GUIDELINES

Method for in vitro determination of UVA protection, 2011

COLIPA

IN VITRO METHOD FOR THE DETERMINATION OF THE UVA PROTECTION FACTOR AND "CRITICAL WAVELENGTH" VALUES OF SUNSCREEN PRODUCTS

Guideline

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Prepared by the COLIPA *In vitro* UV Protection Method Task Force

INTRODUCTION

Sun protection products have long protected against sunlight-induced erythema with the level of performance indicated by the sun protection factor (SPF). Since the SPF number is influenced primarily by UVB wavelengths, however, it is not necessarily a sufficient indicator of a sunscreen product's protection against UVA exposure.

In recent years, the harmful effects of the UVA wavelengths of sunlight have been more thoroughly established. With this understanding arose the need, not only for sun protection products that were effective against UVA wavelengths, but also for a common test method for measuring UVA protection levels.

In seeking to develop an additional method for the separate assessment of a sunscreen product's ability to protect against UVA exposure, it was agreed that an *in vitro* methodology should be the goal, provided that such an *in vitro* method could be shown to correlate with similar data derived from *in vivo* methods of determining UVA protection in human volunteers. In order to achieve this correlation, it was necessary for the test procedure to also take into account any photo-instability in the tested sunscreen product.

The method described in the following sections arises from considerable technical discussion and practical investigation of the dynamics of measuring UVA protection *in vitro*. It is supported by extensive 'ring testing', which established a meaningful correlation with UVA protection factors (UVA-PF) determined on human volunteers using the previously published *in vivo* 'Persistent Pigment Darkening' (PPD) method.

In their Recommendation of 22nd September 2006, on the efficacy of sunscreen products and the claims made relating thereto (2006/247/EC), the European Commission included a requirement for UVA protection comprising both (a) a UVA-PF of 1/3 the SPF as determined by the *in vivo* PPD method or an equivalent degree of protection obtained by any *in vitro* method and (b) an *in vitro* Critical Wavelength value of greater than 370nm, in order to satisfy requirements for broad-spectrum UVB / UVA protection and associated labeling.

This guideline, therefore, provides detailed guidance regarding the instrumentation and procedures used in the *in vitro* method to derive both UVA-PF and Critical Wavelength values. The information and procedures in the following sections should be strictly observed to ensure correct and accurate determination of UVA protection by these metrics.

OBJECTIVE

The objective of this new guideline is to provide a validated *in vitro* method to determine the UVA protection of sunscreen products. Precise specifications and descriptions, based on experimental data, are given to enable determination, either as a UVA-PF or Critical Wavelength value in a reproducible manner, in any laboratory.

Recommendation on use: This *in vitro* method has been developed and validated primarily for liquid and emulsion-type sun protection products. It may be possible to

use it for some powder-containing products, although this should be validated on a product-by-product basis.

PRINCIPLE

This method provides two metrics describing the UVA protection of a sunscreen. Firstly, the method provides *in vitro* UVA protection factors (UVA-PF) which have been shown to correlate well with *in vivo* UVA-PF values derived from the PPD method (the latter being considered as the *in vivo* reference). Secondly, the method also provides a means of calculating Critical Wavelength values.

The test is based on an assessment of the UV transmittance of a thin film of sunscreen sample spread on a roughened substrate after exposure to a controlled dose of UV radiation from a defined UV source. Due to the current lack of inter-laboratory reproducibility of absolute *in vitro* UV measurements, each set of sunscreen transmission data is adjusted by first converting to absorption data (before and after UV exposure) and then by multiplying by a correction coefficient. This coefficient is determined iteratively from the non-exposed sample's absorbance data to provide a calculated *in vitro* SPF value equal to the labelled (*in vivo*) SPF.

The sunscreen sample is exposed to an irradiation dose proportional to an initial UVA protection factor $UVAPF_0$, calculated from the corrected absorbance data of the non-exposed sample.

Both the final *in vitro* UVA-PF and *in vitro* Critical Wavelength value, λ_c, are calculated from the absorbance data of the UV exposed sample.

DEFINITION OF TERMS

***In vitro* UVA protection factor (UVAPF)**
The absolute UVA protection afforded by a suncare product, calculated from the measured *in vitro* transmittance after irradiation and weighted with the PPD action spectrum and with the "standard" output spectrum of a UVA-filtered solar simulator.

***In vitro* UVA protection factor before UV exposure ($UVAPF_0$)**
The *in vitro* UVA Protection Factor measured before sample UV exposure. It is derived from the transmittance curve of the unexposed sample, weighted with the PPD action spectrum and with the "standard" output spectrum of a UVA-filtered solar simulator, after adjustment to the labelled (*in vivo*) SPF.

***In vitro* Sun Protection Factor ($SPF_{in\ vitro}$)**
The absolute protection performance of a suncare product against erythemally-effective UV radiation, calculated from the measured *in vitro* transmittance and weighted with the erythema action spectrum and with the "standard" output spectrum of a UV solar simulator used for SPF testing.

Action spectrum, E(λ) for erythema or P(λ) for PPD

The relative effect of individual wavelengths or wavebands of an exposure source on a specified biological response. Plots of action spectra show the reciprocal of the dose (D) required to produce photo-biological effects at each wavelength or wave-band. Data of the action spectra used in this method are given in Appendix I.

CIE
Commission Internationale de l'Eclairage.

Mean monochromatic absorbance A_λ
The sunscreen absorbance at wavelength λ is related to the sunscreen transmittance T_λ by:

$$A_\lambda = - \log (T_\lambda)$$

Where transmittance T_λ is the fraction of incident irradiance transmitted by the sun-screen film.

Irradiance
Flux per unit area, unit W m^{-2}, always related to a defined range of wavelengths, for example from 290 to 400nm for UVA + UVB irradiance or from 320 to 400nm for UVA irradiance.

Spectral irradiance I(λ) for SPF testing or PPD testing
Irradiance per unit wavelength, I(λ) , unit W m^{-2} nm.

