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Development of a new *in vitro* method to evaluate the photoprotective sunscreen activity of plant extracts against high UV-B radiation

Mélanie Hupel*, Nathalie Poupart, Erwan Ar Gall

Laboratoire d'Ecophysiologie et de Biotechnologies des Halophytes et des Algues Marines (EA 3877 LEBHAM), Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest Iroise, Rue Dumont D'Urville, 29280 Plouzané, Brittany, France

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ABSTRACT

Sunscreen efficiency of biomolecules against UV-B radiation was generally determined *in vitro* by cosmetic methods which are not well-adapted for routine ecophysiological and bio-guidance studies in plant research laboratories. In this article, we propose a new *in vitro* method to evaluate the sunscreen photoprotective activity of plant extracts against high UV-B radiation. Because photosynthetic pigments are one of the first targets of UV-B radiation in plants, the experimental design is based on the ability of the tested substances to limit the degradation of sodium magnesium chlorophyllin (SMC), a derivative compound of natural chlorophyll. SMC photodegradation comparatively to natural chlorophyll and related to temperature, concentration and sample solvent were analyzed in order to optimize the experimental parameters. Then, the method was validated by testing nine standard UV filters used in the European cosmetic industry and by comparing results of their activity with those of a reference *in vitro* procedure. Finally, the method was applied to coastal and marine crude plant extracts. Results have shown that our procedure can be a good alternative to cosmetic methods with a rapid, sensitive and reproducible evaluation of the sunscreen activity of either pure standards or crude plant extracts in small amounts (30 mg).

1. Introduction

Sun radiation constantly impacts the earth with approximately 10% of ultraviolet radiation (UV-R), which is usually divided into three wavelength ranges: UV-C (200–280 nm), stopped in the stratosphere, UV-B (280–315 nm) and UV-A (315–400 nm)[1]. In the last decades, UV-B radiation reached the planet surface with higher intensities, as a consequence of the ozone layer depletion in the upper atmosphere [1,2]. Because of its energetic properties, UV-B radiation is well-known to be the most harmful portion of UV radiation in the biosphere and to induce adverse effects on various organisms [1]. In humans, UV-B radiation induces damages on the human skin, including erythema (or sunburn), cutaneous photoageing and induction of cancers [3,4]. To reduce or prevent the deleterious effects of ultraviolet radiation, different photoprotective measures can be taken, like complete avoidance of sun exposure, seeking shade when disease-inducing wavelengths are relatively intense, wearing protective clothing and using topical sunscreens [4,5]. In plants, UV-B radiation has been shown to damage cell components, including DNA, proteins, lipids and pigments [6,7], to affect physiological processes [8,9] and to impact structure of communities [10]. To protect against UV-B radiation, plants have developed various photoprotective mechanisms such as the production of high levels of antioxidants [6,11] and the synthesis of UV-absorbing compounds [12–14].

The Sun Protection Factor (SPF), which appears as a numeric label on suncare products, is the universal indicator of the photoprotective performance of sunscreens against UV-B radiation. It is generally determined *in vivo* in a group of volunteers by the ratio of the least amount of ultraviolet energy required to produce a minimal erythema on a sunscreen protected skin to the amount of energy required to produce the same erythema on an unprotected skin [5]. In this way, SPF indicates the ability of a sunscreen product to reduce UV-induced erythema. Actually, the US Food and Drug Administration method [15] and the European COLIPA (Comité de Liaison des Industries de la Parfumerie) method [16] are the most widely used *in vivo* procedures for its measurement. However, *in vitro* testing methods have also been developed because they are more rapid, less expensive and above all because they prevent the involvement of human volunteers with the related ethical problems. These methods consist either in the measurement of the spectral transmittance of the sunscreen substance incorporated in a cream emulsion and applied on a synthetic support simulating the human skin [17–22] or in spectrophotometric analysis of dilute solutions of sunscreen products [23,24]. The SPF was determined by mathematical equations after running UV spectra

* Corresponding author. Tel.: +33 2 98 49 88 67; fax: +33 2 98 49 87 72.
E-mail address: melanie.hupel@aliceadsl.fr (M. Hupel).

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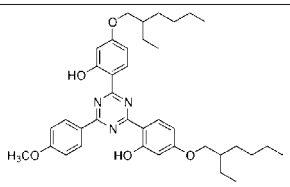
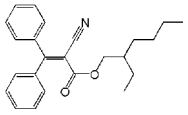
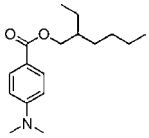
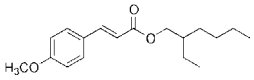
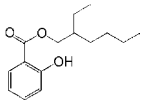
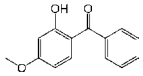
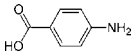
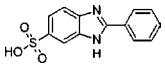
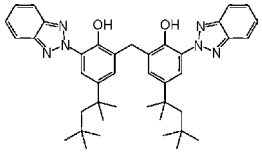
Table 1
Characteristics of the UV-B filters used in this study.

INCI name	Abbreviation	Solubility	Chemical structure
Bis-ethylhexyloxyphenol methoxyphenyl triazine (TinosorbS®)	TS	Ethyl acetate	
Octocrylene	OC	Ethanol	
Octyldimethyl para-aminobenzoic acid	OP	Ethanol	
Octylmethoxycinnamate	OM	Ethanol	
Octylsalicylate	OS	Ethanol	
Oxybenzone	OX	Ethanol	
Para-aminobenzoic acid	PABA	Ethanol	
Phenyl benzimidazole sulfonic acid	PBS	Water pH 7	
Methylene bis-benzotriazolyl tetramethylbutylphenol (TinosorbM®)	TM	Water	

of the sunscreen absorbing film or solution in the spectral limits conventionally accepted by photobiologists and dermatologists for SPF determinations, *i.e.* from 290 to 400 nm [16]. In addition, the photostability of the sunscreen could be evaluated by the values of its SPF before and after an UV irradiation in different solar simulation systems [17,19,21]. Both the reproducibility and accuracy of these procedures in the determination of both the SPF and the photostability of either a single sunscreen substance or a more complex cosmetic formulation can be influenced by several parameters including (i) the type of irradiation system and the target support [25,26]; (ii) the amount of product tested as well as both the uniformity and the thickness of the obtained absorbing film [27,28]; (iii) the components of the cream emulsion in which the sunscreens were incorporated [29]; (iv) the interactive effects between sunscreen substances in the case of testing complex formulations [30,31].

Various natural substances extracted from plants could be potential sunscreen resources because of their ultraviolet ray absorption [12–14] and/or their antioxidant power [11]. Determination of the antioxidant activity of plant extracts is well documented, but the evaluation of their sunscreen capacity is usually limited to both UV spectrum characteristics and concentration of UV-absorbing compounds [12], even though some alternative approaches have been previously investigated [11,32–34]. Marine and coastal plants, *i.e.* seaweeds and angiosperms, are potentially rich sources for original biomolecules, especially antioxidant and sunscreen compounds [35–39]. Indeed, by the strong variations of their environmental conditions (light, salinity, water status, temperature), these plants needed to develop efficient photoprotective mechanisms to survive. More precisely, they are likely to be more or less sensitive to UV radiation in function of (i) the duration of the emersion periods when they live in the intertidal zone; (ii) the degree of their desiccation conditions when they are not regularly inundated by tidal waters but subject to occasional splashes and sprays of salt water from waves. Among known antioxidants and UV-absorbing compounds appear carotenoids and phenols in angiosperms [37–39], phlorotannins in brown seaweeds [36,40] and mycosporine-like amino acids in red seaweeds [12,35].

To better understand both ecological and physiological responses of plants against UV-B radiation and to promote natural UV-absorbing compounds in the cosmetic industry, a new *in vitro* method has been developed for both screening and evaluating the photoprotective activity of plant extracts. Because photosynthetic pigments are the first target of UV-B radiation in plants, the experimental design is based on the ability of the tested substances to absorb UV-B radiation and to limit the degradation of sodium

magnesium chlorophyllin (SMC), a derivative compound of natural chlorophyll (Chl). This novel method has been firstly optimized and validated by testing standard UV filters used in suncare products and then, has been applied to marine and coastal plant extracts.

2. Materials and methods

2.1. Chemicals

Chlorophyll (composed of 90% Chl *a* and 10% Chl *b*) and Sodium Magnesium Chlorophyllin (SMC) were purchased from Carlo Erba (Milan, Italy). SMC corresponds to molecules of chlorophylls in which the phytyl tail has been removed for water solubility. Nine UV-B filters authorized in the European Union were used for the experiments and their characteristics are presented in Table 1. Octocrylene, octyldimethyl para-aminobenzoic acid, octylmethoxycinnamate, octylsalicylate, oxybenzone, para-aminobenzoic acid and phenyl benzimidazole sulfonic acid were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). Bis-ethylhexyloxyphenol methoxyphenyl triazine (TinosorbS®) and methylene bis-benzotriazolyl tetramethylbutylphenol (TinosorbM®) were obtained from Ciba (BASF, Levallois-Perret, France).

