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Factors affecting SPF *in vitro* measurement and correlation with *in vivo* results

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Abstract

OBJECTIVE: The *in vitro* evaluation of SPF is still a problem due to the lack of repeatability and correlation between the *in vitro* and in vivo data and many authors are currently working to develop an internationally harmonized method.(1) Very recently, the use of several ‘‘adjuvant’’ ingredients such as boosters, antioxidants, immuno-modulators, solvents and film forming ingredients have further complicated the pattern for product developers, that should frequently run in vivo test. The aim of this study is to understand if a simple and cheap *in vitro* method could be optimized in order to provide both statistically repeatable and predictive SPF measurement.

METHODS: In vitro SPF assessments were carried out on 75 commercial products. The SPF was measured according to two laboratory methods (A and B), using different substrates (PMMA and surgical tape Transpore^{TM}), quantity of product, spectrophotometers. In order to evaluate if a standard technique of spreading could lead to a statistically reliable result, we applied different spreading pressure (100 g and 200 g). Furthermore, we investigate if other parameters characterizing the product (SPF Category, Filter and Texture) might represent statically significant variables affecting the measures. We then compared the results obtained from in vitro SPF measure of 11 products to *in vivo* SPF, in order to assess the predictivity of in vitro methods.

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RESULTS: Several problems were encountered in confirming the weakness of the *in vitro* procedures. Pressure, SPF Category, Filter and Texture did not affect significantly the results. Overall best results were obtained with the B2 method that in terms of repeatability and predictivity provided statistically better results. Method A with Transpore™ tape showed better *in vitro-in vivo* correlation than Method B with PMMA plates.

CONCLUSION: In our investigation we demonstrated that it is possible for a single laboratory to optimize internal methods and protocols to achieve repeatable and predictive in vitro results, but it is extremely difficult to develop methods reproducible and equally reliable in different laboratories, probably due to "external variables" (eg. environmental, operator), which are difficult to control.

Key words: Claim substantiation, Formulation, Spectroscopy, SPF *in vitro* , Sunscreens

Introduction

Over and incorrect exposure to sunlight has harmful consequences on human skin. Oxidative effects especially due to UV rays are the main cause of aged skin, sunburn and skin cancer. Therefore, sunscreens have become widely used for the prevention of short and long-term skin damage and for this reason consumers are offered a wide range of cosmetic products with protection against UV irradiation.

The sun protection factor (SPF) labelled on sunscreen products determines the amount of protection against the erythema induced by UV radiation. This value is widely recognized by the general public as a measure for the protection offered by a sunscreen preparation against sunburn and the classification of the SPF has to be proved by the manufacturers.

In the European Union sunbathing products are listed as cosmetics: their management must follow the articles of the Regulation EC 1223/2009 of the European Parliament on Cosmetic Products (2). Moreover, regarding the efficacy evaluation, the relevant guideline is the Commission Recommendation 2006/647 on the efficacy of sunscreen products and the claims made relating thereto (3). In this document a particular notice should be placed at the Preliminary Considerations, especially at points 16) and 17) where it clarifies that, in order to ensure reproducibility and comparability of the recommended minimum protection against UVB and UVA radiations, the methods used to evaluate the products must be specific, officially and internationally recognized. At the time of the Commission Recommendation 2006/647 draft, the main reference methods were International Sun Protection Factor Test Method for SPF calculation and the Persistent Pigment

Darkening Method for UVAPF, both based on *in vivo* techniques. Furthermore the Recommendation states that preference should be given to *in vitro* testing methods delivering equivalent results, as *in vivo* methods raise ethical concerns.

According to this, industry increased efforts to develop alternative methods and in 2012 a new method for *in vitro* UVA protection assessment was established and standardized in the ISO 24443:2012(4), whereas for the SPF determination the ISO 24444:2010(5) is still the reference method, despite providing an *in vivo* procedure carried out on human volunteers.

Investigation through the literature in this field highlights that the main concern, about the *in vitro* techniques used for SPF evaluation, is the lack of data to support method reproducibility and correlation to *in vivo* results. (6,7) The *in vivo* measurement remains therefore the ‘‘gold standard’’ and product developers should perform the *in vivo* test during all the phases of the development, not just on the final product, which obviously bring back the attention on costs and the ethical issue.

At the present there are several *in vitro* methods, all used just for screening or developing purposes (8). The first method was proposed by Diffey in 1989 and is still the most accredited reference (9). A sunscreen *in vitro* test is based on spectrophotometric measurement, as absorbance (calculated from trasmittance) or reflectance (10), of a thin film of product applied on suitable UV transparent substrates. The differences in all the methods are basically in the type of plastic support used and the way to apply the sunscreen on it (11,12). Starting from Diffey’s procedure, the supports used can range from plastic perforated surgical tape, as Transpore™ tape, to standardized plastic plates, as Polymethymethacrilate (PMMA) plates, with the amount of product applied varying from 0.7 to 2.0 mg/cm^2. An accurate literature search highlighted that the procedure followed to apply and spread the sunscreen on the plate is a very critical phase of the measure, where the error is increased the most (13). Several factors and variables affect the accuracy and the repeatability of the spectrophotometric measures, such as the different composition of filters (14), the formulation of the sunscreens (15), the thickness and the homogeneity of sunscreen applied (16), the type of spectrophotometer (17), the substrates and their relative roughness (18).

In this study, we first evaluated if a standard technique of spreading could lead to a statistically reliable result, focusing the efforts on controlling spreading pressure. Spreading pressure is sure enough a variable capable to influence consistently the results: according to the pressure exerted, the film thickness obtained on the substrate can vary, it was demonstrated in fact that an increased application pressure reduces the in vitro SPF (19).

Hence, in order to systematically evaluate the influence of spreading pressure on the result, but mostly to understand if the SPF calculated could be predictive of the *in vivo* measurement - the gold standard for regulatory purposes – we performed comparative assessment, using two different in vitro methods and adopting different substrates (Transpore™ Tape and sand blasted PMMA plates). Each method was performed twice on the same product, first using a 100g spreading pressure and later 200g spreading pressure.