Critical Wavelength Value
The critical wavelength λ_c value for the test product is defined as that wavelength where the area under the absorbance spectrum for the irradiated product (obtained using the method described above) from 290nm to λ_c is 90% of the integral of the absorbance spectrum from 290nm to 400nm

MATERIALS AND INSTRUMENTATION

Spectrophotometer (specifications)

The wavelength range measured by the spectrophotometer must span the primary waveband of interest, that is, 290 to 400nm with an increment wavelength step of 1nm.

A spectrophotometer which uses monochromatic illumination and in which the transmitted radiation is not measured through a monochromator should employ a fluorescence rejection filter.

The spectrophotometer input optics should be designed for diffuse illumination and / or diffuse collection of the transmitted irradiance through the roughened PMMA substrate, both with and without the sunscreen layer spread on its surface. An integrating sphere is recommended and, when used, smaller fractional port areas compared with total sphere wall area will lead to greater accuracy. In any case, the spatial response should be close to a cosine response (cosine error smaller than ± 5% for incident angles <70°).

To reduce potential variability between readings and to compensate for the lack of uniformity in product layer, it is recommended that the area of each measurement site should be at least 0.5cm².

The wavelength of the spectrophotometer should be accurate to within 1. This should be checked using a mercury spectral standard lamp or a specially-doped filter and a regular xenon lamp.

The ability of a spectrophotometer to measure transmission or protection factors accurately is limited by the sensitivity of the instrument. The minimum required dynamic range is at least 2.2 absorbance units as determined according to Appendix II. The maximum measured absorbance should be within 90% of the dynamic range of the device used. As a consequence, the spectroradiometer must be designed for efficient stray-light rejection (e.g., as provided by a double monochromator).

The lamp that is used as the source in the measurement of transmittance must emit a continuous spectrum of radiation with no peaks over the measurement range of 290-400nm, and its irradiance should be sufficiently low so that photostability of the product is not unduly challenged (e.g., a xenon flash lamp is a convenient solution). The dose of UV delivered during one measurement cycle should, therefore, not exceed 0.2J/cm². For all wavelengths, irradiance must be at least 100 times higher than the so-called “dark irradiance”, measured when the lamp is switched off.

Monitoring of the UV spectrophotometer

The performance of the spectrophotometer must be checked at regular intervals (recommended at least every month) by the measurement of defined reference materials.

A twofold test is recommended:

- Monitoring the efficiency of the instrument using special PMMA standard plates (see Appendix II A, “Use of PMMA standard plates with incorporated UV filters for checking spectrophotometer performance”)

- Checking the wavelength accuracy of the instrument with an approved standard material (the recommended material is holmium perchlorate, see Appendix II B)

UV source for irradiation of the sunscreen sample

The spectral irradiance at the exposure plane of the artificial UV source (used for irradiation of the sunscreen sample) should be as similar as possible to the irradiance at ground level under a standard zenith sun as defined by COLIPA (1994) or DIN 67501 (1999). The UV irradiance must be within the following acceptance limits (measured in the same plane as the sunscreen sample):

Light source specifications	
Total UV irradiance (290 to 400 nm)	50 – 140 W m^{-2}
Irradiance ratio of $UVA_{(320\ to\ 400\ nm)}$ to $UVB_{(290\ to\ 320\ nm)}$	8 - 22

The reference standard sun has a total irradiance of 51.4 to 63.7 W/m^2 (Colipa 1994 / DIN 67501) and a UVA to UVB irradiance ratio of 16.9 to 17.5.

The device should be able to maintain samples below 40°C (preferably by using cooling trays and / or air-conditioning devices).

An example of an appropriate UV source is the long-arc xenon Atlas Suntest™ insolator, type CPS, CPS+or XLS/XLS+, filtered with its original UV short cut-off filter (Ref: 56052388) combined with the “UV Special Glass” filter (Ref: 56052371), providing a VIS+UVA+UVB spectrum. Technical documentation (including information on various modifications and specifications) is available for these insolators from the suppliers, to help the user in clearly identifying instrument models / filters and how to use them. The insolator should provide regulated and stable UV irradiance at a given power setting. Treated PMMA plates should be placed on a non-reflecting surface during UV exposure.

A water-cooled sample tray for effective cooling of the samples is available from ATLAS Material Testing Technology GmbH (Atlas No. 56052389).

Monitoring of the UV source for irradiation

The emission of the UV source must be checked (at least) annually for compliance with the given acceptance limits by a suitably qualified expert. The inspection should be conducted with a spectroradiometer that has been calibrated against an internationally accepted calibration standard (e.g., endorsed by an institution that is certified by the European co-operation for accreditation EA). On an on-going basis, the emission of the UV source must be monitored radiometrically (e.g. with an integrated UV meter) before every session of usage. In parallel, the radiometer(s) and / or UVA cell(s) (employed for measuring and / or adjusting the irradiance of the UV source at sample level and calculating the UV doses), will be calibrated in terms of UVA irradiance (W m^{-2} UVA, 320-400nm) for the same UV source spectrum, according to the Colipa recommendations given in the Guideline "Monitoring of UV light sources" (2007).

Substrate / Plate

The substrate / plate is the material to which the sunscreen product sample is applied. It must be UV-transparent, non-fluorescent (i.e., give no detectable fluorescence when exposed to UVR, as measured with the spectrophotometer), photostable and inert towards all ingredients of the preparations to be tested. Furthermore, to approximate the application of a thin film of sunscreen product to skin topography, the substrate should have a textured upper surface.

For this method, PMMA plates (polymethylmethacrylate) with one side of the substrate roughened, have proven to be satisfactory. The topography of the recommended PMMA plates has been characterized by 6 roughness parameters and the resulting targets and upper / lower limits of these parameters are described in Appendix III.

The size of the substrate should be chosen such that the application area is not less than 16cm^2 and a square shape is preferred.

The qualitative absorption spectrum of an emulsion applied to the roughened side of these plates, depends strongly on the degree and type of roughening and the physical / chemical properties of the base material. Indeed, the degree of roughness will affect the optical properties (scattering / transmission) of the material itself. A means for checking the quality of these prepared plates, therefore, is important.

