Porcine Ear Skin as a Biological Substrate for in vitro Testing of Sunscreen Performance

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Key Words
Sun protection factor · Pig ear skin · In vitro testing · Surface roughness · Polymethylmethacrylate plates · Sunscreen

Abstract

Purpose: The purpose of the study was to examine the use of skin from porcine ears as a biological substrate for in vitro testing of sunscreens in order to overcome the shortcomings of the presently used polymethylmethacrylate (PMMA) plates that generally fail to yield a satisfactory correlation between sun protection factors (SPF) in vitro and in vivo. Procedures: Trypsin-separated stratum corneum and heat-separated epidermis provided UV-transparent substrates that were laid on quartz or on PMMA plates. These were used to determine surface roughness by chromatic confocal imaging and to measure SPF in vitro of 2 sunscreens by diffuse transmission spectroscopy. Results: The recovered skin layers showed a lower roughness than full-thickness skin but yielded SPF in vitro values that more accurately reflected the SPF determined in vivo by a validated procedure than PMMA plates, although the latter had in part roughness values identical to those of intact skin. Combination of skin tissue with a high roughness PMMA plate also provided accurate SPF in vitro. Conclusions: Besides roughness, the improved affinity of the sunscreen to the skin substrate compared to PMMA plates may explain the better in vitro prediction of SPF achieved with the use of a biological substrate.

Introduction

Over the past few decades, lifestyle habits have undergone substantial changes with a marked trend for outside recreational occupations that have led to generally higher and uncontrolled exposure of people to solar radiation. Although ultraviolet (UV) sun radiation is vital with biological benefits such as the synthesis of vitamin D [1], it is also recognized that excessive exposure to solar radiation has detrimental health issues. UVA and partly UVB rays reach the human epidermis and dermis at an intensity that causes diverse immediate or long-term photodamage, as thoroughly compiled by Matsumura and Ananthaswamy [2].

Besides the appearance of the known erythema mainly as an immediate response to UVB exposure, a major adverse effect is DNA damage [3] that can, in the long run, lead to skin cancer. UVA radiation is mostly responsible for chronic photodamage such as skin pigmentation (age
spots), induction of oxidative stress [4], photoimmuno-
suppression [5], visible effects of premature skin aging [6]
and contribution to skin cancer by generation of radical
oxygen species [7].

Topically applied sunscreens constitute a suitable and
commonly employed measure to protect the skin from
sun damage. To date, the sun protection factor (SPF) is
still the predominant criterion used to describe the degree
of photoprotection afforded by a topical sunscreen. The
only validated procedure for SPF determination is an in
vivo measurement in human volunteers [8] based on ery-
themal response, a biological end point mainly attributed
to UVB radiation. In vivo methods have the drawbacks of
being costly, time-consuming and ethically questionable.
Therefore, there is considerable interest from the indus-
try in developing an in vitro approach to SPF testing.

Although industry players have put a lot of effort in
developing an SPF in vitro technique that correlates with
the clinical SPF in vivo, no attempt undertaken so far has
been validated, and many issues still remain [9]. One ma-
jor influence factor for the successful establishment of a
standard method for SPF in vitro testing is the choice of
a substrate for sunscreen application that best mimics hu-
man skin. The current use of roughened polymethyl-
methacrylate (PMMA) plates for this purpose failed to
yield satisfactory results [9]. The reason for the persisting
discrepancies between in vivo and in vitro data might be
that PMMA plates do not properly imitate human skin.

Attempts to use alternative substrates to better imitate
the skin surface have been reported. Very early studies
with hairless mouse epidermis for in vitro measurements
of SPF with a scanning spectrophotometer provided en-
couraging results [10, 11]. Other workers used human
epidermis as substrate and demonstrated a good correla-
tion between in vitro and in vivo protection factors that
were measured, however, only at one wavelength [12].