The use of Transpore™ Tape, in determining in vitro SPF may be seem obsolete, standing the availability of more standardisable substrates (e.g. PMMA plates). However, during our studies we observed that, more often than supposed, Transpore™ Tape is still used for preliminary screening purposes due to its cheap nature.

We thus included also a method based on Transpore™ Tape, in order to assess whether, by controlling application pressure, it could be possible to improve its performance.

Therefore, the methods proposed were carried out at two different pressures, of 100 g and 200 g, on two different substrates, Transpore™ Tape and sand blasted PMMA plates.

We analyzed 75 commercial sunscreen products, with different SPF label, different types of filter and different formulations. Both the spreading techniques were tested using two different instruments; we then compared the results obtained from *in vitro* SPF measure of 11 products with *in vivo* SPF. An *in vitro* SPF method can be considered adequate and predictive only if the results of the analysis correlate to values obtained from *in vivo* method.

Materials and Methods

Substrates

In vitro approaches consist in applying a thin film of sunscreen product on an artificial substrate and test, via spectrophotometric measures, the amount of UV radiation passing through the film.
Several different artificial substrates are available for this type of analysis; the substrate should be as close as possible to the physical characteristics of the skin.
Among the substrates available for this purpose, the two substrates used in this study to analyze the sunscreen products were Transpore™ tape and PMMA plates.

- **Transpore™ tape:** it is a surgical tape, provided by 3M Company Health Care (Maine, USA). It is used according to the Diffey-Robson method; this tape has a perforated structure and it allows distributing the sunscreen sample in a way similar to the irregular surface of the skin.
- **Sand blasted PMMA plates**: this substrate is easily handled and can be supplied with a reproducible roughness. WW5 PMMA plates have been purchased from Schonberg GmbH (Munich, Germany). The plates, used in this study, have an area of 25 cm^2 and standardized 5 μm roughness. The features of this substrate meet the recommendation of ISO 24443 for in vitro UVA protection assessment.

Sunscreens

Commercial products

All the products were gifted to or purchased by our laboratory from public pharmacies in EU, Canada, US, and Australia. We decided to include products with different SPF labeled values, from 6 to 50+, with different type of filters and texture. In Table I, the list of tested products is reported; for each product the SPF labelled value is shown together with the type of filter (chemical, C, or physical, P or both, CP) and the texture, according to the viscosity (from ‘‘liquid’’ to ‘‘fluid’’, ‘‘creamy’’ and ‘‘paste’’). In the second part of the experiment we performed *in vivo* SPF measurement just on 11 out of the 75 products: the decision followed the purpose to test on human volunteers just formulations of which the exact composition of filters was available to us.

Table I: List of products tested and characteristics.

Product	*SPF Category*	*Labelled SPF*	*Filter*	*Texture*
1	Low	6	C	Liquid
2	Low	6	CP	Fluid
3	Low	10	P	Paste
4	Low	10	C	Fluid
5	Low	10	C	Fluid
6	Low	10	C	Fluid
7	Low	10	C	Liquid
8	Medium	15	C	Fluid
9	Medium	15	CP	Liquid
10	Medium	15	C	Fluid
11	Medium	15	CP	Fluid

12	Medium	15	C	Creamy
13	Medium	15	C	Fluid
14	Medium	15	C	Fluid
15	Medium	15	CP	Fluid
16	Medium	15	C	Liquid
17	Medium	15	P	Fluid
18	Medium	20	P	Paste
19	Medium	20	C	Creamy
20	Medium	20	CP	Fluid
21	Medium	20	C	Creamy
22	Medium	20	P	Fluid
23	Medium	20	P	Creamy
24	Medium	20	CP	Fluid
25	High	30	CP	Creamy
26	High	30	CP	Creamy
27	High	30	CP	Creamy
28	High	30	CP	Fluid
29	High	30	C	Fluid
30	High	30	P	Paste
31	High	30	P	Paste
32	High	30	CP	Fluid
33	High	30	C	Creamy
34	High	30	C	Fluid
35	High	30	CP	Liquid
36	High	30	CP	Fluid
37	High	30	C	Liquid
38	High	30	CP	Fluid
39	High	30	CP	Paste
40	High	30	CP	Paste
41	High	30	C	Fluid
42	High	30	C	Fluid
43	High	40	CP	Fluid
44	High	50	CP	Fluid
45	High	50	P	Paste

46	High	50	C	Creamy
47	High	50	CP	Creamy
48	High	50	P	Fluid
49	High	50	CP	Fluid
50	High	50	CP	Paste
51	High	50	CP	Fluid
52	High	50	CP	Fluid
53	High	50	CP	Fluid
54	Very High	60	CP	Fluid
55	Very High	100+	CP	Liquid
56	Very High	50+	C	Liquid
57	Very High	50+	CP	Creamy
58	Very High	50+	CP	Fluid
59	Very High	50+	CP	Paste
60	Very High	50+	CP	Liquid
61	Very High	50+	CP	Creamy
62	Very High	50+	CP	Creamy
63	Very High	50+	CP	Fluid
64	Very High	50+	CP	Creamy
65	Very High	50+	CP	Fluid
66	Very High	50+	CP	Creamy
67	Very High	50+	CP	Creamy
68	Very High	50+	C	Creamy
69	Very High	50+	CP	Fluid
70	Very High	50+	CP	Fluid
71	Very High	50+	C	Fluid
72	Very High	50+	CP	Fluid
73	Very High	50+	CP	Creamy
74	Very High	50+	CP	Fluid
75	Very High	50+	CP	Liquid

Spectrophotometric measurements

Transmittance and absorbance measurements were carried out with two different spectrophotometers in respect to the two different substrates.