2.2. Plant samples

Three coastal angiosperms were chosen as representative species of different environmental conditions: *Salicornia ramosissima* Woods (Chenopodiaceae, salt marsh), *Matricaria maritima* (L.) (Asteraceae, sand dune) and *Crithmum maritimum* (L.) (Apiaceae, sea cliffs). In addition, three marine seaweed species were selected in function of both their taxonomic position and of their known specific active compounds: *Pelvetia canaliculata* (L.) Decaisne & Thuret (Fucaceae, brown seaweed, phlorotannins), *Porphyra dioica* Brodie & Irvine (Bangiaceae, red seaweed, mycosporine-like amino acids) and *Enteromorpha compressa* (L.) Nees (Ulvaceae, green seaweed). Plants were harvested in coastal sites of North Brittany (France), in summer 2009. Erect parts of freshly collected samples were rapidly cut off, washed with deionized water and freeze-dried. The dry material was ground to yield a fine powder and 5 g were extracted 3 h in the dark with 100 mL of methanol.

2.3. Experimental set-up

The experimental design (Fig. 1) was composed of an irradiation chamber (UV-crosslinker Bio-Link, Vilber Lourmat, France) supplied with six UV-B 312 nm lamps (8 W) and a specific disposal for the exposure of samples.

Each tested sample solution was transferred into a macro UV plastic cuvet (Brand, Germany) which was sealed with a cap. These UV plastic cuvets were chosen to allow the simultaneous testing of large numbers and various types of samples because (i) they are less expensive and fragile than quartz cuvets; (ii) they have a good chemical compatibility with most of the organic solvents. Each UV cuvet containing either SMC or chlorophyll (0.2 g L^{-1} in deionized water and ethyl acetate, respectively) was placed beneath that containing the corresponding tested sample, to constitute one experimental unit. Six experimental units were prepared for each product to be tested and placed in a specific disposal following always the same scheme (Fig. 1), aiming at taking into consideration a possible heterogeneity of irradiation in the chamber. In addition, they were physically kept apart from each other by opaque separators to allow a perfect vertical irradiation and to avoid any lateral diffusion of UV radiation which could affect target pigments.

The total UV irradiance (290–400 nm) was maintained at 60 W m^{-2} (approximately 40 W m^{-2} UV-B (290–320 nm) and

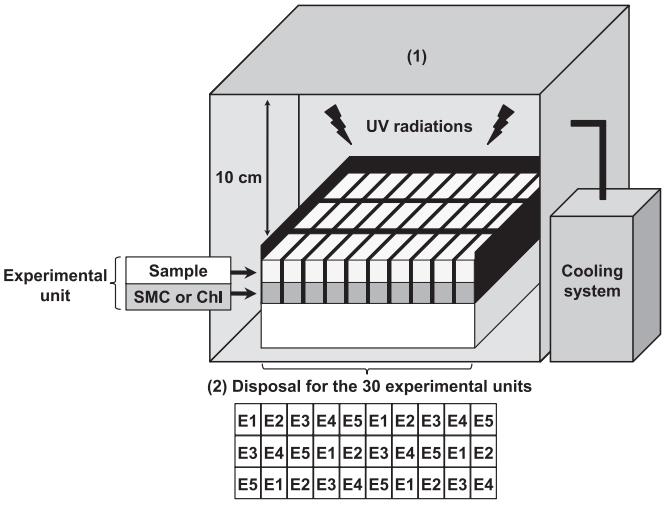


Fig. 1. Scheme of the experimental set-up made up of (1) an irradiation chamber providing 60 W m^{-2} of UV radiations and equipped with an external cooling system (2) a specific disposal for the exposure of 30 experimental units, placed at 10 cm from UV-B lamps thanks to a support (white). The experimental unit was composed of a UV cuvet containing the tested sample placed above a UV cuvet containing either sodium magnesium chlorophyllin or chlorophyll. The typical set location of the experimental units in their disposal is also represented. SMC: sodium magnesium chlorophyllin; Chl: chlorophyll; E1: experimental unit corresponding to tested sample no. 1.

20 W m^{-2} UV-A (320–400 nm)) by placing the experimental units at 10 cm below the surface of UV-B lamps. Spectral irradiance measurements were carried out with a UV-X radiometer (UVP, USA) and the spectral energy distribution of UV-B lamps is presented in Fig. 2. A UV irradiance of 60 W m^{-2} was chosen because (i) it induced an intermediate degradation of SMC in the chamber when no photoprotective compound was used; (ii) it was in the range of the recommended intensities for the UV light source in the COLIPA test method [16,17]. However, the *in vivo* COLIPA method recommends a UV-A/UV-B ratio between 8 and 22 to limit the risk of either excessive skin burning or heating during UV irradiation of human volunteers [16]. This ratio was also applied to *in vitro* tests to get results conforming to SPF [17]. In our method, we have chosen a light source with a UV-A/UV-B ratio of 0.5 to evaluate the photoprotective activity of samples against high UV-B radiation. The duration of exposure was fixed at 2 h (except for kinetics studies), corresponding to the time generally recommended to users between

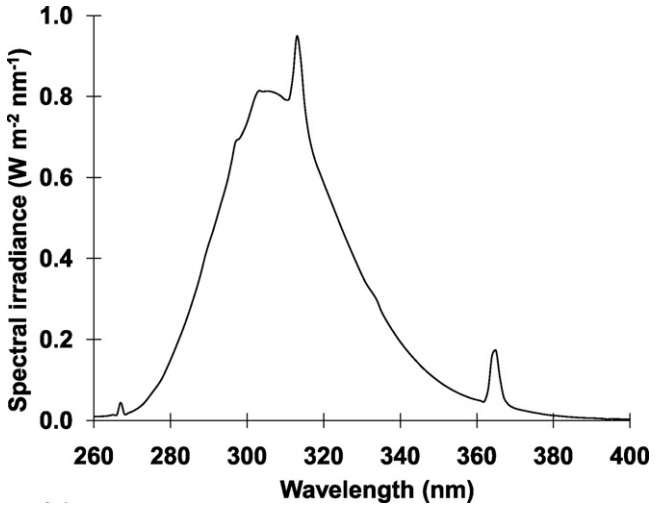


Fig. 2. Spectral energy distribution of the 312 nm lamps.

two successive applications of protective cream to the skin, *i.e.* the minimal period required for both efficiency and photostability of sunscreens.

The irradiation chamber was equipped with an external cooling system to maintain the temperature of the samples below 40 °C as recommended by the COLIPA guideline [17]. In our conditions, measured temperatures at the surface of the experimental units were 28 ± 2 °C during irradiation.

2.4. Photodegradation of target pigments

Previous studies have shown that UV-B radiation induced a strong decrease in both red (600–700 nm) and blue (400–500 nm) absorption bands of chlorophyll solutions [41]. Preliminary tests have shown the same tendency in our UV irradiation conditions for both SMC and chlorophyll (Fig. 3). The absorption maximum in the red band was chosen as a sensible indicator of the photodegradation of both target pigments because it corresponds to chlorophylls only, related to the absorption in the blue band which is also characteristic of carotenoids in plants. So, the photodegradation of both target pigment solutions was evaluated by measuring their absorbance before and after UV-B irradiation at their absorption maximum in the red band, *i.e.* 663 nm for chlorophyll and 650 nm for SMC. Absorbance and spectrum measurements of both SMC and chlorophyll were realized with a Biochrom Libra S12 UV–visible spectrophotometer.

2.5. Determination of optimal experimental conditions

In a first time, photodegradation curves of both SMC and chlorophyll were compared to determine whether responses were homologous or not against UV-B radiation and to justify therefore the use of a synthetic compound instead of the natural one. For these tests, experimental units were composed of an empty cuvet placed above another one containing either SMC or chlorophyll at 0.2 g L^{-1}.

In a second time, photodegradation analyses were performed with different combined parameters to check both the accuracy and the reproducibility of the method: irradiation (presence or absence by covering cuvets with aluminum foil), temperature (irradiation with or without the cooling system), presence of a reference UV-B filter (octylmethoxycinnamate at 0.1 g L^{-1} in ethanol), solvent effect (water, ethanol, methanol and ethyl acetate) and SMC concentration (0.1–0.7 g L^{-1}).

For all these analyses, six experimental units were always placed in the disposal at the same locations and irradiated by 60 W m^{-2} UV radiation during 6 h with an hourly assessment of the absorbance of target pigments.

