, and for the first time, on pig SCE membranes, in addition to full-thickness pig ear skin, and exposed to UVA. Pig skin was used in this study since it shares many similarities to that of human skin including follicular structure, and has been used for in vitro skin penetration of UV filters as well as many other compounds (Jacobi et al., 2007; Weigmann et al., 2009). In addition, the behaviour of the sunscreens was compared with that obtained from their application as a thin film on glass plates, according to our previous method (Damiani et al., 2010).

2. Materials and methods

Two commercial sunscreens currently available on the European market were purchased from local stores and selected on the basis of their equal high SPF (SPF 30), but with a different combination of UV filters, as indicated on the product label, in the following order of appearance and therefore of concentration, as follows: cream A: OMC (ethyhexyl methoxycinnamate, UVB filter), TiO_2 (titanium dioxide, UVA/B filter), BMDBM (butyl methoxydibenzoylmethane, UVA filter); cream B: OCT (octocrylene, UVB filter), BP-3 (benzophenone-3, UVA filter), BMDBM, EHS (ethylhexyl salicylate, UVB filter). All other reagents and solvents were purchased from Sigma–Aldrich Chemical Co. (Milan, Italy).

2.1. Preparation of substrates and products application

Samples of adult human skin (mean age 36 ± 8 years) were obtained from breast reduction operations and treated as previously reported (Puglia et al., 2012). Briefly, subcutaneous fat was carefully trimmed and the skin was immersed in distilled water at $60 \pm 1\,^{\circ}C$ for 2 min, after which SCE were removed from the dermis using a dull scalpel blade (Kligman and Christophers, 1963). Epidermal membranes were dried in a desiccator at ~25% relative humidity. The dried samples were wrapped in aluminium foil and stored at $4 \pm 1\,^{\circ}C$ until use. Previous research work demonstrated the maintenance of SC barrier characteristics after storage under the reported conditions (Swarbrick et al., 1982). Besides, preliminary experiments were carried out in order to assess the barrier integrity of SCE samples by measuring the in vitro permeability of [^{3}H]water through the membranes using the Franz cell method. The value of calculated permeability coefficient (*P*m) for [^{3}H]water agreed well with those previously reported (Bronaugh et al., 1986).

Pig SCE membranes were obtained in a similar way as described above. Briefly, pig ears that had not been scalded, were obtained from freshly killed animals (Large White breed, 9–10 months old) from a local abattoir, and treated in the following way in a cold room: the ears were washed with cold, distilled water and hairs carefully removed using an electric hair clipper for better distribution during sunscreen application. The underlying fatty tissue and cartilage was removed with a scalpel and the full-thickness skin was either treated as described above for human skin to obtain pig SCE membranes or laid out on a polystyrene tray, covered with a plastic bag and stored at $-20\,^{\circ}C$ until ready for use for a period that did not exceed 2 months.

Prior to use, the SCE membranes and full-thickness pig skin were cut into samples 4 cm^2 in size and placed on a petri dish containing filter paper imbibed with a sufficient amount of PBS (phosphate buffered saline) such that only the underside of the skin was in contact with PBS. Sunscreens (15 μl) were then applied to the skin samples using a Microman positive displacement pipette which corresponded to 8 mg (2 mg/cm^2 as recommended by the COLIPA sun protection factor test method (COLIPA, 2006)). The same amount was applied to glass plates of the same dimensions as the skin samples. The sunscreens were spread over the different supports with a gloved finger using a light, circular, rubbing motion for uniform distribution, and left at room temperature in the dark for 20 min. For each cream two samples were always prepared, for and without UVA exposure. In parallel, skin samples without cream were also tested, for and without UVA exposure. Furthermore, human SCE membranes were from two different individuals, therefore one was used for all experiments concerning cream A and the other sample for all those concerning cream B. The same criteria were also used for pig SCE membranes which were from two different animals.