With PMMA plates, the measurements were carried out by a SHIMAZHU UV-2600 provided of integrating sphere ISR 2600 60mm and coupled with a SPF determination software and a PMMA plate with approximately 15 µl of glycerin served as reference. With Transpore™ Tape, the spectrophotometer was a Jasco V530 coupled with a Jasco SPF determination software and an untreated piece of tape used as reference. The choice to use two different instruments follows the idea to compare a traditional low expensive method to a more recent and expensive one. In both methods, the spectra were recorded from 290 nm up to 400 nm with a wavelength increment step set at 1 nm.

***In vitro* method based on spectral measurements**

The products were applied on the two selected substrates with two different pressures, monitored by a scale; all the other conditions were kept identical, such as the operator, the quantity of the product applied according to the substrate and the room temperature. None of the samples were pre-irradiated because testing the photostability was not the aim of the present study.

Method A – Based On Diffey-Robson's Method

The support used is a Transpore™ surgical perforated tape, cut to have an area of 20 cm^2, in which an amount of 0,0400 g ± 0,002 g (2 mg/cm^2) of product is weighed and laid in small spots through all the area. The tape is then positioned on a scale where the spreading phase is carried out with a finger cot and performing a pattern of 6 movements in horizontal, vertical and circular directions, checking the pressure applied in all the movements. For all the products the spreading pressure is first of 100 g (Method A1) and in the second set of tests of 200 g (Method A2). Three tapes were prepared for each product, recording 5 measures each, collecting therefore 15 spectra.

Method B – Based on ISO 24443 procedure

This method incorporates many of the recommendations issued by the ISO 24443:2012 standard for the *in vitro* determination of UVA protection.

The support used is a PMMA (poly methyl methacrylate) plastic plate with an area of 25 cm^2 and standardized 5µm roughness, in which an amount of 0.0320 g ± 0,0005 g (1,3 mg/cm^2) of product is weighed and laid in small spots through all the area. The plate is then positioned on a scale where the spreading phase is carried out performing with pre-saturated finger cot a sequence of 6

movements in horizontal, vertical and circular direction, checking the pressure applied throughout the spreading.(21) For all the products the spreading pressure is first of 100 g ± 15 g (Method B1) and in a second analysis of 200 g ± 15 g (Method B2). Before the measurement the sample lies for a minimum of 15 minutes in a dark place, allowing the evaporation of volatile components. Three plates were prepared for each product, recording 5 measures each, collecting therefore 15 spectra.

Calibration of the *In vitro* SPF

The calibration of the operator and/or the device is controlled by the use of a reference sunscreen formulation with SPF 16.(22) The SPF test results for the reference has to lie between 14 and 18, otherwise the test has to be considered invalid and should be repeated.

The test is performed in triplicate for both the substrates, PMMA plate and Transpore™ Tape, and for both the methods, 100 g and 200 g of pressure.

***In vitro* SPF calculation**

The following definition was used for the *in vitro* Sun Protection Factor (SPF) calculated from the spectral absorbance characteristics described above(23):

$$\boldsymbol{In\ vitro\ SPF} = \frac{\int_{\lambda=290\ nm}^{\lambda=400\ nm} \boldsymbol{E(\lambda)I(\lambda)d(\lambda)}}{\int_{\lambda=290\ nm}^{\lambda=400\ nm} \boldsymbol{E(\lambda)I(\lambda)10^{-A(\lambda)}d(\lambda)}}$$

where:

E(λ) = erythema action spectrum (CIE-1987) at wavelength λ .

I(λ) = spectral irradiance received from the UV source at wavelength λ .

A(λ) = monochromatic absorbance of the test product layer at wavelength λ

d(λ)= wavelength step (1 nm).

The *in vitro* SPF could be calculated through sunscreens UV Transmittance, T(λ), applying the same equation with A(λ) = - Log[T(λ)].

***In vivo* evaluation of products**

The *in vivo* method according to ISO 24444:2010 standard and European Recommendation 647/2006 was applied to determine the SPF value for 11 selected sunscreen products. As previously stated, for ethical reasons only products available with their exact composition were tested on human volunteers. In this study, 10 subjects per product, female and male, with age ranging from 20

to 35 years, were tested. Their skin photo type was chosen with sun sensitivity categories of type I, II and III according to Fitzpatrick. All volunteers had been informed in detail before signing a written declaration of consent.

A skin area on the back, 35 cm^2 was irradiated with different UV irradiation doses, so that the unprotected minimal erythema dose (MEDu) was determined.

For *in vivo* SPF determination, a Multiport UV Solar Simulator Model 601, 150 W, was used, which emits ultraviolet radiation in the region between 290 and 400 nm from 6 independent outputs. Each output is adjusted on scalar UV doses, set according to parameters tabulated by the instrument.

All sunscreen products were applied in a thin film of 2,00 ± 0,05 mg/cm^2 in the selected area on the back. The product distribution was reached by a gentle massage using a finger cot, at least 15 minutes before the irradiation started. 20 ± 4 hours after the UV exposure, MEDp was determined. The SPF was then calculated as described in the ISO 24444:2010 standard.

$$SPF_i = \frac{MED_{protected\ skin}}{MED_{unprotcted\ skin}} = \frac{MED_p}{MED_u}$$

The SPF result for the test product and for the reference sunscreen formulation is calculated as the arithmetical mean of all valid individual SPF values.

Criteria of interpretation

The evaluation of the repeatability of the data was carried out taking as reference the ISO Standard 24443 parameters, where the document states that, starting from "s", the following calculations is to be done:

$$c = \frac{t \times s}{\sqrt{n}}$$

so that *CI 95%* = *SPF + c* to *SPF* − *c*.

$$CI\ [\%] = \frac{100 \times c}{SPF}$$

Where *c* is the confidence interval (95% confidence level), *s* is best estimate of population standard deviation, *t* is the T statistic and *n* is the number of measures.

The ISO standard established that the obtained CI [%] value should not be greater than *17%*, otherwise the result should be considered not statistically significant. The same criteria of

interpretation are listed also for *in vivo* SPF in the ISO standard 24444 and were therefore applied for *in vivo* data collected in this study.