The recommended PMMA plates have been characterized by coating 20 plates with 15µl of glycerine and measuring transmission levels at three different wavelengths in the range from 290 to 400nm to determine reference values. These values are given for reference and guidance in Appendix III.

METHOD

General procedure

Step 1: *In vitro* transmission measurement of the sunscreen product spread on a PMMA plate, prior to UV irradiation. Acquisition of initial UV spectrum with $A_0(\lambda)$ data.

Step 2: Mathematical adjustment of the initial UV spectrum using coefficient "C" (see calculation below) to achieve an *in vitro* SPF (0% UV dose) equal to the labelled SPF (*in vivo*). $UVAPF_0$ is calculated using $A_0(\lambda)$ and C.

Step 3: A single UV dose D is calculated, proportional to $UVAPF_0$.

Step 4: UV exposure of the same sample as in step 1, according to the calculated UV dose D.

Step 5: *In vitro* transmission measurement of the sunscreen product after UV exposure. Acquisition of second UV spectrum with $A(\lambda)$ data.

Step 6: Mathematical adjustment of the second spectrum (following UV exposure) according to the same C coefficient, previously determined in step 2. Calculation of the *in vitro* UVA protection factor UVA-PF after irradiation using $A(\lambda)$ and C and calculation of the Critical Wavelength value from $A(\lambda)$ data.

Transmission measurements through the untreated plate

It is first necessary to determine the transmission of UV radiation through the reference plate. Prepare a 100% transmission reference sample by spreading a few microliters of glycerine or another appropriate UV-transparent substance on the roughened side of a substrate plate. The dose of glycerine used should be such that the entire surface is completely covered, but with no excess (approximately 15µl for a 50 x 50mm plate).

Sample application

Sunscreen product is applied to the roughened PMMA plate (roughened side uppermost) by weight, at an application rate of 1.3mg/cm² (actual quantity applied to the plate).

The sunscreen should be applied as a large number of small droplets of approximate equal volume, distributed evenly over the whole surface of the plate. A positive-displacement automatic pipette is well-suited to this purpose. To ensure the correct application rate, it is recommended that a method of validating the amount of product applied should be adopted (e.g., weighing the pipette before and after dispensing the product and / or weighing the plate before and immediately after applying the product). It is essential to limit possible product evaporation during the weighing process.

After application (and check-weighing, if employed), the sunscreen product is spread immediately over the whole surface using light strokes with a fingertip “pre-saturated” with the product. Spreading should be completed in a two phase process. First, the product should be distributed over the whole area as quickly as possible (less than 30 seconds) without pressure. Then the sample should be rubbed into the rough surface using pressure. The second phase should take between 20 and 30 seconds.

This treated sample should then be allowed to equilibrate for at least 15 minutes in the dark at ambient temperature to help facilitate formation of a standard stabilised product film.

Transmission measurements through the product-treated plate

The product-treated plate is placed in the light-path of the measurement device’s UV source and a mean value for transmission of UV radiation through the sample (by using monochromatic absorbance data measured on different sub-sites within the plate or by measuring the whole plate) is determined from 290 to 400nm at each 1nm step.

Number of determinations

Each sunscreen sample to be tested should be spread onto at least three PMMA plates. Each plate should be measured at a number of different sites to ensure that a total area of at least 2cm² is measured. The single spot area should exceed 0.5cm².If the spot size is 0.6cm^2, therefore, then at least 4 measurements (on different areas) are required so that the total measurement area exceeds the necessary 2cm².

UV exposure using the UV source

For exposure to a UV source as defined above, the incident irradiance should be measured in the plane of the treated plate surface. If a radiometer is used to make the measurement, the measured irradiance may need to be corrected using a correction factor. The correction factor can be derived from measuring the same source using a calibrated spectroradiometer. A correction factor determined in this way should be added to cell E27 of the Colipa UVA Method Excel calculation spreadsheet.

Sample temperature should be maintained below 40°C. The PMMA substrate plates should be supported firmly by a device which can be easily cooled (e.g., an extruded polysterene block or a similar device). The UV source should be of sufficient size to contain all the PMMA plates and should have a matt, dark background behind each plate to reduce the risk of any back exposure. Ensure that the UV source is not switched off while placing samples under the lamp.

To note, it is important that laboratory personnel working with the UV source must be protected adequately against UV radiation (e.g., by the use of protective glasses, gloves, etc.). No bare skin should be exposed to light from the Xenon source.

If a Suntest ^{TM} is used as an appropriate UV source, place the Suntest case on two 15cm high blocks on the bench and remove its bottom plate. To avoid switching the lamp off during measurements, keep the door closed (to maintain the security switch) and place the mounted samples in the light beam under the Suntest case, using an adjustable stand to ensure that the mounted plates are in the same original plane as the Suntest bottom plate.

Transmission measurements after UV expsosure

It is recommended that, as far as possible, transmission measurements after UV exposure are conducted on exactly the same plate locations as those measured before.

CALCULATIONS

The final UVA-PF and Critical Wavelength values are the mean of values derived from individual plates.

Calculation of the $SPF_{in\ vitro}$ for each plate

$$SPF_{in\ vitro} = \frac{\int_{\lambda=290nm}^{\lambda=400nm} E(\lambda) * I(\lambda) * d\lambda}{\int_{\lambda=290nm}^{\lambda=400nm} E(\lambda) * I(\lambda) * 10^{-A_0(\lambda)} * d\lambda} \quad (1)$$

where:

$E(\lambda)$ = Erythema action spectrum (CIE- 1987) (see Appendix I)

$I(\lambda)$ = Spectral irradiance of the UV source (SSR for SPF testing) (see Appendix I)

$A_0(\lambda)$ = Mean monochromatic absorbance measurements per plate of the test product layer *before* UV exposure

$d\lambda$ = Wavelength step (1 nm)

Calculation of the adjusted in vitro $SPF_{in\ vitro,adj}$ and determination of the coefficient of adjustment "C"