The aim of the present work was to investigate the use
of skin from porcine ears as a biological substrate for in
vitro testing of sunscreen performance. The pig ear skin
was compared to PMMA plates that are currently the in-
dustry standard for the in vitro measurement of SPF. Por-
cine skin is already extensively employed in pharmaco-
logical and toxicological research as an in vitro model of
human skin because of the high degree of similarity be-
tween the two tissues [13, 14]. A number of studies em-
ploying the pig as in vitro model of human tissue have
been summarized by Simon and Maibach [15]. These
studies report on the overall anatomical and physiological
resemblance between the pig and man. The likeness of the
stratum corneum (SC) between the skin of the porcine ear
and human skin encompasses several aspects. Corneo-
cytes of pig skin have a polygonal shape [16, 17] and size
[17, 18] which are close to the morphological examina-
tions reported for human corneocytes [17, 19]. Moreover,
thickness [13, 14, 16, 17], barrier function [13] and pen-
etration properties [20] of the SC have been found to be
allogenic in the pig and human.

A UV-transparent substrate, which is a prerequisite
for transmittance measurements, was obtained by isolat-
ing only the upper skin layers of pig ears using 2 different
preparation methods. In a first step, we characterized the
recovered upper skin layers with respect to thickness and
roughness and compared the results to data available for
human skin. In a second step, we measured the SPF in
vitro of 2 distinctive sunscreens using the different por-
cine skin substrates and a standardized solar irradiance
profile. The results were compared to SPF in vitro ob-
tained with PMMA plates and to the SPF in vivo of the
individual sunscreens and evaluated with respect to sub-
strate properties that are relevant for a proper prediction
of SPF.

### Materials and Methods

#### Chemicals and Equipment

The following reagents were used: trypsin 2.5% (10×) liquid
(Gibco, Zug, Switzerland); sodium chloride, sodium hydroxide
1 M, monobasic sodium phosphate and trypsin inhibitor from gly-
cine max. (soybean) 10,000 U/mg (Sigma-Aldrich, St. Gallen, Swit-
zerland); Tinosorb® S, Tinosorb® M, Uvinul® A Plus and Uvinul® MC80 (BASF AG, Ludwigshafen, Germany);
Eusolex 232 (Merck, Darmstadt, Germany).

Quartz plates were obtained from Helma Analytics (Zumikon,
Switzerland), PMMA plates from Helioscreen Laboratories (Mar-
seille, France), Schönberg Kunststoffe (Hamburg, Germany)
and Shiseido Irica Technology (Kyoto, Japan), and Petri dishes from
Nunc (Roskilde, Denmark).

The following equipment was used: electric shaver (Favorita II
GT104, Aesculap, Germany), epilator (Silk-épil7 Xpressive Pro,
Braun, Germany), dermatome (Air Dermatome, Zimmer Inc.,
UK), water purification equipment (Arium 61215, Sartorius, Goet-
tingen, Germany), Raman confocal laser scanning microspec-
trometer (Alpha 500R, WITec, Ulm, Germany), surface texture
analysis instrument (Altisurf® 500, Altimet SAS, Thonon-les-
Bains, France), UV transmittance analyzer (Labsphere UV-2000S,

#### Preparation of Biological Substrate

Ears of freshly slaughtered pigs were obtained from the local
slaughterhouse (Basel, Switzerland) no more than a few hours
postmortem. The study did not require the approval of the ethics
committee of animal research as the ears were taken from pigs not
specifically slaughtered for the purpose of this study. The ears were
washed under running tap water, shaved and epilated. The full-

DOI: 10.1159/000358273

Sohn/Korn/Imanidis
thickness skin of the dorsal side was removed from the underlying cartilage using a scalpel and served as the starting material for further preparation. Two different methods were used for tissue preparation. The methods and the used support materials are summarized in Table 1.