With the final aim to comparatively evaluate the method, in terms of repeatability, we considered the mean CI% for all the valid data (CI% < 17%) for each method and the values obtained were statistically compared by a two-way analysis of variance (ANOVA), taking Method and Pressure as variables.

Later we evaluated if the other parameters characterizing the product (Category, Filter and Texture) could represent statically significant variables in comparison to Method and Pressure.

Results and Discussion

In the following table (Table II) are listed all the results obtained from the experiment. The columns *in vitro* SPF, SD and CI% obtained from the spectrophotometric measurements. The final row reports the mean CI% calculated on the valid samples. As above explained we calculated the CI% to exclude analyses that were not repeatable. The reported *in vitro* SPF is the mean of the values measured. The results are summarized in Table III.

Prod.	*Method A*						*Method B*					
	1 – 100g			*2- 200g*			*1 – 100g*			*2-200g*		
	in vitro SPF	SD	CI %	in vitro SPF	SD	CI(%)	in vitro SPF	SD	CI(%)	in vitro SPF	SD	CI(%)
1	9,63	2,445	12,63%	12,79	2,715	10,55%	6,58	1,07	8,98%	5,19	0,96	10,30%
2	11,71	1,619	6,88%	13,52	1,856	6,83%	9,28	0,38	2,24%	8,90	0,35	2,20%
3	8,74	1,175	6,68%	8,56	2,516	14,61%	5,53	0,61	6,15%	6,69	1,03	8,53%
4	19,79	4,632	11,64%	25,02	4,344	8,63%	11,40	0,89	4,33%	11,92	0,43	2,00%
5	15,44	2,587	8,33%	20,27	5,457	13,39%	14,18	0,97	3,79%	11,36	0,75	3,68%
6	26,10	1,472	2,81%	23,07	2,088	4,50%	11,33	0,56	2,75%	11,65	1,08	5,11%
7	23,88	4,782	9,96%	28,00	3,980	7,07%	14,36	3,77	14,54%	15,13	3,19	11,68%
8	19,12	4,531	11,78%	29,73	4,193	7,01%	23,36	2,80	6,63%	15,43	1,31	4,69%
9	19,50	0,475	1,21%	25,30	4,766	9,37%	15,58	0,91	3,23%	16,11	1,00	3,43%
10	23,05	4,358	9,40%	26,84	7,985	14,79%	12,43	1,49	6,64%	13,75	0,38	1,53%
11	29,80	3,752	6,26%	28,43	7,894	13,81%	14,11	0,82	3,21%	21,42	4,29	11,08%
12	4,99	1,246	12,43%	8,77	2,551	14,47%	6,46	0,34	2,88%	5,88	0,21	1,98%
13	25,28	4,308	8,48%	19,99	3,716	9,25%	12,23	2,08	9,44%	12,55	2,06	9,07%
14	21,08	1,638	3,86%	18,99	5,251	13,75%	16,73	1,86	6,15%	14,20	1,25	4,88%
15	22,88	1,505	3,27%	25,88	2,788	5,36%	32,50	3,29	5,60%	26,40	1,54	3,24%
16	39,91	5,362	6,68%	35,10	9,678	13,71%	28,59	1,70	3,29%	21,88	2,19	5,53%
17	2,85	0,258	4,51%	3,82	0,476	6,20%	3,92	0,35	4,91%	5,97	0,43	3,98%
18	5,04	0,437	4,31%	5,67	1,242	10,90%	3,65	0,57	8,58%	5,68	0,95	9,23%
19	28,94	2,995	5,15%	34,37	5,782	8,37%	25,84	2,94	6,30%	30,72	4,09	7,37%
20	38,16	7,957	10,37%	42,74	5,896	6,86%	38,68	5,90	8,44%	31,54	2,34	4,10%