2.2. Irradiation source and protocol

A commercial UVA sun lamp, Philips Original Home Solarium (model HB 405/A: Groningen, Holland) equipped with a 400 W ozone-free Philips HPA lamp, UV type 3 was used for UVA irradiation. The output was measured with a UV Power Pack Radiometer (EIT Inc., Sterling, MA) while the emission spectrum was checked using a StellarNet portable spectroradiometer (Tampa, FL). The lamps emission spectrum has been reported elsewhere (Venditti et al., 2008), and shows that of the total light emitted between 300 and 400 nm, <1.5% is below 320 nm, hence the UV source is essentially a UVA one. The lamp was always pre-run 10 min to allow the output to stabilize. The petri dish containing the samples were then placed on ice at a distance of 20 cm from the light source and irradiated for 10 min which corresponded to a UVA dose of 183 kJ/m^2. This dose is approximately equivalent to 60 min of sunshine at the French Riviera (Nice) in summer at noon (Seite et al., 1998). For cream A, a kinetic analysis was also carried out between time 0 and 10 min, for monitoring product stability every 2.5 min, within the selected time course. For each irradiated sample, a non-irradiated one serving as control was kept in the dark for 10 min at room temperature.

2.3. Optical absorption spectra

The irradiated and control samples were then placed in beakers containing 15 ml ethyl acetate/ethanol 2:1 (v/v), covered with aluminium foil and magnetically stirred for 30 min, except for the beakers containing glass plates which were manually shaken every 10 min, to allow for maximum extraction of the UV filters and organic material (mainly lipids) from the sunscreens and skin samples. From this organic solution, 50 μl was added to 2450 μl ethyl acetate in a quartz cuvette and the absorption spectra were measured on a Varian Cary 50 UV–visible spectrophotometer (Agilent Technologies Italia S.p.A., Italy) against a blank containing ethyl acetate.

2.4. Evaluation of lipid peroxidation

The remaining organic solutions were transferred into 25 ml recovery flasks and evaporated under vacuum by Rotavapor. To the residue, 2 ml of TBA–TCA–HCl solution (0.375% TBA = thiobarbituric acid, 15% (w/v) TCA = trichloroacetic acid, 0.2 M HCl) was added followed by 10 μl of 20 mM BHT (butylated hydroxytoluene). The samples were heated in a water bath for 30 min at 90 °C to allow for the reaction between aldehydic breakdown products and TBA to take place. The samples were then cooled, transferred to eppendorf tubes, centrifuged and the absorbance of the pink chromophore of the supernatant was measured at 532 nm and compared to that of a calibration curve of 1,1,3,3-tetraethoxypropane reacted with TBA solution (Buege and Aust, 1978). The absorbance was expressed as TBARS (TBA reactive substances), related to that of the respective amount of malondialdehyde in the calibration curve.

Appropriate controls were carried out throughout all the experiments described above, and the data reported represent average values from at least three independent experiments. Statistical analysis was performed using Student's *t*-test.

3. Results and discussion

The spectral behaviour of the two sunscreens with same SPF was investigated when spread onto glass plates, full-thickness pig ear skin or human/pig SCE membranes before and after 10 min UVA irradiation. This irradiation time, corresponding to a UVA dose of 183 kJ/m^2 (approximately equivalent to a Standard Erythemal Dose, SED = 4) (Gonzalez et al., 2007), is a physiologically relevant dose of UVA, achievable during an approximate 60 min sunshine exposure at midday in summer in the Mediterranean. From the literature, it is reported that if a sunscreen is photounstable, it starts to degrade rather rapidly when exposed to the sun (Gonzalez et al., 2007). The process is dose-dependent and it appears that the most pronounced photodegradation is induced already by low UV doses (Gasparro, 1985; Berset et al., 1996; Tarras-Wahlberg et al., 1999). Hence in our study, this single time/dose condition for final analysis was chosen. Concomitantly, the lipid peroxidation levels before and after UVA exposure were also examined on all the samples.