Method 1: Isolation of SC by Trypsin Treatment (Modified Method after Kligman and Christophers [21])

Sheets of full-thickness skin were dermatomed to a thickness of around 500 μm. This tissue was immediately used or stored at −20 °C until further use. After washing with water puriﬁed by reverse osmosis, the dermated skin was laid ﬂat with the SC facing upward on ﬁlter papers saturated with trypsin solution (0.5% in phosphate buffer at pH 7.4) in a glass Petri dish and stored for 4 h at 37 °C in a saturated vapor atmosphere. The digestion occurred from the dermis end of the tissue, ensuring that the SC remained undamaged. The top layer representing the SC was carefully removed using forceps and washed with puriﬁed water. Compared to Kligman and Christophers [21], the recovered SC slice was additionally immersed in trypsin inhibitor solution (0.01% in phosphate buffer at pH 7.4) for 2 h at 37 °C to stop the enzymatic reaction. The tissue was washed again with puriﬁed water and kept in phosphate buffer. Finally, pieces of SC were placed flat either on quartz plates or on PMMA SPF Master PA-01 plates for SPF in vitro measurement, or on polystyrene Petri dishes for thickness analysis. When a SC specimen was laid on PMMA plates, vacuum was applied to prevent air enclosure between the SC and the plate. The plates with the SC were stored at 4 °C in a desiccator over saturated sodium chloride solution (relative humidity of 80%) until use.

Method 2: Isolation of Epidermal Membrane by Heat Treatment

The sheet of full-thickness skin was immediately used or stored at −20 °C until further use. The skin was thawed if necessary at room temperature and immersed in a water bath at 60 °C for 60 s. Subsequently, the epidermal membrane was separated from the dermis by gentle peeling off [22]. The isolated epidermal membrane was then laid on quartz plates for SPF in vitro measurement or on polystyrene Petri dishes for thickness analysis. The prepared samples were stored at 4 °C in a desiccator over saturated sodium chloride solution until use.

Skin Tissue Thickness Measurement

Raman confocal laser scanning microspectroscopy (Alpha 500R, WITec) was employed for tissue thickness measurement. Raman spectra were recorded from 0 to 4,000 cm⁻¹ (spectral grating of 600 g/mm, spectral resolution of 3 cm⁻¹ per pixel) using a 532-nm excitation laser source, a Nikon EPI plan 100× 0.95 NA objective and an integration time of 1 s. The equipment permitted an x-y resolution of 340 nm and a z resolution of 500 nm. This technique combines Raman spectroscopy with confocal microscopy allowing a depth analysis of the sample.

The thickness of the isolated tissue was assessed by scanning the samples over a line of 40 μm in the x direction (with 120 points per line) and over a depth of 40 μm in the z direction (with 240 lines per image). The measurements were conducted in cluster analysis modus with the WITec control software. The raw data were evaluated with WITec Project Plus 2.04 software.

PMMA Plates

Three types of PMMA plates served as synthetic UV-transparent substrate. The plates are roughened on one side to mimic skin surface and differ in their manufacturing process and topographical property (Table 2).

Surface Topographical Assessment

We carried out surface topographical measurements of skin specimens laid on quartz or PMMA plates and of the PMMA plates by chromatic confocal imaging based on the white light chromatic aberration principle using the Alimsurf 500 instrument. This allowed noncontact surface topography measurement and analysis. The employed optical sensor allowed an axial resolution (z) of 5 nm and a lateral resolution (x–y) of 1.1 μm. The motorized x-y table permitted scanning of samples in the millimeter range based on which the 3-dimensional microtopographical surface structure of the samples was reconstructed.

In this study, an area of 5 × 5 mm was scanned in 0.5-μm increment steps and the arithmetical mean height over an area, Sa (equation 1, ISO 25178 guideline [23]), was selected as a representative measure of skin surface topography [24]. This measure was also used for the PMMA plates.

\[
S_a = \frac{1}{L_x L_y} \int_0^{L_x} \int_0^{L_y} |Z(x, y)| \, dxdy, 
\]

(1)

where Lx and Ly are the lengths in the x and y directions, respectively. Z(x, y) is the altitude of the sampling point measured from the sampling surface. Use of an areal parameter such as Sa for describing surface texture better serves the needs of the present study compared to, for example, the roughness over a profile (e.g. Ra in the ISO 4287 guideline [25]) which has also been used to describe the skin surface.