2.6. Testing UV-B filters and plant extracts

Each UV-B filter or plant extract was dissolved in an appropriated solvent at four different concentrations (0.01 g L^{-1}, 0.1 g L^{-1},1 g L^{-1} and 10 g L^{-1}), transferred into UV cuvets, which were then placed above others containing SMC. Experimental units were irradiated during 2 h with the experimental conditions defined in Section 2.5. The protective effect of the tested samples was evaluated by the resulting kinetic parameters of SMC degradation. In parallel, the UV spectrum (200–400 nm) of UV-B filters and plant extracts was monitored before and after 2 h of irradiation. Then, different parameters were calculated after normalization of areas defined under the extinction curve to the range of wavelengths involved:

- UV-B_{120}/UV-B_0 ratio to evaluate the photostability of samples, with UV-B_0 and UV-B_{120} the total absorption between 290 and 320 nm before and after irradiation. A ratio close to 1 means high stability.
- UV-B/UV-A ratio that defines the potential sunscreen performance of the sample in the UV-B range (290–320 nm) in relation to its performance in the UV-A range (320–400 nm). A ratio lower than 1, equal to 1 or higher than 1 suggests preferential protection against only UV-A radiation, both UV-A and UV-B radiation and only UV-B radiation, respectively.
- Critical wavelength (λ_c), which is the wavelength between 290 and 400 nm below which 90% of the cumulative area of the absorbance curve resides. A sunscreen with a critical wavelength of 370 nm or greater denotes balanced protection throughout both the UV-B and UV-A ranges [17].

2.7. Statistical analyses

Statistical analyses were performed using the Statgraphics Centurion XV software. Distributional normality of data was evaluated using the Shapiro–Wilk test and homogeneity of variances with a Cochran's test followed by a transformation of data when it was necessary. Regression analyses, one-way ANOVA (followed by a Fisher's least significant difference test (LSD)), Student's *t*-tests, Mann–Whitney tests and Pearson correlations were carried out at a 95% confidence level.

3. Results and discussion

3.1. Photodegradation of target pigments

Statistical analyses have shown that the photodegradation of both SMC and chlorophyll followed apparent first order kinetics (Fig. 4) and could be described by an exponential regression ($r^2 > 0.99$) with the equation:

$$A_{(t)} = A_{(o)} \cdot e^{-kt}$$

where $A_{(o)}$ and $A_{(t)}$ refer to the absorbance of either SMC or chlorophyll before and after UV-B exposure, k is the degradation rate constant and t is the irradiation time (min).

Exponential degradation of chlorophyll under UV-B radiation was in agreement with previous studies [41–43]. Variations of chlorophyll and SMC absorbance under UV-B radiation were strongly correlated (Pearson, $r = 0.97$; $p < 0.001$), whereas the loss in absorbance was twice faster for chlorophyll than for SMC ($k = 0.014$ and 0.007, respectively). These differences in the photodegradation rate might be explained by a higher chemical stability of SMC against UV-B radiation due to its modified structure. However, SMC was retained as target molecule of UV-B radiation because its higher hydrosolubility made easier the preparation of the experimental units and results have shown that its chemical structure, spectroscopic properties and photodegradation curve were close to those of chlorophyll.

To evaluate the protective effect of samples, the half-life time of SMC ($t_{50\%}$), which is the time necessary for a 50% decrease of initial absorbance, could be calculated as follows:

$$t_{50\%} = \frac{\ln(2)}{k}$$

3.2. Accuracy of the experimental set-up

3.2.1. Effect of temperature

SMC photodegradation curves related to temperature were obtained after 6 h of UV irradiation and overall data were tested by regression analyses ($r^2 > 0.99$) (Fig. 5). The degradation rate constants (k) were calculated from data at $t = 0$ and $t = 120$ min and compared by Student's *t*-tests. The effect of temperature on the

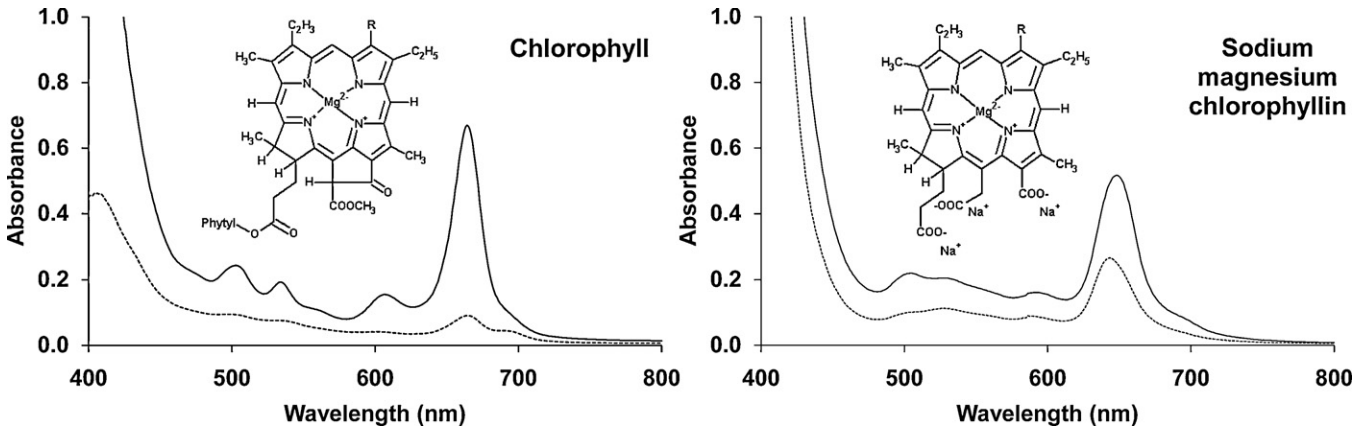


Fig. 3. Chemical structure of both chlorophyll and SMC with associated visible spectra before (bold line) and after (dotted line) 2 h of UV irradiation (60 W m^{-2}).

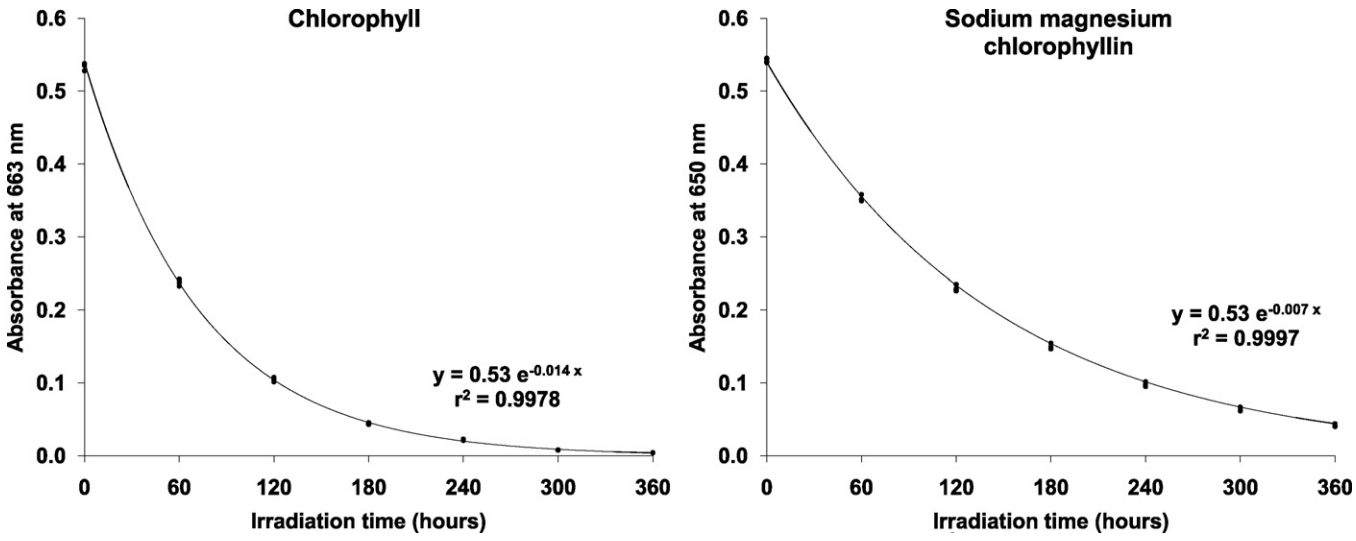


Fig. 4. Photodegradation kinetics of both chlorophyll and sodium magnesium chlorophyllin (0.2 g L^{-1}) obtained after 6 h of UV radiation (60 W m^{-2}) without any photoprotective compound. For each target pigment, six experimental units were tested and all values of absorbance were plotted on the graph. In addition, mean tendencies curves obtained after regression analyses at a 95% confidence level were indicated for each target pigment.