C is the coefficient of adjustment, iteratively determined to adjust the calculated *in vitro* SPF value to the labelled (*in vivo*) SPF value. Ring test data show that when the value of 'C' falls within the range of 0.8 to 1.2, the UVAPF that is calculated is consistent and reliable. Larger or smaller values of 'C' could possibly distort the UV absorbance profile of the test sample and consequently may have an impact on the accuracy of the UVAPF determined. Consequently, it is recommended that the value of 'C' should fall within the range of 0.8 to 1.2. If 'C' falls outside this range, then the

operator may optionally review their product application and spreading procedure, in order to establish whether a modification to their technique might help achieve this target range for the value of 'C'. The amount of product applied to the plate, however, must not be adjusted to bring C within this range

$$SPF_{in\ vitro,adj} = SPF\ label = \frac{\int_{\lambda=290nm}^{\lambda=400nm} E(\lambda) * I(\lambda) * d\lambda}{\int_{\lambda=290nm}^{\lambda=400nm} E(\lambda) * I(\lambda) * 10^{-A_0(\lambda)*C} * d\lambda} \quad (2)$$

Where:
$E(\lambda)$,$I(\lambda)$,$A_0(\lambda)$ and $d\lambda$ are defined in equation (1).

Calculation of $UVAPF_0$

$UVAPF_0$ is calculated for each plate individually.

$$UVAPF_0 = \frac{\int_{\lambda=320nm}^{\lambda=400nm} P(\lambda) * I(\lambda) * d\lambda}{\int_{\lambda=320nm}^{\lambda=400nm} P(\lambda) * I(\lambda) * 10^{-A_0(\lambda)*C} * d\lambda} \quad (3)$$

where:

$P(\lambda)$ = PPD action spectrum (see Appendix I)
$I(\lambda)$ = Spectral irradiance of the UV source (UVA 320-400nm for PPD testing; see Appendix I)
$A_0(\lambda)$ = Mean monochromatic absorbance of the test product layer before UV exposure
C = Coefficient of adjustment previously determined in equation (2)
$d\lambda$ = Wavelength step (1 nm)

Calculation of UVA dose "D" for sample irradiation

The single UVA dose D is derived from the $UVAPF_0$ value. To note, the sample is exposed to full-spectrum UV radiation but the dose used is defined by its UVA content.

$$D = UVAPF_0 \times D_0 \quad J\ cm^{-2} \quad (4)$$

D_0 is the unit UVA dose per unit $UVAPF_0$, to be given by the UV source (which has been determined experimentally to give a good correlation between *in vitro* UVAPF and *in vivo* PPD values). This D_0 value has been optimised using data from a multi-centre Ring Study performed by the Colipa In Vitro UV Methods group and is fixed at **1.2**J cm^{-2} UVA.

Calculation of UVAPF of plates after UV irradiation of the sample

$$\text{UVAPF} = \frac{\int_{\lambda=320nm}^{\lambda=400nm} P(\lambda) * I(\lambda) * d\lambda}{\int_{\lambda=320nm}^{\lambda=400nm} P(\lambda) * I(\lambda) * 10^{-A(\lambda)*C} * d\lambda} \quad (5)$$

Where:
P(λ),I(λ),C and dλ are defined in equation (3).
A(λ) is the mean monochromatic absorbance of the test product layer *after* UV exposure.

The UVAPF of an individual plate is calculated from the mean absorbance value from all individual spots. If the coefficient of variation of absorbance between spots exceeds 50% then the plate should be rejected and a new plate prepared.

Calculation of UVAPF

The UVAPF of the product should be the mean of the UVAPF´s of at least three individual plates.

If the coefficient of variation (CoV) between the UVAPF´s of the individual plates exceeds 20%, then further plates should be measured until this CoV threshold is achieved.

Ratio SPF / UVAPF Calculation

Using the *in vivo* sun protection factor (labelled SPF) and the *in vitro* UVA protection factor UVAPF, calculate the ratio below.

$$\text{Ratio} = \frac{SPF_{label}}{UVAPF}$$

Calculation of Critical Wavelength Value

The Critical Wavelength λ_c value for the test product is defined as that wavelength where the area under the absorbance spectrum for the irradiated product (obtained using the method described above) from 290nm to λ_c is 90% of the integral of the absorbance spectrum from 290nm to 400nm, and is calculated in the following way:

A series of absorbance values (dependent on the wavelength increment) are calculated for each of the three separate plates to which the test product has been applied. Absorbance at each wavelength increment (A_λ) is calculated thus:

$A_\lambda = \log (C_\lambda / P_\lambda)$ (6)

where
$$C_\lambda = \sqrt[n]{(c_\lambda[1] \times c_\lambda[2] \times .... \times c_\lambda[n]}$$
$$P_\lambda = \sqrt[n]{(p_\lambda[1] \times p_\lambda[2] \times .... \times p_\lambda[n]} \quad (7)$$

c_λ[n] = arithmetical mean of transmission measurements taken at measurement point n and at wavelength λ for the reference sample (glycerine-treated roughened PMMA plate)

p_λ[n] = arithmetical mean of transmission measurements taken at measurement point n and at wavelength λ for irradiated, sunscreen product treated sample (roughened PMMA plate)

Critical Wavelength λ_c is calculated for each irradiated plate as follows:

$$\int_{290}^{\lambda c} A_\lambda . d\lambda = 0.9 \int_{290}^{400} A_\lambda . d\lambda \quad (8)$$

The final Critical Wavelength value for each tested sunscreen product is the mean of values recorded for each measured, irradiated, product-treated PMMA plate.

REFERENCE SUNSCREEN

The method should be checked regularly by the use of a reference sunscreen formulation to verify the test procedure and, therefore, reference sunscreen formula S2 (as described in Appendix IV) should be used for this purpose. The test results of the reference S2 UVAPF must lie between the upper and lower limits below (as determined from *in vivo* testing results), or the test is deemed invalid and the test must be repeated. A value of 16 should be used as the S2 *in vivo* SPF value for computation purposes.