**Table 1. Skin preparation types used in the study**

<table>
<thead>
<tr>
<th>Skin preparation</th>
<th>Underlaid material</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-separated SC</td>
<td>Quartz</td>
<td>UV transmittance measurement</td>
</tr>
<tr>
<td>PMMA SPF Master PA-01</td>
<td>PMMA SPF Master PA-01</td>
<td>UV transmittance measurement</td>
</tr>
<tr>
<td>Petri dish</td>
<td>Petri dish</td>
<td>Thickness measurement</td>
</tr>
<tr>
<td>Heat-separated epidermal membrane</td>
<td>Quartz</td>
<td>UV transmittance measurement</td>
</tr>
<tr>
<td></td>
<td>Petri dish</td>
<td>Thickness measurement</td>
</tr>
</tbody>
</table>

Porcine Skin for in vitro Testing of Sunscreens

DOI: 10.1159/000358273
Sunscreen Formulations

We tested the in vitro and in vivo performance of 2 oil-in-water (O/W) sunscreens. The filter system and the SPF in vivo of the sunscreens measured in accordance with ISO24444:2010 guidelines [8] are given in Table 3.

Measurement of the SPF in vitro Using Spectral Transmission of Ultraviolet Radiation

SPF in vitro is derived from diffuse transmission spectroscopy measurements based on the model proposed by Sayre et al. [10].

\[
SPF_{in\,vivo} = \frac{\sum_{290\,nm}^{400\,nm} S_{er}(\lambda) \cdot S_{s}(\lambda) \cdot T(\lambda)}{\sum_{290\,nm}^{400\,nm} S_{s}(\lambda) \cdot T(\lambda)},
\]

where \(S_{er}(\lambda)\) is the erythema action spectrum as a function of wavelength \(\lambda\), \(S_{s}(\lambda)\) is the spectral irradiance received from the UV source at wavelength \(\lambda\), and \(T(\lambda)\) is the measured transmittance of the light through a sunscreen film applied on a suitable UV-transparent substrate.

The spectral UV transmittance was recorded from 290 to 400 nm in 1-nm increment steps through a substrate before and after application of a sunscreen using the Labsphere UV-2000S. The linear range of the device was checked by measuring the absorbance of increasing concentrations of the UVB filter ethylhexyl methoxycinnamate in ethanol solutions and plotting the measured absorbance data against the expected absorbance.

The blank transmittance spectrum before application of the sunscreen was recorded for the PMMA plates using the plates covered with glycerin and for the skin substrates using the bare skin specimens on quartz or PMMA SPF Master PA-01 plates without further treatment [22]. For the PMMA plates, a single blank transmittance spectrum was measured in the center of a plate and used for the evaluation of the SPF values of all plates of the same type. For the skin samples, a blank transmittance spectrum was recorded in each single measurement position. We applied 1.8 mg/cm\(^2\) of sunscreen on the skin samples; the amount of sunscreen applied on the PMMA plates is given in Table 2. The application of the sunscreen and the equilibration step were conducted as previously reported [26].

A surface area of substrate of 2.0 × 2.0 cm was used in the SPF measurements. This area was chosen because skin specimens of this dimension could be easily prepared. The impact of the surface area of the substrate on SPF in vitro was assessed by comparing PMMA plates with a size of about 5.0 × 5.0 cm which are routinely used, with plates cut to 2.0 × 2.0 cm.

Statistical Analysis

Statistical analysis was performed using Statgraphics centurion XVI (Statpoint Technologies Inc., Warrenton, Va., USA) software. The statistical significance at the 5% confidence level of the difference between 2 groups was evaluated using the Mann-Whitney test.