21	23,03	3,197	6,90%	22,35	2,854	6,35%	10,02	1,54	8,49%	13,54	1,77	7,22%
22	3,91	0,440	5,60%	3,98	0,305	3,81%	11,38	1,22	5,92%	11,38	1,22	5,92%
23	33,57	4,534	6,72%	31,64	3,178	4,99%	22,76	2,58	6,28%	24,61	1,57	3,53%
24	22,39	1,854	4,12%	25,06	0,779	1,55%	14,17	1,22	4,75%	16,47	2,79	9,39%
25	72,44	15,112	10,38%	62,72	4,649	3,69%	26,51	4,38	9,15%	25,78	3,82	8,20%
26	51,20	7,412	7,20%	70,74	6,259	4,40%	47,50	3,07	3,57%	42,22	0,86	1,13%
27	37,43	10,208	13,56%	31,04	7,956	12,75%	41,94	2,28	3,00%	40,53	2,12	2,90%
28	26,53	3,799	7,12%	22,96	4,362	9,45%	39,63	4,99	6,97%	19,52	3,42	9,72%
29	34,65	4,972	7,14%	33,23	6,381	9,55%	35,57	16,18	25,20%*	32,08	8,14	14,05%
30	6,16	1,994	16,09%	6,90	1,035	7,46%	6,35	0,35	3,02%	5,59	0,25	2,48%
31	6,35	2,029	15,90%	8,63	2,494	14,38%	6,29	0,22	1,93%	7,34	0,77	5,77%
32	35,79	6,226	8,65%	54,89	4,691	4,25%	30,37	5,61	10,23%	40,76	4,42	6,00%
33	50,37	0,618	0,61%	42,80	10,533	12,24%	56,02	15,04	14,86%	57,02	6,37	6,19%
34	33,31	2,376	3,55%	33,31	3,411	5,09%	32,75	3,10	5,24%	27,07	1,82	3,73%
35	26,38	5,153	9,72%	36,85	3,342	4,51%	12,04	0,38	1,76%	12,03	0,37	1,72%
36	32,97	3,821	5,76%	39,02	4,998	6,37%	31,76	7,55	13,17%	30,03	4,02	7,41%
37	35,13	7,203	10,20%	39,13	7,157	9,10%	24,74	1,60	3,58%	34,40	8,88	14,30%
38	50,11	20,223	20,07%*	64,29	19,906	15,40%	65,36	2,93	2,48%	62,68	6,54	5,78%
39	47,49	4,192	4,39%	44,27	0,482	0,54%	47,43	5,78	6,75%	42,39	2,71	3,54%
40	33,84	2,821	4,15%	30,81	2,345	3,79%	58,33	6,06	5,76%	38,17	3,45	5,01%
41	32,85	14,193	21,49%*	36,13	19,021	26,18%*	40,58	12,54	17,11%*	29,21	2,84	5,38%
42	32,65	4,508	6,87%	49,30	2,071	2,09%	35,66	4,21	6,54%	38,12	5,88	8,54%
43	51,92	13,165	12,61%	55,67	1,892	1,69%	52,66	7,53	7,92%	47,67	2,77	3,22%
44	25,33	8,282	16,26%	25,19	6,442	12,72%	37,20	3,65	5,43%	39,94	3,07	4,26%
45	4,98	0,873	8,71%	5,25	0,860	8,15%	6,76	1,78	14,56%	4,77	0,33	3,83%
46	31,13	5,519	8,82%	35,69	4,105	5,72%	47,61	4,86	5,65%	55,61	1,95	1,94%
47	37,26	6,313	8,43%	51,62	5,310	5,12%	41,19	1,24	1,67%	56,70	7,15	6,98%
48	6,95	1,230	8,80%	5,54	0,606	5,44%	31,45	6,70	11,80%	28,62	4,53	8,77%
49	41,70	6,238	7,44%	51,08	10,139	9,87%	48,02	5,96	6,88%	48,41	3,56	4,08%
50	69,63	16,576	11,84%	57,16	13,41223	11,67%	22,32	1,64	4,06%	33,79	5,29	8,68%
51	39,67	4,296	5,39%	39,63	6,813	8,55%	51,04	1,96	2,13%	46,20	4,55	5,46%
52	49,35	16,320	16,45%	58,52	7,212	6,13%	52,80	4,64	4,87%	64,68	21,01	17,99%*
53	47,52	1,575	1,65%	83,22	14,009	8,37%	44,50	3,47	4,32%	53,69	5,79	5,98%
54	44,26	11,098	12,47%	56,72	7,985	7,00%	74,86	3,67	2,71%	59,68	6,86	6,36%
55	14,43	6,593	22,72%*	29,44	6,783	11,46%	31,24	4,16	7,37%	19,16	2,52	7,28%
56	23,09	6,567	14,14%	31,73	4,505	7,06%	26,34	6,15	12,93%	27,15	2,33	4,74%
57	59,93	9,196	7,63%	62,50	10,346	8,23%	52,60	4,84	5,10%	70,80	7,42	5,81%
58	65,55	10,530	7,99%	73,38	13,238	8,97%	33,20	6,65	11,09%	57,85	3,71	3,55%
59	52,81	6,647	6,26%	55,95	8,130	7,23%	47,90	6,43	7,43%	56,45	3,94	3,86%
60	51,27	2,590	2,51%	43,34	1,511	1,73%	51,44	5,19	5,59%	54,38	4,26	4,34%
61	32,04	8,606	13,36%	54,22	5,804	5,32%	62,44	4,98	4,42%	59,18	3,71	3,47%
62	25,68	4,999	9,68%	38,15	8,220	10,72%	63,87	4,89	4,24%	62,42	3,59	3,19%
63	30,72	3,855	6,24%	33,55	7,555	11,20%	30,55	5,83	10,57%	42,41	2,67	3,48%
64	69,35	11,970	8,58%	58,13	6,137	5,25%	53,66	3,04	3,14%	47,96	11,39	13,15%
65	51,28	4,526	4,39%	45,78	3,510	3,81%	36,03	2,98	4,58%	35,31	2,85	4,47%
66	33,15	3,499	5,25%	32,49	1,951	2,99%	29,87	1,36	2,53%	20,80	1,44	3,84%

67	66,97	9,864	7,32%	67,15	0,895	0,66%	50,46	5,70	6,26%	60,88	7,29	6,64%
68	45,62	19,560	21,32%*	52,51	6,901	6,54%	67,43	6,12	5,03%	18,29	1,16	3,51%
69	26,78	4,967	9,22%	26,25	6,871	13,02%	59,58	4,00	3,72%	65,52	4,98	4,21%
70	25,27	4,599	9,05%	45,54	2,488	2,72%	44,30	7,46	9,33%	53,38	2,02	2,09%
71	40,02	3,272	4,07%	60,04	7,000	5,80%	45,81	3,95	4,77%	58,30	2,51	2,38%
72	28,34	4,839	8,49%	29,27	7,423	12,61%	43,71	12,75	16,15%	42,16	13,11	17,22%*
73	29,02	4,124	7,07%	29,05	2,807	4,81%	20,50	5,60	15,14%	34,45	1,21	1,95%
74	70,74	9,566	6,73%	49,69	5,609	5,61%	65,05	1,92	1,63%	61,94	4,34	3,88%
75	39,13	8,482	10,78%	42,98	7,763	8,98%	17,21	0,90	2,91%	28,94	7,57	14,48%
Mean CI% of valid samples			**8,01%**			**7,79%**			**6,27%**			**5,64%**

Table II: analytical and statistical results. * indicates invalid CI% values.

	Method			
	A1	**A2**	**B1**	**B2**
Not Valid	4	1	2	2
Valid	71	74	73	73
Mean CI%	8.01 %	7.79 %	6,67 %	5.64 %

Table III. Summary of results.