Fig. 1 shows the absorbance profiles of sunscreen A before and after UVA exposure when applied onto the different supports. First, similar spectral profiles and behaviour can be observed irrespective of the support on which the sunscreen was spread. The levels of absorbance measured were also similar, although slightly lower absorbance levels were detected from the glass slides. Although similar quantities were applied on all the supports, during spreading with the gloved finger sunscreen penetrates into skin whereas on glass it does not, hence slightly more sunscreen may be retained on the gloved finger during spreading. After extraction in ethyl acetate/ethanol, slightly lower absorbance levels may therefore be expected from samples spread onto the glass support. Second, the figures show that there is a remarkable decrease in spectral absorbance after UVA exposure in all cases, throughout the whole UVA/UVB range, which implies that sunscreen A is highly photounstable, in accordance with our previous results on this same cream (Damiani et al., 2010). However, since cream A proved to be remarkably degraded at the final time point chosen, a kinetic analysis was also carried out on glass plates for this product in order to obtain information on the kinetics of this process. As Fig. 1A shows, the absorbance loss is dose/time-dependent with 50% loss in the initial absorbance being reached after only 5 min irradiation in the both the UVA/UVB regions, whereas a further loss, approximately 80%, was observed in the UVA region after 10 min irradiation. This behaviour is in agreement with the observations of Maier et al. who detected a greater, significant dose- and wavelength-dependent decrease in the UVA protective capacity compared to the UVB one, of seven sunscreens out of the sixteen tested (Maier et al., 2001). Gonzalez et al. also noted a dose-dependent decrease in three photounstable sunscreens exposed to natural sunlight, where almost 100% loss in absorbance in the UVA region was achieved after 30–90 min of exposure, depending on the sunscreen tested. Loss in the UVB region was also observed, but less than for the UVA one (Gonzalez et al., 2007).

The decline in spectral absorbance is due to the presence of BMDBM and OMC. Both these UV-filters when studied as single ingredients mixed with petroleum jelly and exposed to UVA, showed a significant reduction in absorbance which was rapid and dose-dependent for BMDBM (Tarras-Wahlberg et al., 1999). However, even their combination within a formulation has long been known to be a photounstable one if adequate stabilizing molecules are not present (Damiani et al., 2007; Diffey et al., 1997; Dondi et al., 2006; Gonzalez et al., 2007; Sayre et al., 2005). This is in line with a recent study by Hojerova et al. (2011) who showed that among 15 commercial sunscreens all labelled with the same SPF 20 exposed to natural sunlight, 7 were photounstable in the total UV range and each contained the combination of OMC and BMDBM. Dondi et al. also observed significant degradation of two well-known and popular commercial sunscreens both containing these two popular UV-filters. They ascribed the loss in absorbance and hence of UV protection in the two sunscreens to cycloaddition reactions between these two filters which yield a mixture of potentially toxic diketones (Dondi et al., 2006). Furthermore, the performance of sunscreen A may also be affected by the TiO_2 particles present as physical UV filter, because if they still retain some photocatalytic activity, they may interact with other active ingredients of the sunscreen leading to rapid photodegradation (Wakefield et al., 2004).

The spectral behaviour of sunscreen B is reported in Fig. 2 and in this case too the spectral profiles and performance are similar regardless of whether the sunscreen was spread on glass, full-thickness pig skin or pig/human SCE membranes. Slightly lower absorbance levels were detected from the glass slides in accordance with what was observed and discussed above for sunscreen A. However, strikingly different from sunscreen A is its remarkable photostability throughout the whole UV range. Although this sunscreen contains BMDBM which is known to be inherently photounstable (Schwack and Rudolph, 1995), it is sufficiently stabilized by the presence of OCR, a well known, effective stabilizer of BMDBM and also in part by BP-3 (Bonda, 2005). One other important feature that can be observed when comparing the spectral profiles of sunscreens A and B, is that despite having the same SPF, they not only have significantly different shapes but they also display different levels of absorbance throughout the UV range, therefore providing different levels of UV protection (sunscreen B > sunscreen A). This discrepancy among sunscreens with same SPFs but with different UV spectral profiles was also found in the 15 commercial sunscreens recently tested by Hojerova et al. (2011) and by