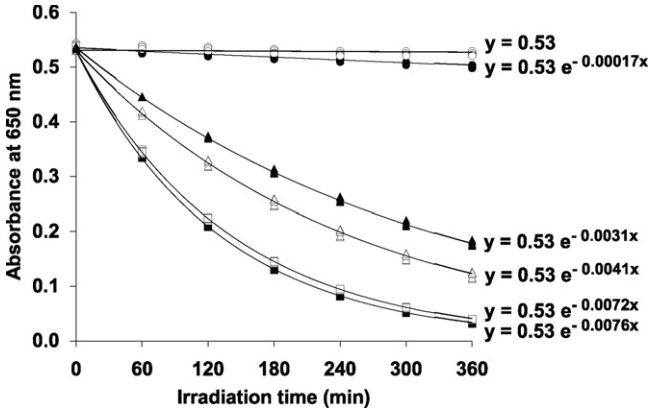


Fig. 5. Influence of irradiation, temperature and UV-B filter (OM at 0.1 g L^{-1}) on the photodegradation rate of SMC at 0.2 g L^{-1}. (□) Irradiation (60 W m^{-2}) + cooling system (standard conditions); (■) irradiation + no cooling system (temperature above 40 °C); (○) no irradiation (cuvettes covered with aluminum foil) + cooling system; (●) no irradiation + no cooling system; (△) irradiation + UV-B filter + cooling system; (▲) irradiation + UV-B filter + no cooling system. For each condition, six experimental units placed in the middle of the disposal were tested and all values were plotted on the graph. In addition, mean tendencies curves obtained after regression analyses at a 95% confidence level were indicated for each condition.

degradation rate of SMC was tested by covering or not cuvets with aluminum foil, to avoid UV irradiation, and by using or not the external cooling system. With the presence of the external cooling system (*ca.* 30 °C), results showed no degradation of non-irradiated SMC ($A_{650\,nm} = 0.53$; $k = 0$) compared to irradiated SMC ($k = 0.0072 \pm 0.0001$) (Fig. 5). The same experiment was realized by removing the external cooling system (*ca.* 40 °C) and statistical analyses pointed out significant differences in k constants for both irradiated ($k = 0.0072 \pm 0.0001$ with cooling system *vs.* $k = 0.0076 \pm 0.0002$ without cooling system; Student's t-test, $p < 0.001$) and non-irradiated samples ($k = 0$ with cooling system *vs.* $k = 0.00017 \pm 0.00001$ without cooling system; Mann–Whitney, $p = 0.003$). These observations suggested that a temperature of 40 °C had an additive effect on the degradation of SMC and justified the use of the external cooling system for the experiments (Fig. 5). Another irradiation experiment was performed with a UV-B filter (OM at 0.1 g L^{-1}) above the SMC cuvet. Results have shown strong statistical differences between tests in either the presence ($k = 0.0031 \pm 0.0001$) or the absence ($k = 0.0041 \pm 0.0001$) of the external cooling system (Student's t-test, $p < 0.001$). So, the temperature could have an influence on the degradation of both SMC and UV-B filter, which underlines the interest of an external cooling system.

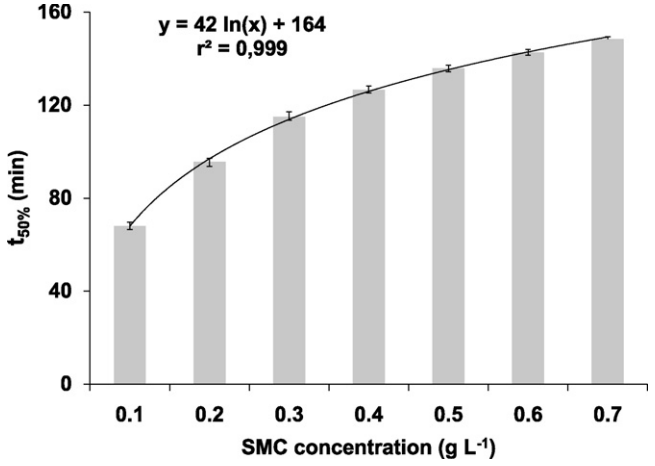


Fig. 6. Influence of SMC concentration on the half-life time of SMC ($t_{50\%}$) after 2 h of UV irradiation (60 W m^{-2}). Each bar is the mean ± standard deviation (n = 6) and regression curve between $t_{50\%}$ and SMC concentration was added.

3.2.2. Effect of SMC concentration

The influence of SMC concentration was tested from 0.1 g L^{-1} ($A_{650\,nm\,(0)}$ = 0.2) to 0.7 g L^{-1} ($A_{650\,nm\,(0)}$ = 2.0) (Fig. 6). Photodegradation curves were obtained after 6 h of UV irradiation for each concentration (exponential regression, r^2 > 0.99, data not shown) and the half-life time of SMC ($t_{50\%)}$ was calculated from overall data. Regression analysis has pointed out a logarithmic relation (r^2 = 0.999) between the concentration of SMC and its $t_{50\%}$. As irradiation time was fixed at 2 h, we have chosen a SMC concentration for which $t_{50\%}$ was less than 2 h, *i.e.* in the range of 0.1–0.3 g L^{-1}, to appreciate noticeably the photoprotective effect of samples. So, a mean SMC concentration at 0.2 g L^{-1} was used for the method, corresponding to an average $t_{50\%}$ of 95 min.

After these initial tests, the standard conditions retained in our method was a 2-h UV irradiation at 60 W m^{-2}, a temperature below 40 °C (cooling system) and a concentration of SMC at 0.2 g L^{-1}.

3.2.3. Effect of solvent

Some solvents absorb UV wavelengths and can interfere with ultraviolet absorbance of sunscreens [44]. For this reason, the solvents used to dissolve UV-B filters and plant extracts were tested for their potential effects on the $t_{50\%}$ of SMC (Fig. 7). ANOVA analysis demonstrated an effect of solvent (p < 0.001) for ethanol, methanol and ethyl acetate in comparison with control condition (empty cuvet). No effect of water was detected whereas alcoholic solvents and ethyl acetate induced a 20 min and a 15 min increase of the $t_{50\%}$, respectively.

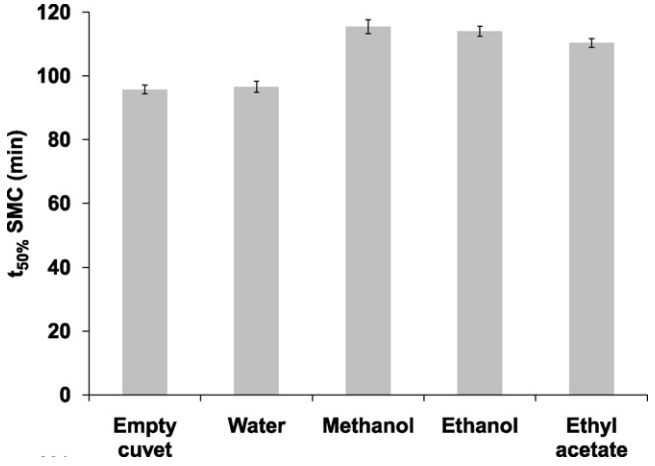


Fig. 7. Influence of solvent on the half-life time of SMC ($t_{50\%}$) after 2 h of UV irradiation (60 W m^{-2}). Each bar is the mean ± standard deviation (n = 6) and samples sharing a common letter are not significantly different (ANOVA, Fisher's LSD test, p = 0.05).

Consequently, we recommend two steps for the evaluation of the photoprotective activity in our method: a first 2 h UV-B irradiation with only solvents (at the future location of samples in the disposal), followed by a 2-h UV-B irradiation with the tested samples (compound + solvent). Then, the effective photoprotective activity of potentially photoprotective compounds can be expressed as the additional half-life time of SMC occurring without any influence of the corresponding solvent ($\Delta t_{50\%}$):

$$\Delta t_{50\%} = t_{50\%\,\text{sample}} - t_{50\%\,\text{solvent}}$$

where $t_{50\%\,\text{sample}}$ is the half-life time of SMC with sample and $t_{50\%\,\text{solvent}}$ the half-life time of SMC with the solvent only.

Following our method, the $\Delta t_{50\%}$ may be considered as a useful index to classify the tested compounds in two categories: those which allow an increase of the initial half-life time of SMC of more than 2 h ($\Delta t_{50\%}$ > 120 min; good photoprotection) *versus* those which allow an increase of less than 2 h ($\Delta t_{50\%}$ < 120 min; poor photoprotection).

3.3. UV-B filters

First, the photostability and photoprotective activity of UV-B filters authorized in the European cosmetic industry were tested to check the adequacy of our method with data obtained from other procedures. Whereas a large number of studies describe the performances of commercial sunscreen formulations with various proportions of combined filters [*e.g.* 21, 24], few data are available on both the photostability and the SPF of single sunscreens. However, some researchers in pharmaceutics (Faculty of Pharmacy, Nantes, France) have obtained data for single sunscreens with an *in vitro* method based on transmission measurements through creams containing UV filters and applied onto PMMA plates [19,20,30,31]. The light source used for the photostability analyses of the filters provides 50 W m^{-2} of UV radiation with a UV-A/UV-B ratio of 15. This procedure was optimized following the recommendations given by the reference COLIPA *in vitro* method [17] and was chosen for comparison with our results.

3.3.1. Spectral properties and photostability

Analyses of initial UV-B/UV-A ratios have shown a predictive preferential protection against (i) only UV-B radiation (290–320 nm) for PBS (15.22), PABA (10.02), OP (3.92), OS (3.81), OM (3.02) and OC (1.72); (ii) both UV-B and UV-A radiation (290–400 nm) for OX (1.14); (iii) only UV-A radiation (320–400 nm) for TS (0.54) and TM (0.36) (Table 2). Moreover, both UV absorption maxima (λ_{max}) and initial critical wavelength (λ_{c0}) pointed out a potential protection in both UV-B and UV-A for TM (λ_{max} = 306 and 348 nm; λ_c = 385 nm) and TS (λ_{max} = 310 and 340 nm; λ_c = 364 nm) as well as partially in UV-A for OC (λ_c = 343 nm). The protection in UV-A for TS, TM, OC and OX was confirmed by a stability of the critical wavelength after 2 h of UV irradiation. A significant decrease of the UV-B/UV-A ratio was observed for PABA, OP and TS after irradiation (Student's t-test of paired samples, p < 0.05), suggesting a more important loss of absorption in the UV-B domain than in the UV-A domain, whereas it seemed to be the opposite for OS, OC and OM (Table 2).