Starting from the statistical results presented in Table IV, we were able to highlight that just the method exerts some influence on the results, being statistically significant ($p<0.00001$). Method B showed mean CI% values lower than Method A (Figure 1) confirming, as expected, that the use of PMMA plates give more reproducible results than the tape, regardless of the pressure applied to spread the product. The pressure indeed does not influence the analysis in a statistically significant way ($p > 0,05$) and comparing different application pressures on the same substrate, no statistically significant difference subsists in terms of repeatability.

	SS	**df**	**MS**	**F**	**p**
Intercept	13954.89	1	13954.89	1086.439	0.000000
Method	276.41	1	276.41	21.520	**0.000005**
Pressure	13.28	1	13.28	1.034	**0.310037**
Method*Pressure	3.00	1	3.00	0.234	**0.629118**
Error	3686.40	287	12.84		

Table IV. Statistical analysis Mean CI% vs Method and Pressure: the mean CI% values were statistically compared by a two-way analysis of variance (ANOVA), taking Method and Pressure applied as variables.

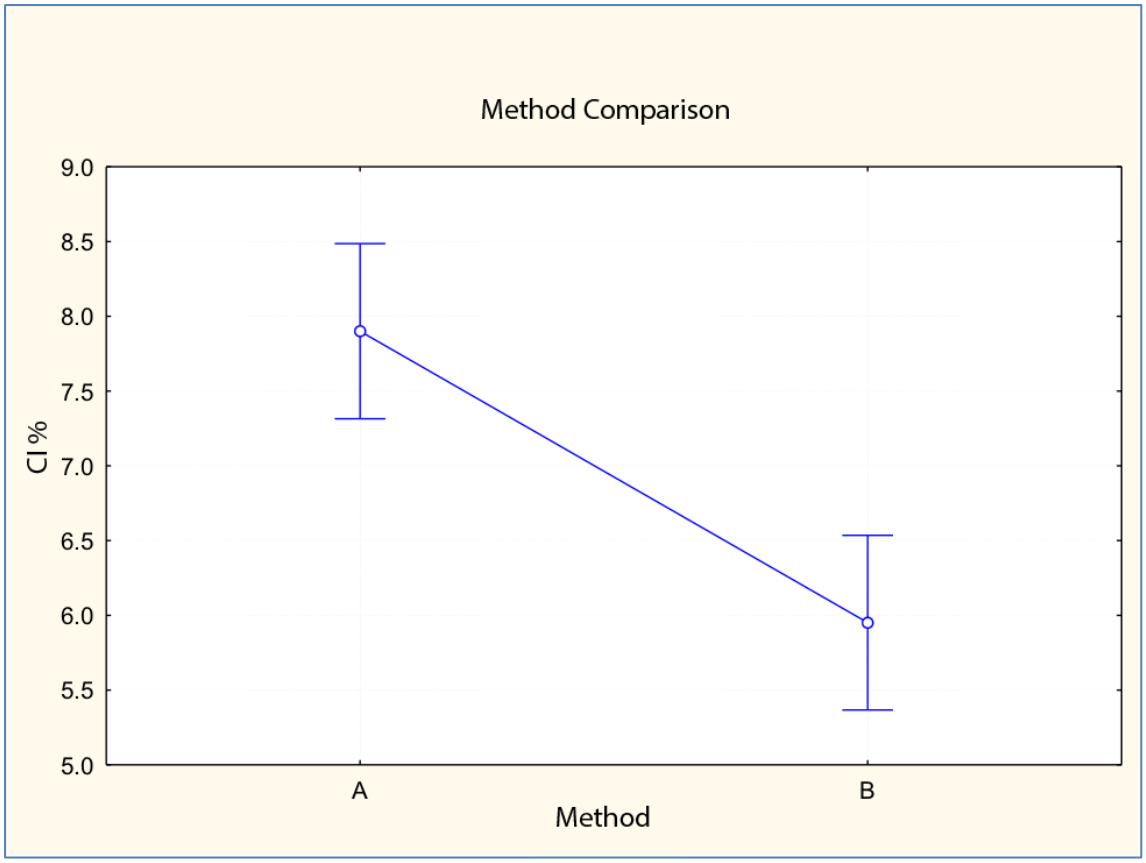

Figure 1: Comparison between mean CI% obtained with Method A to the one obtained with Method B: vertical segments show confidence interval (95%).

Secondly the samples tested were grouped according to the three variables Category, Filter and Texture indicated in Table I. Tables V, VI and VII summarize the results for each method, following the grouping.

	SPF Category			
	Low *(7)*	***Medium*** *(16)*	***High*** *(29)*	***Very High*** *(22)*
Method A, 100g	8,42%	6,53%	8,80%	8,06%
Method A, 200g	9,37%	8,86%	7,45%	6,90%
Method B, 100g	6,11%	5,93%	6,20%	6,67%
Method B, 200g	6,21%	5,66%	5,89%	5,08%

Table V: Distribution of the tested products by SPF category and corresponding mean CI% for each method.

	Filter		
	Chemical C *(23)*	***Physical P*** *(9)*	***Combination CP*** *(43)*
Method A, 100g	7,88%	8,59%	7,95%
Method A, 200g	8,87%	8,84%	7,10%
Method B, 100g	6,80%	7,02%	5,85%
Method B, 200g	6,08%	5,78%	5,36%

Table VI: Distribution of the tested products by type of filters and corresponding mean CI% for each method.

	Texture			
	Liquid *(10)*	***Fluid*** *(38)*	***Creamy*** *(18)*	***Paste*** *(9)*
Method A, 100g	8,65%	7,60%	8,18%	8,70%
Method A, 200g	8,35%	7,88%	6,81%	8,75%
Method B, 100g	6,42%	6,31%	5,98%	6,47%
Method B, 200g	7,78%	5,38%	4,94%	5,66%

Table VII: Distribution of the tested products by texture and corresponding mean CI% for each method.

Leaving aside the pressure and only considering the method as significant variable, we studied the statistical relation between the method and SPF category, filter and texture respectively, according to the mean CI% of the analysis (Table VIII, IX and X). We can conclude that also in this case the choice of the method is found to be the only significant variable able to affect the repeatability of the measure.