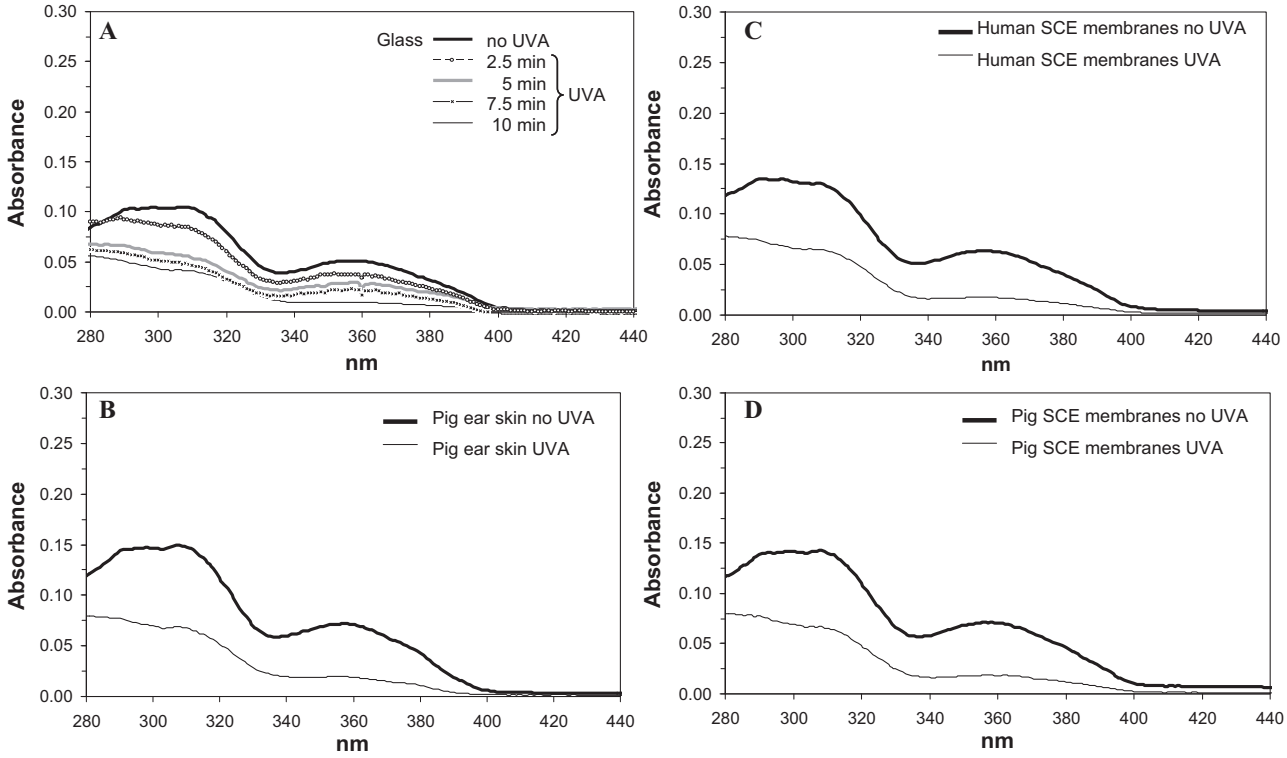

Fig. 1. UV-absorption spectra of sunscreen A spread onto different substrates, before and after UVA exposure, followed by extraction with ethyl acetate/ethanol 2:1. Panel A also shows the absorption spectra monitored at different times of UVA exposure. See Section 2 for experimental details.