In addition to its spectral properties, a filter has to be photostable during 2 h for its use in suncare products. Calculations of UV-B_{120}/UV-B_0 ratios have shown a total stability of TM (1.0) and PBS (0.99), a good stability for OC (0.91), OX (0.88), TS (0.87) and PABA (0.84), a poor stability for OM (0.56) and OP (0.65)

Table 2
Spectral properties (UV absorption maxima (λ_{max}) as well as critical wavelength (λ_c) and UV-B/UV-A ratio before and after 120 min of irradiation) and photostability (UV-B_{120}/UV-B_0 ratio) of UV-B filters tested in this study. For each calculated ratio, values of samples (mean ± standard deviation; $n = 6$) sharing a common letter are not significantly different at 95% confidence level (ANOVA, Fisher's LSD test). For each sample, the occurrence of an asterisk (*) denotes a significant difference between UV-B/UV-A ratios before and after irradiation at a 95% confidence level (Student's *t*-test of paired samples).

Sample	λ_{max}	λ_{c0}	λ_{c120}	$(UV\text{-}B/UV\text{-}A)_0$	$(UV\text{-}B/UV\text{-}A)_{120}$	UV-B_{120}/UV-B_0
OS	307	324	324	3.81 ± 0.02^{d}	$5.19 \pm 0.03^{c\,*}$	0.10 ± 0.00^{h}
PBS	302	317	317	15.22 ± 0.02^{a}	15.22 ± 0.02^{a}	0.99 ± 0.01^{b}
PABA	290	316	316	10.02 ± 0.03^{b}	$8.68 \pm 0.02^{b*}$	0.84 ± 0.01^{e}
OP	310	325	325	3.92 ± 0.02^{c}	$3.56 \pm 0.02^{d*}$	0.65 ± 0.01^{f}
OC	303	343	343	1.74 ± 0.02^{f}	$1.78 \pm 0.03^{f*}$	0.91 ± 0.00^{c}
OX	287/325	346	346	1.14 ± 0.01^{g}	1.14 ± 0.01^{g}	0.88 ± 0.01^{d}
OM	308	329	329	3.02 ± 0.03^{e}	$3.08 \pm 0.02^{e*}$	0.57 ± 0.01^{g}
TS	310/340	364	364	0.57 ± 0.01^{h}	$0.54 \pm 0.02^{h*}$	0.87 ± 0.02^{d}
TM	306/348	385	385	0.36 ± 0.01^{i}	0.36 ± 0.01^{i}	1.01 ± 0.01^{a}

and a very poor stability for OS (0.1) (Table 2). Our results were globally in agreement with half-life time values of filters as calculated in the comparative method, which had demonstrated a total stability for PABA (7500 min), TM (1700 min), OX (1500 min) and PBS (1390 min), a high stability for OC (515 min), a relative poor stability for OM (225 min) and TS (155 min) and a very poor stability for OS and OP (85 min) [19]. In addition, sunscreen formulations containing OM are known to be photolabile, because of cis–trans isomerization under UV irradiation which altered protection parameters, whereas those which incorporate TS, OC or OX are photostable [19,21,45]. However, slight differences can be observed in the photostability of filters between our method and the comparative procedure, that could be due to the spectral emission of the UV light source with different proportions of UV-B and UV-A radiation. Indeed, our method pointed out that increasing the UV-B/UV-A ratio of the light source seemed to have a negative effect on the photostability of PABA and OX and a positive effect on the photostability of TS, related to the comparative method [19]. For further investigations, we have chosen to precise the UV photostability criterion: a tested sample is highly stable with a UV-B_{120}/UV-B_0 ratio ≥ 0.9, poorly stable with a $0.5 \leq$ UV-B_{120}/UV-B_0 ratio < 0.9 and totally unstable with a UV-B_{120}/UV-B_0 ratio < 0.5.

3.3.2. Photoprotective activity

Values of SPF obtained by the comparative method for the UV-B filters used in this study are mentioned in Table 3. At maximum concentrations authorized in the European Union, the following order of activity could be given if we consider the minimum measured SPF: TS (20) > OM (11) > OC–OP (8) > PBS (5) > TM–PABA–OX (3) > OS (2). This order is based on concentrations up to 10 times higher than the maximum tested in our method (TS, OM, OC, TM and OX at 10%; PBS at 8%; PABA and OS at 5%). Our results have shown an effect of the nature of the filter on their photoprotective activity ($\Delta t_{50\%}$ of SMC) for all tested concentrations (ANOVA; $p < 0.05$), which underlines the discriminative power of our method. However, the photoprotective activity was strongly dependent on the concentration of the sample (Table 3). The order of activity of UV-B filters was almost the same at both 1 and 10 g L^{-1} (TM > TS > OM > PBS > PABA ≥ OS ≥ OC–OX > OP). At 0.01 g L^{-1}, TS was more active than TM, whereas OX, OC, OP and PABA were more active than PBS. At 0.1 g L^{-1}, OX, OC and OS presented a higher sunscreen activity than PABA.

For all the UV-B filters, a logarithmic relation seemed to link their concentration to the $\Delta\ t_{50\%}$ ($r = 0.99$ for all the filters) with variable *a* and *b* parameters (Table 3). This observation supposed differences in the efficient concentration (EC) of sunscreen required for minimal protection of SMC ($\Delta t_{50\%} = 120$ min) and a possible loss of sensitivity for the discrimination of the activity of filters at high tested concentrations (10 g L^{-1}). In fact, the progressive loss in the specific activity of a filter with the increase of concentration could be explained by (i) the structure and absorption properties of the sunscreen which are more implicated than the concentration in the photoprotective activity; (ii) an increase of interactive effects between the sunscreen molecules in UV irradiated solutions, which limits progressively their photoprotective activity. Calculation of the efficient concentration of UV-B filter for minimal protection of SMC (EC_{120}) from their respective logarithmic equations, as determined by our method, made possible to order their activity: TS (<0.010 g L^{-1}) > TM (0.011 g L^{-1}) > OM (0.019 g L^{-1}) > OX (0.052 g L^{-1}) > OC (0.064 g L^{-1}) > PBS (0.070 g L^{-1}) > PABA (0.122 g L^{-1}) > OS (0.147 g L^{-1}) > OP (0.400 g L^{-1}). For TS, the efficient concentration cannot be exactly calculated because the $\Delta t_{50\%} > 120$ min at 0.01 g L^{-1}. It appeared that an efficient concentration of 0.15 g L^{-1} was globally necessary to detect a significant activity ($\Delta t_{50\%} = 120$ min) for all the UV-B filters (Table 3). Except for OX, OP and TM, our method has described the same order of activity than the reference method chosen for comparison. The high activity obtained for TM could be explained by its large molecular size and its opaque white color which probably results in both scattering and reflection, in addition to its UV absorbing capacity. OX seemed to be more efficient against higher UV-B/UV-A ratios than in the comparative method because it absorbed strongly in the UV-B band. On the other hand, OP could be more affected by our conditions because of its poor stability. Moreover, filters were tested for sunscreen activity after incorporation in a cream emulsion in the reference procedure, whereas they were in solution and physically separated from SMC in our method. So, differences in the sunscreen activity of filters observed between our method and the reference procedure might be also explained by a matrix effect of cream emulsion. The photoprotective activity of the UV-B filters has also to be relativized by their photostability (TS–TM–OX–OC–PBS > OM–PABA–OP > OS), which placed OS as the least photostable sunscreen and OM as a poorly stable compound, despite of its high activity.

The erythema induced by sunlight UV in unprotected human skin is mainly generated by wavelengths between 295 and 320 nm, with a maximum effectiveness around 308 nm [16]. For this reason, the use of a light source concentrated on UV-B wavelengths, as in our method, must allow a good evaluation of both photoprotective activity and photostability of sunscreens. However, when a filter predominantly protects in the UV-B wavelengths, the erythemal effect by UV-A wavelengths can become important and *vice versa* [16]. Consequently, it could be useful to test both high UV-B/UV-A ratios (65% UV-B and 35% UV-A in our method) and natural high UV-A/UV-B ratio (*i.e.* approximately 90–95% UV-A and 5–10% UV-B radiations in the reference COLIPA method), to better evaluate both the photoprotective activity and the photostability of sunscreens against a large range of UV conditions.