Results and Discussion

Skin Thickness

The thickness of human and porcine ear skin is commonly measured by light microscopy of histological sec-

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Table 2. Characteristics of PMMA plates

<table>
<thead>
<tr>
<th></th>
<th>Helioplate HD6</th>
<th>Schönberg</th>
<th>SPF Master PA-01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Helioscreen</td>
<td>Schönberg</td>
<td>Shiseido Irica</td>
</tr>
<tr>
<td>Manufacturing process</td>
<td>mold injection</td>
<td>sandblasting</td>
<td>mold injection</td>
</tr>
<tr>
<td>Surface size as supplied, cm</td>
<td>4.7 × 4.7</td>
<td>5.0 × 5.0</td>
<td>5.0 × 5.0</td>
</tr>
<tr>
<td>Surface size adjusted for the study, cm</td>
<td>2.0 × 2.0</td>
<td>2.0 × 2.0</td>
<td>2.0 × 2.0</td>
</tr>
<tr>
<td>Roughness given by the supplier (Ra or Sa), μm</td>
<td>Ra = 4.5</td>
<td>Ra = 5.9</td>
<td>Sa = 17.8</td>
</tr>
<tr>
<td>Amount of sunscreen for SPF in vitro testing, mg/cm(^2)</td>
<td>1.3</td>
<td>1.3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 3. Tested sunscreens

<table>
<thead>
<tr>
<th>Sunscreen designation</th>
<th>SPF in vivo (mean ± SD)</th>
<th>Contained UV filter, weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EHM C</td>
</tr>
<tr>
<td>O/W No. 1</td>
<td>27.5 ± 7.6</td>
<td>5</td>
</tr>
<tr>
<td>O/W No. 2</td>
<td>19.9 ± 5.8</td>
<td>–</td>
</tr>
</tbody>
</table>

EHMC = Ethylhexyl methoxycinnamate; BEMT = bis-ethylhexyloxyphenol methoxyphenyl triazine; MBBT = methylene bis-benzotriazolyl tetramethylbutylphenol; DHHB = diethylaminohydroxyhexyl benzoate; EHT = ethylhexyl triazone; PBSA = phenylbenzimidazole sulfonic acid.

The SPF in vivo was evaluated in accordance with ISO24444:2010 guidelines with n = 5.
tions of stained skin biopsies using formalin-paraffin or freezing preparations [14, 27]. The thickness of SC was measured by tape stripping requiring determination of the amount of removed corneocytes [28]. Such procedures are generally time-consuming and may introduce artifacts due to preparation or data evaluation. A noninvasive method based on confocal Raman spectroscopy that required no tissue preparation was introduced for measuring SC thickness in vivo in human volunteers [29]. This was based on the fact that water content remains constant in the viable epidermis [30].

In the present investigation, we employed a procedure for assessing the thickness of trypsin-separated and of heat-separated skin using also confocal Raman microspectroscopy. The Raman spectra acquired for the skin samples and polystyrene Petri dish are shown in figure 1. The Raman spectrum of the skin was identical for the trypsin separation and the heat separation procedure (data not shown). Raman profiles of skin and the polystyrene of the Petri dish differed noticeably (fig. 1). As an example, a peak that is characteristic of the skin is detectable at 1,650–1,690 cm\(^{-1}\) corresponding to the amide I band [31]. This amide I band is absent in the polystyrene of the Petri dish.

A cluster analysis was performed by the software in which the number of clusters was set equal to 3. This analysis detected spectral differences between the materials as a function of depth. Figure 2 shows the result of this analysis.

A clear differentiation between air, skin tissue and polystyrene is evident. From this representation, estimation of thickness of the skin specimens was possible after correction by multiplying the extent of the optical skin layer with the ratio of refractive index of SC to air, being equal to 1.55.

The trypsin separation and the heat separation procedures gave skin layer thicknesses of about 5.9 μm (n = 2) and 14 μm (n = 2), respectively. Both procedures allowed the separation of an upper skin layer from the full thickness skin; the heat separation, however, led to the recovery of a thicker tissue layer than the trypsin separation, which was consistent with results of other authors [21]. This is because the trypsin procedure enables recovering the SC exclusively, whereas the heat procedure leads to the recovery of almost the entire epidermis.