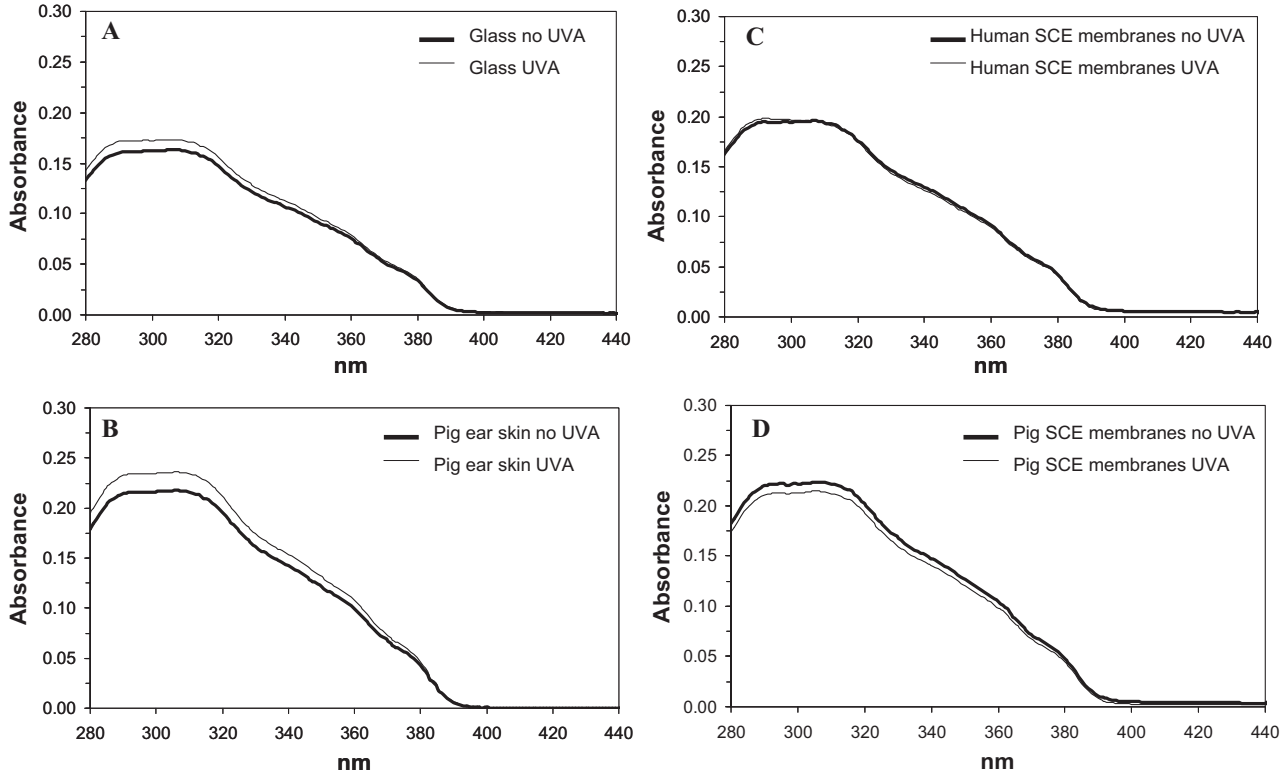

Fig. 2. UV-absorption spectra of sunscreen B spread onto different substrates, before and after UVA exposure, followed by extraction with ethyl acetate/ethanol 2:1. See Section 2 for experimental details.

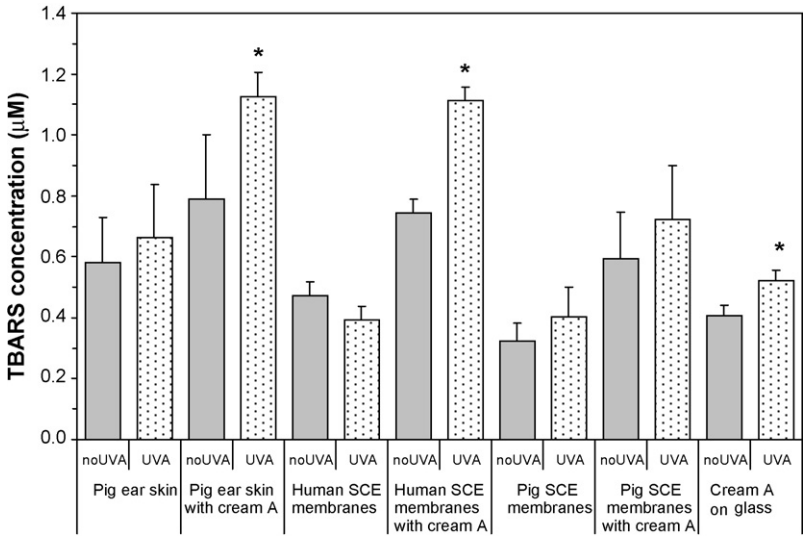

Fig. 3. TBARS levels obtained from the different skin samples before and after UVA exposure and in the presence or absence of sunscreen A. For comparison, TBARS levels obtained from sunscreen A spread onto glass plates before and after UVA exposure, are also reported. See Section 2 for experimental details. $^{*}p < 0.05$ versus respective unexposed control.