Parameter	Lower Limit	Upper Limit
UVA-PF	10.7	14.7

TEST REPORT

The test report on the determination of the UVA protection factor or the Critical Wavelength value of a suncare product should contain at least the following information:

a) Detailed description of the instruments used

b) Detailed information on the substrate (manufacturer, batch code and reference date)

c) Information on the exact quantity applied

d) Individual data of the transmittance measurements for the sample and blank reference

e) Statement of the labeled *in vivo* sun protection factor

f) Constant C

g) UVA dose used

h) Coefficient of calibration of the radiometer versus spectroradiometer

i) Temperature at the plate level during the exposure

j) Critical Wavelength values calculated for each suncare product

k) Statement of the values calculated for the UVAPF, the ratio SPF/UVAPF and statistical data (e.g. number of measurements, standard deviation)

l) Results (mean, standard deviation) obtained for the reference sunscreen S2

REFERENCES

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The European Cosmetic Toiletry and Perfumery Association, COLIPA (2007): Method for the *in vitro* determination of UVA protection provided by sunscreen products, ref 2007a

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APPENDIX I

PPD and erythema action spectra and UVA and UV-SSR spectral irradiances

Wavelength nm	PPD action spectrum	Erythema action spectrum	UV-SSR source $W.m^{-2}nm^{-1}$	UVA radiation source $W.m^{-2}nm^{-1}$
290	-	1.0000E+00	8,7410E-06	-
291	-	1.0000E+00	1,4500E-05	-
292	-	1.0000E+00	2,6589E-05	-
293	-	1.0000E+00	4,5745E-05	-
294	-	1.0000E+00	1,0057E-04	-
295	-	1.0000E+00	2,5894E-04	-
296	-	1.0000E+00	7,0349E-04	-
297	-	1.0000E+00	1,6776E-03	-
298	-	1.0000E+00	3,7268E-03	-
299	-	8.0538E-01	7,9381E-03	-
300	-	6.4863E-01	1,4782E-02	-
301	-	5.2240E-01	2,5136E-02	-
302	-	4.2073E-01	4,1755E-02	-
303	-	3.3884E-01	6,2231E-02	-
304	-	2.7290E-01	8,6903E-02	-
305	-	2.1979E-01	1,2160E-01	-
306	-	1.7701E-01	1,6148E-01	-
307	-	1.4256E-01	1,9892E-01	-
308	-	1.1482E-01	2,4827E-01	-
309	-	9.2470E-02	2,8937E-01	-
310	-	7.4473E-02	3,3580E-01	-
311	-	5.9979E-02	3,8715E-01	-
312	-	4.8306E-02	4,3105E-01	-
313	-	3.8905E-02	4,8842E-01	-
314	-	3.1333E-02	5,1215E-01	-
315	-	2.5235E-02	5,5668E-01	-
316	-	2.0324E-02	5,9568E-01	-
317	-	1.6368E-02	6,2562E-01	-
318	-	1.3183E-02	6,5653E-01	-
319	-	1.0617E-02	6,8787E-01	-
320	1.000E+00	8.5507E-03	7,2362E-01	4,8434E-06
321	9.750E-01	6.8865E-03	7,3706E-01	8,4661E-06
322	9.500E-01	5.5463E-03	7,6771E-01	1,3558E-05
323	9.250E-01	4.4668E-03	7,9546E-01	2,0742E-05
324	9.000E-01	3.5975E-03	7,9867E-01	3,0321E-05
325	8.750E-01	2.8973E-03	8,2900E-01	4,2940E-05
326	8.500E-01	2.3335E-03	8,4352E-01	5.7382E-05

327	8.250E-01	1.8793E-03	8,5593E -01	7,6011E-05
328	8.000E-01	1.5136E-03	8,7912E-01	9,8450E-05
329	7.750E-01	1.4125E-03	8,9506E-01	1,2153E-04
330	7.500E-01	1.3646E-03	9,0097E-01	1,5055E-04
331	7.250E-01	1.3183E-03	9,1609E-01	1,8111E-04
332	7.000E-01	1.2735E-03	9,4342E-01	2,1323E-04
333	6.750E-01	1.2303E-03	9,4442E-01	2,4435E-04
334	6.500E-01	1.1885E-03	9,4315E-01	2,8332E-04
335	6.250E-01	1.1482E-03	9,5706E-01	3,1863E-04
336	6.000E-01	1.1092E-03	9,6627E-01	3,5892E-04
337	5.750E-01	1.0715E-03	9,7713E-01	3,9795E-04
338	5.500E-01	1.0351E-03	9,7698E-01	4,3869E-04
339	5.250E-01	1.0000E-03	9,9667E-01	4,7781E-04
340	5.000E-01	9.6605E-04	9,9387E-01	5,1977E-04
341	4.938E-01	9.3325E-04	1,0069E+00	5,6084E-04
342	4.876E-01	9.0157E-04	1,0118E+00	5,9985E-04
343	4.814E-01	8.7096E-04	1,0114E+00	6,3844E-04
344	4.752E-01	8.4140E-04	1,0214E+00	6,7395E-04
345	4.690E-01	8.1283E-04	1,0251E+00	7,1232E-04
346	4.628E-01	7.8524E-04	1,0328E+00	7,4679E-04
347	4.566E-01	7.5858E-04	1,0344E+00	7,7836E-04
348	4.504E-01	7.3282E-04	1,0395E+00	8,1797E-04
349	4.442E-01	7.0795E-04	1,0269E+00	8,4269E-04
350	4.380E-01	6.8391E-04	1,0454E+00	8,7535E-04
351	4.318E-01	6.6069E-04	1,0419E+00	9,0444E-04
352	4.256E-01	6.3826E-04	1,0398E+00	9,2876E-04
353	4.194E-01	6.1660E-04	1,0392E+00	9,4861E-04
354	4.132E-01	5.9566E-04	1,0428E+00	9,7333E-04
355	4.070E-01	5.7544E-04	1,0457E+00	9,8625E-04
356	4.008E-01	5.5590E-04	1,0353E+00	1,0094E-03
357	3.946E-01	5.3703E-04	1,0393E+00	1,0281E-03
358	3.884E-01	5.1880E-04	1,0266E+00	1,0446E-03
359	3.822E-01	5.0119E-04	1,0353E+00	1,0617E-03
360	3.760E-01	4.8417E-04	1,0371E+00	1,0783E-03
361	3.698E-01	4.6774E-04	1,0254E+00	1,0861E-03
362	3.636E-01	4.5186E-04	1,0230E+00	1,0978E-03
363	3.574E-01	4.3652E-04	1,0162E+00	1,0948E-03
364	3.512E-01	4.2170E-04	9,9840E-01	1,0995E-03
365	3.450E-01	4.0738E-04	9,9602E-01	1,1001E-03
366	3.388E-01	3.9355E-04	9,6745E-01	1,0927E-03
367	3.326E-01	3.8019E-04	9,6479E-01	1,0866E-03
368	3.264E-01	3.6728E-04	9,3892E-01	1,0824E-03
369	3.202E-01	3.5481E-04	9,1910E-01	1,0712E-03
370	3.140E-01	3.4277E-04	8,9768 E-01	1,0484E-03
371	3.078E-01	3.3113E-04	8,7252E-01	1,0263E-03
372	3.016E-01	3.1989E-04	8,4731E-01	9,9533E-04
373	2.954E-01	3.0903E-04	8,1229E-01	9,7028E-04