Table 3
Results of $\Delta t_{50\%}$ (min) of sodium magnesium chlorophyllin obtained after 2 h of UV irradiation for UV-B filters at each tested concentration (mean ± standard deviation; $n = 6$). For each sample, the efficient concentration ($g\,L^{-1}$) required for $\Delta t_{50\%} = 120$ min (EC_{120}) was determined from equations linking the photoprotective activity ($\Delta t_{50\%}$) to the tested concentration (regression analyses; $r = 0.99$). Literature data on sunscreen performances of UV-B filters (SPF range) at maximum authorized concentrations in the European Union are noticed (values in brackets) [19,20,30,31]. At a given concentration, values of $\Delta t_{50\%}$ sharing a common letter are not significantly different at a 95% confidence level (ANOVA, Fisher's LSD test).

Sample	Concentration ($g\,L^{-1}$)				Correlation of $\Delta t_{50\%}$ (y) with concentration (x)	EC_{120} ($g\,L^{-1}$)	SPF range
	0.01	0.1	1	10			
OS	15 ± 3^{i}	120 ± 6^{f}	183 ± 7^{f}	273 ± 8^{e}	$y = 189.8 + 36.4 \ln x$	0.147	2–3 (5%)
PBS	24 ± 3^{h}	149 ± 6^{d}	244 ± 10^{d}	332 ± 12^{d}	$y = 237.5 + 44.1 \ln x$	0.070	5–14 (8%)
PABA	30 ± 3^{g}	112 ± 5^{g}	200 ± 11^{e}	274 ± 14^{e}	$y = 195.0 + 35.6 \ln x$	0.122	3–14 (5%)
OP	52 ± 7^{f}	87 ± 5^{h}	137 ± 5^{g}	184 ± 7^{g}	$y = 137.4 + 19.0 \ln x$	0.400	8–11 (8%)
OC	70 ± 7^{e}	140 ± 6^{e}	185 ± 9^{f}	233 ± 12^{f}	$y = 183.8 + 23.2 \ln x$	0.064	8–15 (10%)
OX	80 ± 5^{d}	142 ± 5^{e}	180 ± 6^{f}	244 ± 10^{f}	$y = 188.2 + 23.1 \ln x$	0.052	3–6 (10%)
OM	91 ± 7^{c}	185 ± 7^{c}	274 ± 7^{c}	353 ± 10^{c}	$y = 269.2 + 37.9 \ln x$	0.019	11–14 (10%)
TS	134 ± 5^{a}	417 ± 6^{b}	569 ± 9^{b}	682 ± 16^{b}	$y = 593.9 + 93.9 \ln x$	<0.010	20–37 (10%)
TM	122 ± 5^{b}	522 ± 7^{a}	971 ± 7^{a}	1472 ± 15^{a}	$y = 997.0 + 195.5 \ln x$	0.011	3–7 (10%)

3.4. Plant extracts

3.4.1. Spectral properties and photostability

Analyses of initial UV-B/UV-A ratios have shown a predictive preferential protection against UV-A radiations (320–400 nm) for all methanol plant extracts (ratios < 1) (Table 4). The initial critical wavelength (λ_{c0}) was close to 370 nm for all tested samples and pointed out a potential balanced protection throughout both the UV-B and UV-A ranges. Values of UV absorption maxima (λ_{max}) were in agreement with this result for the three angiosperm plant extracts (290 and 330 nm for *Salicornia ramosissima* (SR); 302 and 325 nm for both *Crithmum maritimum* (CM) and *Matricaria maritima* (MM)), as well as for both extracts of the red seaweed *Porphyra dioica* (PD) and the green seaweed *Enteromorpha compressa* (EC) (280 and 334 nm) (Table 4). The extract of the brown seaweed *Pelvetia canaliculata* (PC) absorbed strongly in UV-C but weakly in both UV-B and UV-A (λ_{max} = 220 and 270 nm). After 2 h of irradiation, no variation of both the UV-B/UV-A ratio (Student's t-test of paired samples, $p > 0.05$) and the critical wavelength was observed for the angiosperms and EC, suggesting no loss in their ability to protect against both UV-A and UV-B. However, calculations of UV-B_{120}/UV-B_0 ratios have shown a poor stability for all of these extracts: EC (0.50), SR (0.67), CM (0.66) and MM (0.73) (Table 4). These results let suppose that some molecules, absorbing between 290 and 400 nm, were degraded during the exposure without modifying the global spectral profile of the corresponding extracts. A decrease in the critical wavelength was detected for PD and PC extracts after irradiation, associated with a strong increase of the UV-B/UV-A ratio for PC (0.49 ± 0.01 at $t = 0$ min and 0.86 ± 0.02 at $t = 120$ min; Student's t-test of paired samples, $p < 0.001$) and a slight decrease of the UV-B/UV-A ratio for PD (0.40 ± 0.01 at $t = 0$ min and 0.37 ± 0.01 at $t = 120$ min) (Table 4). This result suggested that the UV absorption spectra of these seaweed extracts were modified after UV irradiation. For PD extract, it seems that an important degradation of both UV-A absorbing molecules (λ_{c0} = 380 nm and λ_{c120} = 360 nm) and UV-B absorbing molecules (UV-B_{120}/UV-B_0 = 0.60) occurred, with a slight modification of the spectral profile. On the other hand, modifications observed for PC were due to a strong increase in the UV-B absorption domain (UV-B_{120}/UV-B_0 = 4.21), opposed to a strong decrease in the UV-C absorption domain (data not shown). These data suggest that the initial pool of UV-C absorbing compounds found in the PC extract could be modified by UV-B radiation and form new molecules able to absorb UV-B radiation.

These spectral properties results let suppose the presence of polyphenolic compounds in the plant extracts tested as (i) flavonoids and phenol acids in SR, CM and MM because of both the absorption between 300 and 400 nm and previous studies confirming the occurrence of high contents in these species [37–39]; (ii) phlorotannins in PC, by a similar UV absorption profile as the phloroglucinol standard [40]. The maximal absorption at 334 nm measured for EC could correspond to the production of phenols, as already measured in different species of the genus *Enteromorpha* [46]. Moreover, the presence of mycosporine like-amino acids can be suspected in both PD and EC, since porphyra 334 and shinorine, which both absorb at 334 nm, occur in high amounts in *Porphyra* species [12,35] and have already been found in Ulvaceae [35].

3.4.2. Photoprotective activity

While a large number of studies exists on the determination of the photoprotective activity of various cosmetic formulations, few data are available on methods applied to plant extracts [11,32–34]. In our study, we have applied our method to marine and coastal plants because their extracts can contain high amounts of UV photoprotective compounds, produced in response to their extreme environmental conditions.

Results showed that a concentration of 0.01 $g\,L^{-1}$ was not sensitive enough to detect any activity in all plant extracts (data not shown), as well as a concentration of 0.1 $g\,L^{-1}$ for PD and EC (Table 5). As in the case of UV-B filters, a logarithmic relation seems to link the concentration of extract to the $\Delta t_{50\%}$ ($r = 0.99$)

Table 4
Spectral properties (UV absorption maxima (λ_{max}), as well as both critical wavelength (λ_c) and UV-B/UV-A ratio before and after 120 min of irradiation) and photostability (UV-B_{120}/UV-B_0 ratio) of plant extracts tested in this study. For each sample, values of the different ratios (mean ± standard deviation; $n = 6$) sharing a common letter are not significantly different at 95% confidence level (ANOVA, Fisher's LSD test). For each sample, the occurrence of an asterisk (*) denotes a significant difference between UV-B/UV-A ratios before and after irradiation at a 95% confidence level (Student's t-test of paired samples). PC: *Pelvetia canaliculata*; PD: *Porphyra dioica*; EC: *Enteromorpha compressa*; SR: *Salicornia ramosissima*; CM: *Crithmum maritimum*; MM: *Matricaria maritima*.

Sample	λ_{max}	$\lambda_{c\,0}$	$\lambda_{c\,120}$	$(UV\text{-}B/UV\text{-}A)_0$	$(UV\text{-}B/UV\text{-}A)_{120}$	UV-B_{120}/UV-B_0
PC	220/270	390	381	0.49 ± 0.01^{d}	0.86 ± 0.02^{a} *	4.21 ± 0.03^{a}
PD	280/334	380	360	0.40 ± 0.01^{f}	0.37 ± 0.01^{f} *	0.60 ± 0.03^{d}
EC	280/334	390	390	0.47 ± 0.01^{e}	0.47 ± 0.01^{e}	0.50 ± 0.02^{e}
SR	290/330	375	375	0.53 ± 0.01^{c}	0.53 ± 0.01^{d}	0.67 ± 0.01^{c}
CM	302/325	365	365	0.71 ± 0.01^{b}	0.71 ± 0.01^{c}	0.66 ± 0.03^{c}
MM	302/325	365	365	0.73 ± 0.01^{a}	0.73 ± 0.01^{b}	0.77 ± 0.02^{b}

Table 5
Results of $\Delta t_{50\%}$ (min) of sodium magnesium chlorophyllin obtained after 2 h of UV irradiation for plant extracts at each tested concentration (mean ± standard deviation; $n = 6$). For each sample, the efficient concentration ($g\,L^{-1}$) required for $\Delta t_{50\%} = 120$ min (EC_{120}) was firstly determined from equations linking the photoprotective activity ($\Delta t_{50\%}$) to all tested concentrations (regression analyses; $r = 0.99$) ($EC_{120\,(1)}$) and then from results obtained only at 0.1 $g\,L^{-1}$ and 1.0 $g\,L^{-1}$ ($EC_{120\,(2)}$). At a given concentration, values of $\Delta t_{50\%}$ sharing a common letter are not significantly different at a 95% confidence level (ANOVA, Fisher's LSD test). PC: *Pelvetia canaliculata*; PD: *Porphyra dioica*; EC: *Enteromorpha compressa*; SR: *Salicornia ramosissima*; CM: *Crithmum maritimum*; MM: *Matricaria marítima*.