Lejeune et al. who tested two SPF 15 sunscreens on a reconstructed skin model (Lejeune et al., 2008).

The photostability of sunscreens was also tested by evaluating the extent of lipid peroxidation following UVA exposure, of the lipid constituents of the sunscreens and possibly of the skin samples. In fact, the most abundant UV wavelengths present in sunlight are UVA ones (>95%) and these are well known to promote and propagate lipid peroxidation via generation of ROS much more efficiently than UVB ones (Morliere et al., 1995; Polte and Tyrrell, 2004). By measuring TBARS levels, one has an indirect estimate of any UVA and/or UV-filter-induced ROS generated in the systems under study. Figs. 3 and 4 document the TBARS levels obtained from the different skin samples before and after UVA exposure and in the presence or absence of sunscreens A and B, respectively. TBARS generated from the sunscreens spread onto glass plates are also reported for comparison. In general, one can observe that TBARS are higher in skin samples with sunscreens than without, due to the contribution of TBARS deriving from the sunscreen alone. From Fig. 3 one can observe that sunscreen A spread on glass is prone to undergo photo-oxidation as documented by the significant increase in TBARS upon UVA exposure, as previously observed (Damiani et al., 2010). This is in analogy with the photoinstability observed in the UV absorbance spectra (Fig. 1): any BMDBM not involved in cyclo-addition with OMC, can undergo cleavage leading to ROS which contribute to increasing TBARS levels following UVA exposure. Any ROS generated by photoactivation of TiO_2 may also play a part in this increase (Carlotti et al., 2009). When sunscreen A was spread onto all the skin samples, an increase in TBARS was observed with respect to the non-irradiated control which was significant for pig ear skin and human SCE membranes, but not for pig SCE membranes. The increase observed is likely due to the photo-oxidation of the sunscreen as observed on the glass plates. Furthermore, any photo-products generated upon UVA exposure of the sunscreen may interact with the lipid components of the skin samples which could also contribute to increasing the TBARS levels. However, the TBARS assay cannot discriminate between

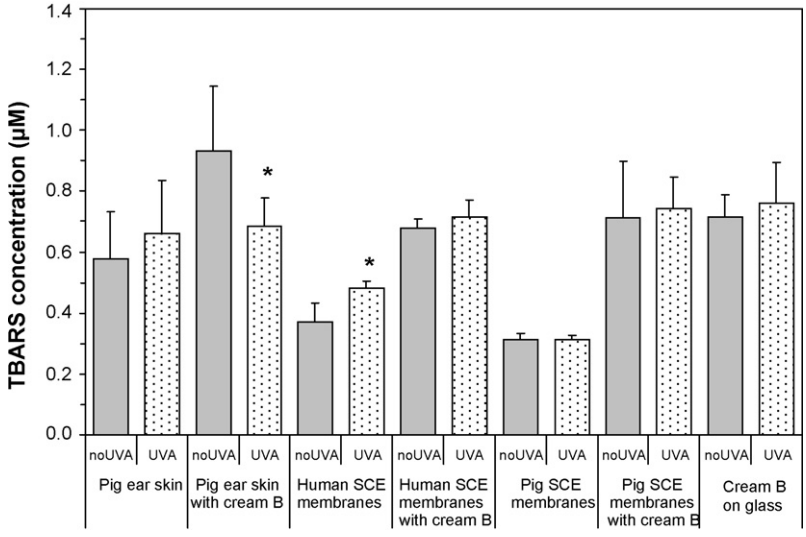

Fig. 4. TBARS levels obtained from the different skin samples before and after UVA exposure and in the presence or absence of sunscreen B. For comparison, TBARS levels obtained from sunscreen B spread onto glass plates before and after UVA exposure, are also reported. See Section 2 for experimental details. $^{*}p < 0.05$ versus respective unexposed control.

break-down products of lipid peroxidation deriving from the sunscreen and those deriving from the skin samples. Despite this, of note is the fact that increased TBARS can be detected from UVA + sunscreen exposed skin when a photounstable sunscreen is used, despite the fact that all sunscreens nowadays contain antioxidants. This is undesirable because not only is the photoprotective efficiency of the sunscreen reduced, but also photo-induced lipid peroxidation in the sunscreen may lead to potentially toxic breakdown products which remain on the skin for as long as the sunscreen is present. At worst, they may interact with skin components and/or with other co-formulated sunscreen ingredients.