374	2.892E-01	2.9854E-04	7,8396E-01	9,3674E-04
375	2.830E-01	2.8840E-04	7,4156E-01	9,0571E-04
376	2.768E-01	2.7861E-04	7,1485E-01	8,7573E-04
377	2.706E-01	2.6915E-04	6,6874E-01	8,4279E-04
378	2.644E-01	2.6002E-04	6,2796E-01	8,0575E-04
379	2.582E-01	2.5119E-04	5,8633E-01	7,6129E-04
380	2.520E-01	2.4266E-04	5,3413E-01	7,1053E-04
381	2.458E-01	2.3442E-04	4,9253E-01	6,6551E-04
382	2.396E-01	2.2646E-04	4,4824E-01	6,1146E-04
383	2.334E-01	2.1878E-04	3,9321E-01	5,5611E-04
384	2.272E-01	2.1135E-04	3,4283E-01	4,9899E-04
385	2.210E-01	2.0417E-04	2,9853E-01	4,4337E-04
386	2.148E-01	1.9724E-04	2,5666E-01	3,8764E-04
387	2.086E-01	1.9055E-04	2,1479E-01	3,3630E-04
388	2.024E-01	1.8408E-04	1,7998E-01	2,8681E-04
389	1.962E-01	1.7783E-04	1,4860E-01	2,4081E-04
390	1.900E-01	1.7179E-04	1,1927E-01	2,0115E-04
391	1.838E-01	1.6596E-04	9,4026E-02	1,6399E-04
392	1.776E-01	1.6032E-04	7,2734E-02	1,3107E-04
393	1.714E-01	1.5488E-04	5,5317E-02	1,0282E-04
394	1.652E-01	1.4962E-04	4,0099E-02	7,8975E-05
395	1.590E-01	1.4454E-04	2,8849E-02	5,9753E-05
396	1.528E-01	1.3964E-04	2,0678E-02	4,4546E-05
397	1.466E-01	1.3490E-04	1,3998E-02	3,2594E-05
398	1.404E-01	1.3032E-04	9,5104E-03	2,3015E-05
399	1.342E-01	1.2589E-04	6,1938E-03	1,5806E-05
400	1.280E-01	1.2162E-04	4,1718E-03	1,0446E-05

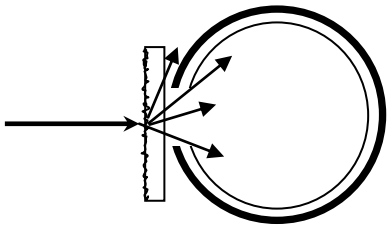
APPENDIX II

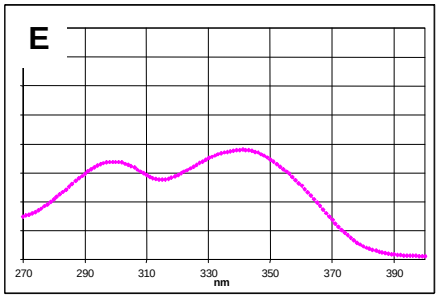
A) Use of PMMA standard plates with incorporated UV filters for checking spectrophotometer performance

These calibration plates contain UV-absorbing substances and are made in such a way as to match the absorption spectra of a range of common commercial sunscreens. The plates (supplied by Schonberg GmbH – order number 951) are cut from a large sheet of a standard cast, UV-stabilised Plexiglas® which helps ensure the same optical properties for each plate). . This casting process enables a very homogeneous distribution of UV absorbing material within the PMMA. The plates are roughened on one side by a standardised sandblasting procedure (glasspearls, 90-150µm, 30cm, 6 bar).

Because of their stable and standardised absorption and diffuse-scattering properties, they are very suitable as “reference emulsions" to check and compare spectrophotometric instruments used for *in vitro* determination of UV protection, for intra- as well as inter-laboratory purposes.

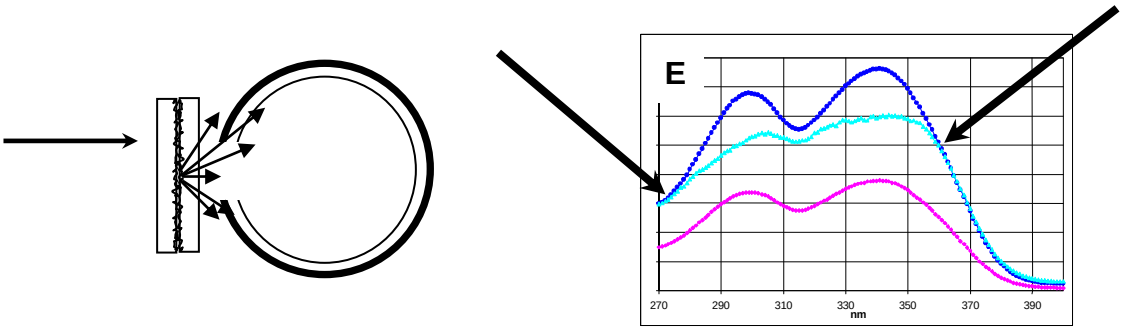
The **first extinction-measurement** through one plate only should be performed in the same manner as with a normal PMMA plate with a sunscreen film applied, i.e., the roughened, scattering surface should be orientated to the incident beam with the polished back of the plate positioned as near as possible to the aperture of the integrating sphere:





The **second measurement** should be performed with **two** plates, orientated with the roughened surfaces toward one another, to ensure that the position of the scattering surface is identical to that of the first measurement. Ideally, the UV attenuation (absorption and scattering) measured through the two plates by the spectrophotometer should be equal to exactly twice the UV attenuation of a single plate (upper spectrum in diagram, below). Practically, many spectrophotometers equipped with an integration device, will exhaust their linear measurement range at a certain degree of attenuation (middle spectrum). The points where the flanks of the “upper” and the “middle” spectra diverge (arrows), determine the instrument's upper range of measurement in the UVA- and UVB-region.

Note: When measuring with a glycerine-covered PMMA-plate as a reference (as described in main method), the instrument's efficiency at around 300nm will decrease by approximately 0.2 absorption units.



In summary, several pieces of data can be derived from these two fast and simple measurements:

- wavelength comparability between different instruments (measurement 1)
- quantitative comparability, measuring a Standard "sunscreen" (measurement 1)
- dynamic linear range of the instrument (measurement 2)

The plates can also be used for routinely checking an instrument's performance.

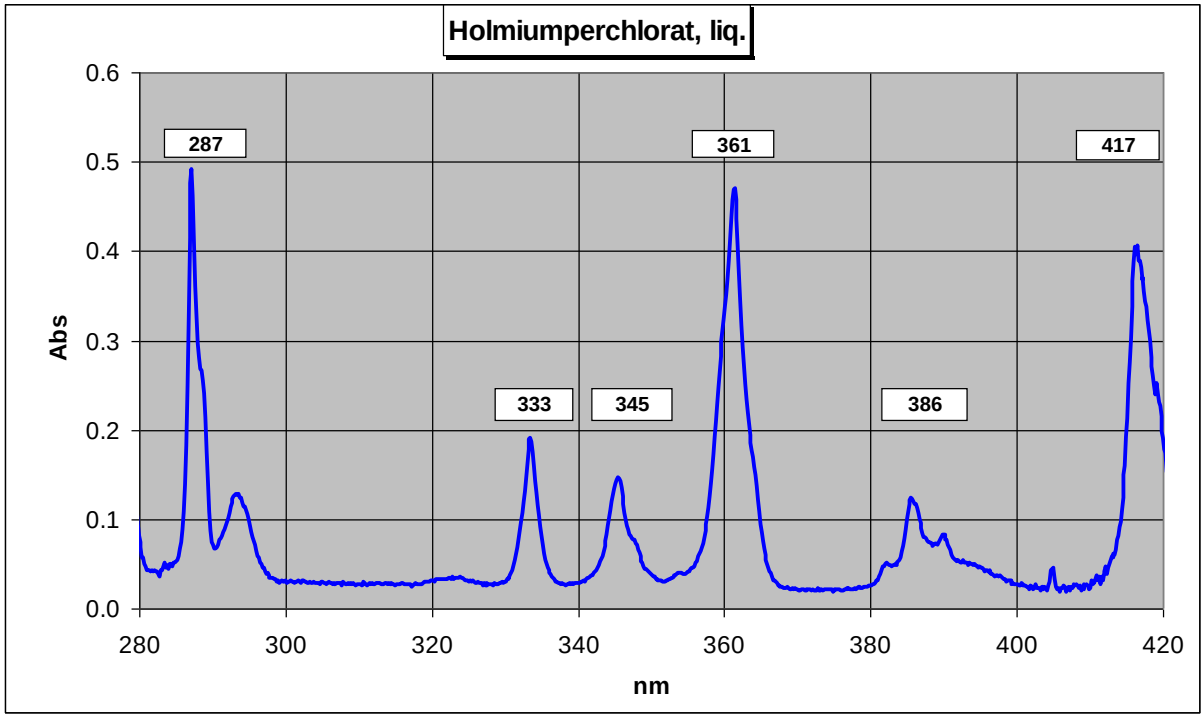
B) Use of the holmium perchlorate for instrument control purposes

The positions of the six relevant spectral bands of a holmium perchlorate solution, rounded to integer wavelengths, are listed in the table below.

Each certified standard is provided with a unique calibration table.

Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
287	333	345	361	386	417

According to the European Pharmacopoeia, deviation of the measured bands from the reference values in a UV range should not exceed 1nm for any spectrophotometer. An example of a measured calibration spectrum is shown below:



APPENDIX III

PMMA Test Plate Surface Specifications

1. Test Plate Type

A polymethylmethacrylate (PMMA) plate with a surface roughness that has tightly-defined surface parameters (see below) was qualified for use in this *in vitro* UVA test method via ring testing. It was found that plates which had a moulded surface topography provided the lowest degree of intra- and inter-batch variation.

2. Roughness of substrate

Measure the surface topography of the substrate, covering a surface area of at least 10mm x 5mm in 15µm intervals. Although contact mechanical profilometers could be employed for this measure, plates for ring-testing in the development of this test were measured using non-contact surface topographic analysis (based on white light chromatic aberration), chosen for its excellent resolution in the vertical and horizontal axis (10nm and 1µm respectively). It is recommended that, likewise, a reliable system is selected to provide the best possible vertical and horizontal resolution to enable sensitive measures of the roughness parameters below.

Surface topography parameters: Surface topography parameters should be calculated as the mean of the profiles analysed (R_a, R_v, R_{dq}, A1, SSc, Vw).