	SS	df	MS	F	p
Intercept	10147.49	1	10147.49	792.2758	0.000000
Method	198.10	1	198.10	15.4666	**0.000106**
Filter	52.13	2	26.07	2.0352	**0.132548**
Method*Filter	0.32	2	0.16	0.0125	**0.987610**
Error	3650.29	285	12.81		

Table VIII: Statistical analysis Mean CI% vs Method and Filter: the mean CI% values were statistically compared by a two-way analysis of variance (ANOVA), taking Method and Filter as variables.

	SS	df	MS	F	p
Intercept	10880.58	1	10880.58	845.9816	0.000000
Method	212.98	1	212.98	16.5594	**0.000061**
Texture	54.66	3	18.22	1.4167	**0.238079**
Method*Texture	7.73	3	2.58	0.2004	**0.896095**
Error	3639.80	283	12.86		

Table IX: Statistical analysis Mean CI% vs Method and Texture: the mean CI% values were statistically compared by a two-way analysis of variance (ANOVA), taking Method and Texture as variables.

	SS	df	MS	F	p
Intercept	11014.85	1	11014.85	848.3435	0.000000
Method	239.50	1	239.50	18.4460	**0.000024**
SPF Category	20.45	3	6.82	0.5251	**0.665355**
Method*SPF Category	7.92	3	2.64	0.2033	**0.894036**
Error	3674.46	283	12.98		

Table X: Statistical analysis Mean CI% vs Method and SPF Category: the mean CI% values were statistically compared by a two-way analysis of variance (ANOVA), taking Method and SPF Category as variables.

An analogue analysis was performed considering pressure in relation to three variable (SPF category, filter and texture) and, as expected; no statistically significant combination of parameters was highlighted (statistical data not showed).

Correlation between *in vitro* and *in vivo* data

Because the only officially accepted method for SPF determination is the *in vivo* procedure, we next compared the SPF *in vitro* results of 11 selected products with their SPF *in vivo*, to prove the accuracy of the methods, independently from that reported in the label of the products. The results are summarized in Table XI.

	In vivo		*In vitro A1*		*In vitro A2*		*In vitro B1*		*In vitro B2*	
Prod.	***SPF***	***CI%***	***SPF***	***CI%***	***SPF***	***CI%***	***SPF***	***CI%***	***SPF***	***CI%***
1	6,1	15,6	9,63	12,63	12,79	10,55	6,58	8,98	5,19	10,30
2	6,9	15,5	11,71	6,88	13,52	6,83	9,28	2,24	8,9	2,20
15	18,8	9,9	22,88	3,27	25,88	5,36	35,5	5,60	26,4	3,24
20	20,3	7,5	38,16	10,37	42,74	6,86	38,68	8,44	31,54	4,10
35	35,1	12,5	26,38	9,72	36,85	4,51	12,04	1,76	12,03	1,72
36	30,6	9	32,97	5,76	39,02	6,37	31,76	13,71	30,03	7,41
43	41,2	4,6	51,92	12,61	55,67	1,69	52,66	7,92	47,67	3,22
49	52	7,6	41,7	7,44	51,08	9,87	48,02	6,88	48,41	4,08
59	62,8	11,5	52,81	6,26	55,97	7,23	47,9	7,43	56,45	3,86
65	56,1	10,5	51,28	4,39	45,78	3,81	36,03	4,58	35,31	4,47
75	61,2	5,1	39,13	10,78	42,98	8,98	17,21	2,91	28,94	14,48

Table XI: SPF results of 11 selected product from *in vivo* and *in vitro* methods.

To evaluate a possible linear correlation of *in vitro* and *in vivo* data, we calculated the Pearson index, reported in Table XII.

	Mean	Dv.Std.	r(X,Y)	r^2	t	p	N
vivo	35.55455	20.85969					
A1	34.41545	15.32343	0.849327	**0.721356**	4.826934	**0.000938**	11
vivo	35.55455	20.85969					
A2	38.38909	15.15401	0.842200	**0.709301**	4.686132	**0.001142**	11
vivo	35.55455	20.85969					
B1	30.51455	16.62740	0.509246	**0.259332**	1.775159	**0.109611**	11
vivo	35.55455	20.85969					
B2	30.07909	16.67036	0.743459	**0.552731**	3.334982	**0.008730**	11

Table XII: Pearson correlation of in vitro and in vivo results.

Surprisingly, method A gives best statistically correlated results between *in vivo* and *in vitro* data. From these results it can be pointed out that *in vitro* SPF obtained with Method A significantly correlates with *in vivo* result for both the pressures applied, being in such way a measure independent from pressure exerted by the operator. The same cannot be confirmed for Method B, where only the data set obtained applying 200 g pressure (Method B2) is statistically correlated with the *in vivo* data. Furthermore, it should be observed that, regardless the previous data presented on repeatability, the Methods A do indeed better correlate to in vivo SPF.