In contrast, the results reported in Fig. 4 for sunscreen B are in line with its UV absorbance spectral behaviour: no increase in TBARS levels are detected either when spread onto glass or on skin/SCE samples. Indeed, when spread onto pig ear skin exposed to UVA, a significant decrease in TBARS was observed. Furthermore, for exposed human SCE membranes, a significant increase in TBARS was observed which was reduced when the same SCE membranes were exposed to UVA with cream B. This is the ideal behaviour that all sunscreens should possess when spread onto skin and exposed to UVA.

When full-thickness pig ear skin was exposed to UVA in the absence of sunscreen, no significant increase in TBARS was observed, and this is the main trend detected even in the other skin samples studied (Figs. 3 and 4). This does not imply that skin is not damaged by UVA exposure, but rather, that the assay used might not be sufficiently sensitive to detect small amounts of breakdown products of skin lipid peroxidation. In Fig. 4, however, a significant increase in human SCE membranes was noted, differently from the results on the human SCE membranes reported in Fig. 3. As mentioned earlier, the human SCE samples were from two different individuals, hence it is likely that they respond differently when exposed to UVA. Worthy of mention is also the fact that the basal TBARS levels in full-thickness pig ear skin are higher than those detected from human and pig SCE membranes. This is expected since full thickness skin consists of stratum corneum, viable epidermis and dermis, thus basal lipid levels are expected to be higher, compared to SCE membranes which comprise the epidermis and the stratum corneum (Huong et al., 2009).

4. Conclusion

Overall, from the comparative study presented and discussed above on the photostability assessment of sunscreens exposed to UVA spread onto glass plates or on skin, the following points can be highlighted. First, the results indicate that sunscreen performance consequent to UVA exposure, appears to be independent of whether it is spread onto a non-biological and chemically inert substrate such as glass, or on biological substrates such as skin/SCE membranes. Stokes and Diffey also assessed the photostability of 4 sunscreen products, in terms of SPF, by applying them to excised human epidermis or quartz plates. Although they found significant differences between the results obtained on the two substrates (but not between application thickness, 1 or 2 mg/cm^2, or whether the UV source was natural sunlight or a solar simulator), they concluded that since the ranking of the four products in terms of their photostability was the same for both substrates, either quartz plates or a human epidermis substrate can be used successfully to compare the photostabilities of different sunscreen products (Stokes and Diffey, 1999). This conclusion is therefore in strong agreement with the findings of the present study. Second, the results show that despite the same SPF, sunscreen performance and photostability can be very different. Having the same SPF does not necessarily imply the same absorbance profile and the same level of protection. Our findings are in agreement with those of Lejeune et al. who determined the biological damage after standard daily ultraviolet radiation in human reconstructed skin (assessed by histology, vimentin immunostaining for dermal fibroblasts and analysis of matrix metalloprease-1 secretion) in the presence of two commercial sunscreens with same SPF of 15 but different transmission profiles over the UVA range. The product with higher absorbance profile over the UVA range leads to less skin alterations (Lejeune et al., 2008). This underlines the importance of assessing both spectral profile and photostability of sunscreen products prior to marketing in order to guarantee broad-spectrum protection for consumers. Thirdly, the data obtained on human SCE membranes are similar to those on pig SCE membranes, indicating the suitability of these latter membranes as a model for human skin. In fact, pig skin is accepted and validated as a model by the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) because of the remarkable correlation between human and pig ear skin data (Huong et al., 2009), and the findings reported here further reinforce this. Because of the limited availability of human SCE membranes, pig SCE membranes may be considered a suitable alternative for photostability testing. However, since the results obtained from sunscreens spread onto skin membranes, akin to the more realistic conditions of use in vivo, do not substantially differ from those obtained on glass plates, these latter may be used for rapid, inexpensive, efficacy screening of photostability of sunscreen products. We believe that photostability testing should be a mandatory requirement for safer sunscreen protection products, since our results, in agreement with those of others, clearly show that some are still far from perfect.

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