2.1 Plate roughness parameters

PMMA plates should have surface topography characteristics that meet the following measurement targets and ranges, measured using instrumentation of the type referenced above:

Target Roughness with Upper and Lower Limits

Parameter	R_a	R_v	R_{dq}	A1	Ssc	Vvv
Target value	4.853	13.042	11.122	239.750	0.033	1.044E-6
Upper Limit	5.170	13.669	12.411	284.256	0.046	1.663E-06
Lower Limit	4.535	12.414	9.833	195.244	0.020	4.248E-07

3. Plate Optical characteristics

3.1 Transmittance Specifications

The transmission properties of representative samples of each lot of PMMA plates should be tested to assure compliance. The rough surface of the test plate should be treated with either pure glycerine or a modified glycerol solution (see below):

Ingredients for modified glycerol solution %w/w

Glycerol BP/USP/JP 90.0
Sodium Lauryl Sulphate (SLS) solution 10.0 (1% SLS solution in water)

3.2 Method

4.2.1 Prepare a standard PMMA blank plate by applying approximately 15mg of pure glycerol or modified glycerol solution as a thin continuous film to the rough side of the plate. The slide should be even and transparent after treatment. Wipe away any excess glycerol / glycerol solution with a bare fingertip.

4.2.2 Place the plate in the light path of a UV spectrophotometer and measure transmittance (using an air reference) from 290nm to 400nm.

3.3 Limits

Treated plate transmission values should be:

290nm >60 %T
300nm >69 %T
320nm >81 %T

[1] M. Pissavini, S. Marguerie, A. Dehais, L. Ferrero and L. Zastrow, Characterising Roughness: A New Substrate to Measure SPF. *Cosmet. Toiletries*, Sept 09, 56-62, 2009.

APPENDIX IV

Reference Sunscreen S2

1. Mean UVAPF and acceptance limits for reference sunscreen formulations

Reference Sunscreen Formulation	Mean SPF	Mean UVAPF	Acceptance Range	
			Lower limit	Upper limit
S2	16.0	12.7	10.7	14.7

2. S2 Formula

	Ingredient	%w/w
Phase 1 (Aqueous)		
	water	62.445
	propylene glycol	1.000
	xanthan gum	0.600
	Carbomer Ultrez	0.150
	disodium EDTA	0.080
Phase 2. (Oil)		
	octocrylene	3.000
	butylmethoxy dibenzoylmethane	5.000
	ethylhexyl methoxycinnamate	3.000
	bis-ethylhexyloxyphenol	2.000
	methoxyphenyl triazine	
	cetyl alcohol	1.000
	steareth-21	2.500
	stearth-2	3.000
	dicaprylyl carbonate	6.500
	decyl cocoate	6.500
	phenoxyethanol and	1.00
	methylparaben and	
	ethylparaben and	
	butylparaben and	
	propylparaben	
Phase 3		
	cyclopentasiloxane	2.000
	triethanolamine	0.225

3. Manufacturing Process

(i) Heat Phase 1 and Phase 2 separately to 75°C
(ii) Slowly add Phase 2 to Phase 1 with continual stirring
(iii) Cool to 40°C with continual stirring
(iv) Add Phase 3 to combined Phases 1 and 2 with continual stirring
(v) Compensate for any evaporative water-loss and homogenise

4. Specifications

(i)	Colour:	white to slightly yellow
(ii)	pH:	6.5 +/- 0.5
(iii)	Density:	0.96 – 1g/cm^3
(iv)	Viscosity:	7000 to 12000 (Brookfield DV-II, Helipath Mobile, spindle set B, 20rpm, time of assessment 60sec)

5. Storage and expiry

12 months at 20°C from the date of manufacture, in a light-proof container.

6. Analysis

6.1 Principle

The formulation is sampled gravimetrically, solubilised in ethanol, filtered and then subjected to HPLC on a micro-particulate silica gel column, using a water / ethanol mobile phase. The concentrations of the analytes in the sample are determined by quantification against a mixed external standard solution of analyte raw materials.

6.2 Reagents

(i) Absolute Ethanol, HPLC grade
(ii) Ultra-pure Water, HPLC grade
(iii) Phosphoric Acid 85% p.a.
(iv) Ethyl Hexyl Methoxycinnamate
(v) Butyl Methoxydibenzoylmethane
(vi) Octocrylene
(vii) Bis-EthylHexyloxyphenol Methoxyphenyl Triazine

6.3 Apparatus

6.3.1 HPLC Injector Column

Injection Volume	10.0µL
Type	Symmetry Shield C18, 5µm
Length	150mm
I.D.	4.6mm
Flow rate	1.2µmL/min

6.3.2 Eluant

A – Ultrapure Water acidified with Phosphoric Acid
B – Absolute Ethanol, HPLC grade

6.3.3 Gradient

0-12min	37% A + 63% B
12-22min	100% B

22-25min	100% B
25-26min	37% A + 63% B
26-30min	37% A + 63% B

6.3.4 Detector

Type	UV
Wavelength	312nm

6.3.5 Data

Quantification	Peak Area

6.4 Method

(i) Using an analytical balance, weigh approximately 50mg of formulation (to the nearest 0.1mg) into a 25ml volumetric flask.

(ii) Dilute to volume with Ethanol.

(iii) Agitate using a vortex mixer and, in the case of a non-liquid formulation, sonicate with an ultrasonic bath until completely homogenised.

(iv) Filter through a 0.45µm PVDF disc filter.

(v) Analyse the sample and mixed working standard by Reverse-Phase HPLC.

6.5 Quality control

6.5.1 Analyse a sample of HPLC mobile phase and a vehicle control (if available, prepared by the same method), by Reverse Phase HPLC, to confirm the absence of interfering chromatographic peaks.

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6.5.2 Calculate the coefficient of variation of the analysis peak areas (to be performed in triplicate).

6.6 Calculations

$$\text{Analyte \% w/w} = \frac{M \times h \times 2.5}{P \times H}$$

where

M = Weight in mg of analyte
P = Weight in mg of sample
h = Area of analyte peak for sample
H = Area of analyte peak for standardisation

6.7 Acceptance Criteria

The analytical results are acceptable if the following are achieved:

(i) The standard coefficient of variation is equal to or less than 2.5%.
(ii) The recovery value is 95 - 105% of the formula quantity.
(iii) No interfering chromatographic peaks are found in the vehicle control or working solvent.