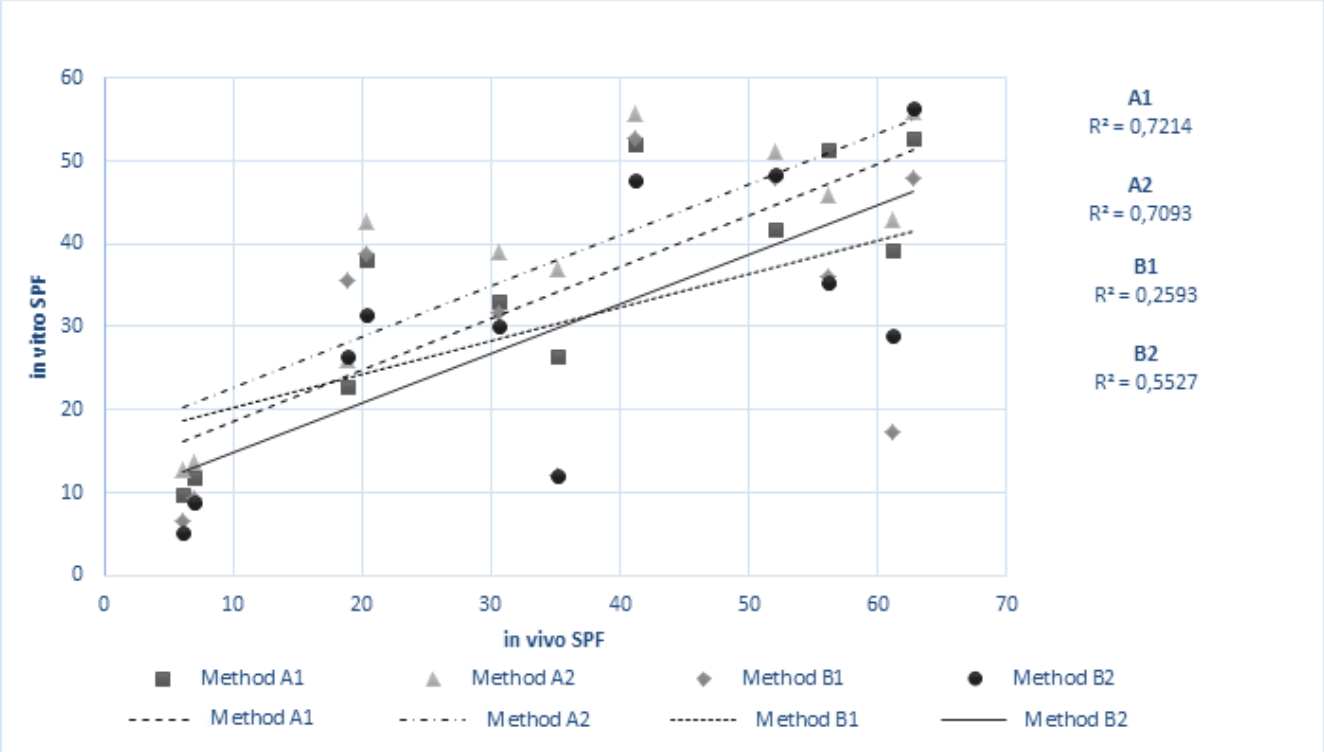

Figure 2: *In vitro*-in vivo correlation.

Conclusion

Repeatability and *in vivo* correlation of *in vitro* SPF (accuracy) measurements, together with reproducibility, are a puzzling issue already discussed by other authors (24). Furthermore, even if a method appears to be statistically valid and interesting, we must consider that the SPF analysis is not a merely academic exercise, but mostly a manufacturer's must. In this regard, a method must be not only statistically significant, but also predictive and easy to apply to a routine complex development process that deals with ever-complex formulations.

Starting from conflicting results, observed during our routine measurements, the purpose of this study was to evaluate if a standard technique of spreading could statistically improve the results in terms of repeatability and if the SPF calculated could be predictive of the *in vivo* measurement, which is the standard method for SPF determination of sunscreen products. As stated in the introduction our concern was to elaborate a simple method, applicable not just in scientific investigations, but also by manufacturers with limited equipment.

Data collected and analyzed showed that the standardization of the pressure applied to spread the product on the substrate does not lead to significant improvements of the data variability.

As confirmed by other authors, the choice of the substrate is much more critical and PMMA plates gives, in terms of repeatability, better results than the ‘‘old fashioned’’ Transpore™ Tape, although it also has shown acceptable values of CI%, with results well below the 17% threshold.

As is known, the PMMA plates provide more repeatable data, being a more standardized substrate in terms of composition and surface roughness, while the main limit of the Transpore™ tape is precisely the impossibility to obtain a support with unique characteristics: surface can vary greatly from one batch to another. In this case it is necessary to rely on the experience and know-how of the laboratory to make small adaptations of the protocol in order to correct the differences due to the substrate and this makes impossible the definition of a standard method valid and applicable in any laboratory.

Other variables as SPF category, filters or texture, resulted not statistically significant for the determination of *in vitro* SPF and therefore it was not possible to identify a better method according to these subsets of products.

Meanwhile, the results obtained from the linear correlation of *in vitro* SPF and the in vivo data obtained applying ISO 24444 were completely unexpected: for the 11 products selected the best correlation could be obtained with Method A, using the Transpore™ Tape and, moreover, the correlation was not influenced by the operator's pressure.

A significant correlation with *in vivo* test was found for Method B (using PMMA plates) just for the 200 g pressure of application.

Based on the results of our investigation, we can conclude that the Method B2 is the most reliable according to the data repeatability and accuracy. Nevertheless, the method A with Transpore™ tape can still be considered an *in vitro* method predictive of the *in vivo* SPF during the research and development phases of the solar product, especially in laboratories with limited financial resources and limited equipment.

Further work is currently ongoing to compare influence of different kind of formulations, frequently used by product developers, in order to devise possible modifications to the methods proposed, to obtain better correlation to *in vivo* test results.

The present study was conducted entirely in our laboratory, in highly standardized operating conditions with regard to the operator, environmental conditions, the substrates used and the instruments, in order to evaluate whether the application pressure of the sample on the substrate and the intrinsic characteristics of the formulations could affect the result. As the considered variables did not provide significantly relevant results, it may be assumed that the difficulty in defining a single protocol for the determination of sunscreen *in vitro* SPF could be due to "external variables" (eg. environmental, operator), which are more difficult to control in different laboratories.

This also explains the evidence that it is possible for a single laboratory to optimize internal methods and protocols to achieve repeatable and predictive *in vitro* results, as we demonstrated in this work, while it is extremely difficult to make methods reproducible and equally reliable in different laboratories.

For these reasons, as concluded by other authors (25), we support the need of a common room for discussion, to compare methods between different laboratories in order to devise a common protocol, which takes into consideration the environmental variables.

-Acknowledgements

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