Sample	Concentration ($g\,L^{-1}$)			Correlation of $\Delta t_{50\%}$ (y) with concentration (x)	$EC_{120\,(1)}$ ($g\,L^{-1}$)	$EC_{120\,(2)}$ ($g\,L^{-1}$)
	0.1	1	10			
PC	85 ± 7[a]	481 ± 7[b]	965 ± 8[a]	$y = 511.8 + 191.0 \ln x$	0.13	0.127 ± 0.002[b]
PD	*	175 ± 9[e]	878 ± 7[c]	–	–	–
EC	*	54 ± 6[f]	656 ± 8[e]	–	–	–
SR	38 ± 6[c]	337 ± 7[d]	597 ± 6[f]	$y = 323.6 + 121.4 \ln x$	0.19	0.186 ± 0.006[d]
CM	68 ± 5[b]	438 ± 7[c]	813 ± 7[d]	$y = 439.7 + 161.9 \ln x$	0.14	0.139 ± 0.004[c]
MM	86 ± 6[a]	494 ± 7[a]	922 ± 7[b]	$y = 501.0 + 181.5 \ln x$	0.12	0.121 ± 0.004[a]

* No activity detected.

for PC, SR, CM and MM between 0.1 $g\,L^{-1}$ and 10 $g\,L^{-1}$. Like for UV-B filters as well, increasing concentrations of these plant samples decrease their sunscreen specific activity, which could be explained by a negative effect of possible intermolecular interactions on the sunscreen efficiency. For EC and PD, it seems that this logarithmic relation would be only adequate for concentrations between 1 $g\,L^{-1}$ and 10 $g\,L^{-1}$. This observation underlines the fact that active compounds are potentially present in EC and PD but need to be more concentrated to be efficient. The order of activity ($\Delta t_{50\%}$) was globally PC–MM > CM > SR > PD ≥ EC at both 0.1 $g\,L^{-1}$ and 1 $g\,L^{-1}$, whereas PD was more active than CM and EC was more active than SR at 10 $g\,L^{-1}$ (Table 5).

The aim of this study was to test only crude extracts to see if our method was sensitive to screen rapidly the photoprotective status of a plant and to propose a procedure to classify their sunscreen activity. Among the extensive literature on active plant extracts, it appears that concentrations between 0.01 and 10 $g\,L^{-1}$ are generally used for testing their biological activity, as realized in our study. Moreover, a compound is considered as highly active when its required concentration for minimal activity is the lowest in the ranges of tested concentrations. So, we can consider that the methanol extract of EC was totally inactive ($\Delta t_{50\%} = 54$ min at 1 $g\,L^{-1}$), whereas PD seemed to have a medium photoprotective activity, with $\Delta t_{50\%} > 120$ min at 1 $g\,L^{-1}$ ($\Delta t_{50\%} = 170$ min) (Table 5). For the other extracts, calculation of the efficient concentration required for a minimal protection of SMC (EC_{120}) were based on logarithmic equations obtained from overall data ($EC_{120\,(1)}$). In addition, efficient concentrations were calculated only from data obtained at 0.1 $g\,L^{-1}$ and 1 $g\,L^{-1}$ ($EC_{120\,(2)}$) because (i) a logarithmic relation always links the concentration of active crude extracts to the corresponding $\Delta t_{50\%}$ between these two concentrations ($\Delta t_{50\%} = b + a \ln(\text{concentration})$); (ii) these concentrations are in the range of the concentrations generally used for the screening of biological activity of plant extracts; (iii) a concentration of 10 $g\,L^{-1}$ requires large amount of samples and seems few sensitive in our method. The equation used for calculation of $EC_{120\,(2)}$ was:

$$EC_{120(2)} = e^{(120-b)/a}$$

where b corresponds to the $\Delta t_{50\%}$ obtained for a given plant extract at 1 $g\,L^{-1}$ and a was obtained after resolution of the logarithmic equation with a $\Delta t_{50\%}$ obtained for the same plant extract at 0.1 $g\,L^{-1}$.

Calculation of the efficient concentration with this equation was only applicable if both an activity was detected at 0.1 $g\,L^{-1}$ and $\Delta t_{50\%} > 120$ min at 1 $g\,L^{-1}$. Moreover, UV-B irradiation has to be realized in three steps (SMC + solvent, SMC + sample at 0.1 $g\,L^{-1}$ and SMC + sample at 1 $g\,L^{-1}$) with tested samples at always the same location in the experimental disposal.

Calculations of $EC_{120\,(1)}$ and $EC_{120\,(2)}$ have shown similar results and have evidenced the same order of activity: MM > PC > CM > SR (Table 5). All these plant extracts denoted a significant high photoprotective activity which corresponds to approximately 10–20 times less activity than a pure molecule like TS (<0.01 $g\,L^{-1}$), the most active UV-B filter in our study. If we correlate these results with those obtained on the photostability ($UV\text{-}B_{120}/UV\text{-}B_0$), we can propose the following order for the photoprotective activity of the tested marine and coastal plant extracts: MM > PC > CM > SR > PD > EC. The UV-B absorption of the PC extract was strongly modified during the exposure ($UV\text{-}B_{120}/UV\text{-}B_0 = 4.21$) and it would be interesting to test the new formed extract for both its photostability and photoprotective activity. The high sunscreen activity found in methanol crude extracts of PC, MM, CM and SR could be due to the occurrence of phenol compounds, whereas the potential activity found for PD could be due to mycosporine like-amino acids, which would be better extracted in other conditions. However, these assumptions need to be confirmed by further investigations, especially by testing purified extracts with the method used in this study.

So, we propose the following procedure to classify the activity of crude plant extracts:

(1) If $\Delta t_{50\%} > 120$ min at 0.1 $g\,L^{-1}$, the plant extract is very highly photoprotective and it is not necessary to test a concentration of 1 $g\,L^{-1}$.
(2) If $\Delta t_{50\%}$ at 0.1 $g\,L^{-1} < 120$ min $< \Delta t_{50\%}$ at 1 $g\,L^{-1}$, the plant extract is highly photoprotective and EC_{120} can be calculated from data at 0.1 and 1 $g\,L^{-1}$ to classify the activity of extracts (PC, SR, CM and MM).
(3) If no activity is detected at 0.1 $g\,L^{-1}$ but $\Delta t_{50\%} > 120$ min at 1 $g\,L^{-1}$, the plant extract is potentially photoprotective (PD).
(4) If no activity is detected at 0.1 $g\,L^{-1}$ and $\Delta t_{50\%} < 120$ min at 1 $g\,L^{-1}$, the plant extract is not active (EC).

This procedure could be also applied to both purified extracts and pure UV-B filters, but it seems more accurate to test firstly a concentration of 0.01 $g\,L^{-1}$ which could be sufficient to evaluate the activity of some isolated compounds (Table 3). Thereby, we have determined that an amount of only 30 mg was enough to test both pure compounds and crude extracts with our method.

Finally, the classification of the photoprotective activity of the samples has to be adjusted with the UV photostability criterion: a tested sample is highly stable with a $UV\text{-}B_{120}/UV\text{-}B_0$ ratio ≥ 0.9, poorly stable with a $0.5 \leq UV\text{-}B_{120}/UV\text{-}B_0$ ratio <0.9 and totally unstable with a $UV\text{-}B_{120}/UV\text{-}B_0$ ratio <0.5.

4. Conclusion

The aim of this study was to develop a new *in vitro* method for screening and evaluating rapidly the sunscreen photoprotective activity of small amounts of plant extracts against high UV-B radiation. In plants, chlorophyll molecules localized in internal tissues are one of the first targets of UV-B radiation and UV absorbing compounds can be produced in epidermal cells to limit their photodegradation. The hierarchical vertical location of protecting epidermal compounds above sensitive photosynthetic pigments in plants has served as model for the development of our experimental design. In our method, sodium magnesium chlorophyllin (SMC) was chosen as UV-B target because (i) it was hydrosoluble and was used easily; (ii) its spectral properties and photodegradation kinetics were close to natural chlorophyll. The photodegradation kinetic of SMC was evaluated under the effect of several factors (UV-B intensity, temperature, concentration, sample solvent) to both optimize the experimental parameters and establish a recommended procedure for the use of our method. Then, validation of the method was realized by testing standard sunscreens used in the European cosmetic industry. Comparisons of the results obtained by an *in vitro* procedure close to the COLIPA reference method have shown differences in both the photostability and photoprotective activity of some filters, suggesting that irradiation with high UV-B/UV-A ratios could give supplementary information on the real photoprotective potential of a sample. In addition, extracts of marine and coastal plants were tested because (i) these plants live in extreme conditions and their extracts were likely to be highly photoprotective; (ii) UV-B absorbing compounds have already been found in their tissues; (iii) results would allow to validate the method for extracts of both seaweeds and higher plants. We have chosen to realize methanol extracts because this solvent is usually used in the screening of biological activities in plants, for its ability to extract a large number of various polar and relatively apolar compounds. In seaweeds, results have shown that the methanol extract of the brown species *Pelvetia canaliculata* was highly active related to a low photoprotective activity in the red species *Porphyra dioica* and no activity in the green species *Enteromorpha compressa.* On the contrary, all extracts of angiosperms (*Salicornia ramosissima*, *Crithmum maritimum* and *Matricaria maritima*) seemed highly active. However, the potential sunscreen activity and the nature of the photoprotective compounds in these marine plants have to be confirmed by testing purified methanol extracts and/or other solvent systems.

In summary, the developed method was successful to evaluate rapidly, sensitively and at a lower cost the sunscreen activity of small amounts of either pure or crude samples (30 mg) against high UV-B radiation. It could be applied for both promoting natural UV-absorbing compounds in the cosmetic industry, as well as in ecological and physiological studies for evaluating the photoprotective responses of plants against UV-B radiation. Further investigations would be interesting by testing the activity of the samples under other UV conditions, with both our experimental design and reference procedures, to extend the scope of our method. Finally, we have to notice that sunscreens evidenced with this new method have to be supplementary tested for their phototoxicity on human cells and their protective effect against UV indirect damages, as free radical generation, before to be potentially used in suncare products [47].

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References

[1] N.D. Paul, D. Gwynn-Jones, Trends Ecol. Evol. 18 (1) (2003) 48–55.
[2] F.R. de Gruijl, J.C. van der Leun, CMAJ 163 (7) (2000) 851–855.
[3] Y. Matsumura, H.N. Ananthaswamy, Toxicol. Appl. Pharmacol. 195 (2004) 298–308.
[4] European Union, Off. J. Eur. Union L 265 (2006) 39–43.
[5] S. Lautenschlager, H.C. Wulf, M.R. Pittelkow, Lancet 370 (2007) 528–537.
[6] F. Hollósy, Micron 33 (2002) 179–197.
[7] A. Holzinger, C. Lütz, Micron 37 (2006) 190–207.
[8] L.A. Franklin, R.M. Forster, Eur. J. Phycol. 32 (3) (1997) 207–232.
[9] M.M. Caldwell, L.O. Björn, J.F. Bornman, S.D. Flint, G. Kulandaivelu, A.H. Teramura, M. Tevini, J. Photochem. Photobiol. B: Biol. 46 (1998) 40–52.
[10] K. Bischof, I. Gómez, M. Molis, D. Hanelt, U. Karsten, U. Lüder, M.Y. Roleda, K. Zacher, C. Wiencke, Rev. Environ. Sci. Biotechnol. 5 (2006) 141–166.
[11] F. Bonina, M. Lanza, L. Montenegro, C. Puglisi, A. Tomaino, D. Trombetta, Int. J. Pharm. 145 (1996) 87–91.
[12] C.S. Cockell, J. Knowland, Biol. Rev. 74 (3) (1999) 311–345.
[13] J. Rozema, L.O. Björn, J.F. Bornman, A. Gaberščik, D.-P. Häder, T. Trošt, M. Germ, M. Klisch, A. Gröniger, R.P. Sinha, M. Lebert, Y.-Y. He, R. Buffoni-Hall, N.V.J. de Bakker, J. van de Staaij, B.B. Meijkamp, J. Photochem. Photobiol. B: Biol. 66 (2002) 2–12.
[14] A.E. Solovchenko, M.N. Merzlyak, Russ. J. Plant Physiol. 55 (6) (2008) 719–737.
[15] Food and Drug Administration, Federal Register 64 (1999) 27666–27693.
[16] Comité de Liaison des Industries de la Parfumerie (COLIPA), Guidelines for International Sun Protection Factor Test Method, 2006.
[17] Comité de Liaison des Industries de la Parfumerie (COLIPA), Method for the *in vitro* determination of UVA protection provided by sunscreen products, COLIPA Guideline, 2009.
[18] B.L. Diffey, J. Robson, J. Soc. Cosmet. Chem. 40 (1989) 127–133.
[19] C. Couteau, A. Faure, J. Fortin, E. Paparis, L.J.M. Coiffard, J. Pharm. Biomed. Anal. 44 (2007) 270–273.
[20] C. Couteau, M. Pommier, E. Paparis, L.J.M. Coiffard, Pharmazie 62 (2007) 449–452.
[21] J. Akrman, L. Kubáč, H. Bendová, D. Jírová, K. Kejlova, Int. J. Cosmet. Sci. 31 (2009) 119–129.
[22] M. Pissavini, L. Ferrero, Cosm. Toil. 118 (2003) 63–72.
[23] E.A. Dutra, D.A.G.C. Oliveira, E.R.M. Kedor-Hackmann, M.I.R.M. Santoro, J. Pharm. Sci. 40 (2004) 381–385.
[24] J.S. Azevedo, N.S. Viana Jr., C.D. Vianna Soares, Il Farmaco 54 (1999) 573–578.
[25] R.M. Sayre, J. Stanfield, A.J. Bush, D.L. Lott, Photodermatol. Photoimmunol. Photomed. 17 (2001) 278–283.
[26] S. Scalia, M. Mezzena, A. Bianchi, Int. J. Cosmet. Sci. 32 (2010) 55–64.
[27] U. Heinrich, H. Tronnier, D. Kockott, R. Kuckuk, H.M. Heise, Int. J. Cosmet. Sci. 26 (2) (2004) 79–89.
[28] S.M. Kim, B.H. Oh, Y.W. Lee, Y.B. Choe, K.J. Ahn, J. Am. Acad. Dermatol. 62 (2010) 218–222.
[29] H. Maier, G. Schauberger, K. Brunnhofer, H. Hönigsmann, J. Invest. Dermatol. 117 (2001) 256–262.
[30] S. Alami-El Boury, C. Couteau, L. Boulande, E. Paparis, L.J.M. Coiffard, Int. J. Pharm. 340 (2007) 1–5.
[31] C. Couteau, R. Chammas, B. Alami-El Boury, E. Choquenet, L.J.M. Paparis, Coiffard, J. Dermatol. Sci. 50 (2008) 159–161.
[32] R. Aquino, S. Morelli, A. Tomaino, M. Pellegrino, A. Saija, L. Grumetto, C. Puglia, D. Ventura, F. Bonina, J. Ethnopharmacol. 79 (2002) 183–191.
[33] J.G. Avila Acevedo, C.M.C. Castañeda, F.J.C. Benitez, D.A. Durán, V.R. Barroso, C.G. Martínez, L.J.L. Muñoz, C.A. Martínez, A. Romo de Vivar, Fitoterapia 76 (2005) 301–309.
[34] P. Khazaeli, M. Mehrabani, Iran J. Pharm. Res. 7 (1) (2008) 5–9.
[35] A. Gröniger, R.P. Sinha, M. Klisch, HäderF D.-P., J. Photochem. Photobiol. B: Biol. 58 (2–3) (2000) 115–122.
[36] D.S. Bhakuni, D.S. Rawat, Bioactive Marine Natural Products, Springer, New York, 2005.
[37] L. Meot-Duros, C. Magne, Plant Physiol. Biochem. 47 (1) (2009) 37–41.
[38] D.L. McKay, J.B. Blumberg, Phytother. Res. 20 (2006) 519–530.
[39] M.H. Rhee, H.-J. Park, J.Y. Cho, J. Med. Plants Res. 3 (2009) 548–555.
[40] M.A. Ragan, K.W. Glombitza, in: F.E. Round, D.J. Chapman (Eds.), Progress in Phycological Research, Biopress Ltd., London, 1986, pp. 130–132.
[41] J. Zvezdanović, T. Cvetić, S. Veljović-Jovanović, D. Markovic, Radiat. Phys. Chem. 78 (2009) 25–32.
[42] P. Cuny, J.-C. Romano, B. Beker, J.-F. Rontani, J. Exp. Mar. Biol. Ecol. 237 (1999) 271–290.
[43] S. Santabarbara, Arch. Biochem. Biophys. 455 (2006) 77–88.
[44] L.E. Agrapidis-paloympis, R.A. Nash, N.A. Shaath, J. Soc. Cosmet. Chem. 38 (1987) 209–221.
[45] B. Herzog, M. Wehrle, K. Quass, Photochem. Photobiol. 85 (2009) 869–878.
[46] K. Ganesan, K. Suresh Kumar, P.V. Subba Rao, Innovat. Food Sci. Emerg. Tech. (2010), doi:10.1016/j.ifset.2010.11.005.
[47] E. Venditti, T. Spadoni, L. Tiano, P. Astolfi, L. Greci, G.P. Littarru, E. Damiani, Free Radic. Biol. Med. 45 (2008) 345–354.