The thickness obtained via trypsin separation was smaller than previously published data on SC thickness of porcine ears using 2-photon microscopy [16], quantitative tape stripping [28], cryoscanning electron microscopy [32] or common histological examination [14]. This difference may be explained by the water evaporation occurring during the storage and equilibration of the skin specimens over salt solution in our experiment. This step was required for the subsequent SPF measurements (see below). These results demonstrate that the developed method makes it possible to measure the thickness of isolated skin layers at multiple locations in a fast, exact and convenient manner requiring no special preparation.
Surface Topographical Assessment

Different methods have been used for roughness measurement of human skin such as topographical analysis using the digital stripe projection technique [24], a stylus profilometry on skin replica [33], 3-dimensional optical in vivo topography analysis [34] and confocal scanning laser microscopy [35]. A nonexhaustive list of the invasive, semi-invasive and noninvasive methods is given in Corcuff and Piérard [36]. The ideal system to assess the real topography of skin should allow a noncontact measurement, a spatial resolution in the micrometer range, a range of measurement covering the amplitude of the skin relief, a 3-dimensional reconstruction and the collection of the data in a reasonable time. Most of these recommendations were fulfilled by the white light aberration principle of measurement used in the present study.

Sa roughness parameter values of the different substrates are reported in table 4. Sa of full-thickness skin of porcine ears had a value of about 22 μm. This result is in accordance with the data available for human skin roughness [35]. An illustration of the surface of full-thickness pig ear skin is given in figure 3. Figure 3 illustrates the differences in altitude (micrometer range on scale) and the highly organized architecture of the skin surface including the v-shaped furrows. This pattern is characteristic also of human skin as shown using optical laser profilometry [36] or scanning electron microscopy [30]. These results hence confirm that full-thickness skin of the porcine ear presents the same surface architecture as human skin.

Table 4. The arithmetical mean height Sa of the surface of selected substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sa, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-thickness pig ear skin¹</td>
<td>21.7</td>
</tr>
<tr>
<td>Human skin²</td>
<td>22</td>
</tr>
<tr>
<td>Heat-separated epidermal membrane on quartz plate³</td>
<td>2.56±0.74</td>
</tr>
<tr>
<td>Trypsin-separated SC on quartz plate⁴</td>
<td>1.26±0.20</td>
</tr>
<tr>
<td>Trypsin-separated SC on PMMA SPF Master PA-01⁵</td>
<td>19.2</td>
</tr>
<tr>
<td>PMMA Helioplate HD⁶</td>
<td>6.07±0.03</td>
</tr>
<tr>
<td>PMMA Schönberg plate⁵</td>
<td>6.05±0.51</td>
</tr>
<tr>
<td>PMMA SPF Master PA-01⁵</td>
<td>22.23±1.90</td>
</tr>
</tbody>
</table>

¹ n = 1 ear. ² Literature values. ³ n = 13 ears (61 single measurements). ⁴ n = 3 ears. ⁵ n = 3 plates.

Fig. 2. Visualization of cluster evaluation obtained from Raman spectral differences corresponding to air (top black zone), skin tissue (white zone) and polystyrene (bottom black zone). Vertical coordinate corresponds to depth in z direction (40 μm). a Trypsin-separated skin. b Heat-separated skin.
The roughness parameter Sa of the isolated tissue layers decreased compared to full-thickness tissue to 1.26 and 2.56 μm for trypsin-separated and heat-separated porcine skin, respectively. A 3-dimensional representation of the surface of a heat-separated sample is shown in figure 4. This figure illustrates that the typical topographical relief of full-thickness skin was lost as a result of the preparation procedure. The topographical relief of the full-thickness skin is principally characterized by clusters separated by invaginations resembling valleys also referred to as furrows which are extensions of the SC into the epidermis and can reach down into the basal layer [16]. By removing the connective tissue (dermis), these valleys disappear resulting in a flatter skin surface and a loss of skin roughness. Additionally, the trypsin-separated SC which is thinner than the heat-separated epidermal membrane (about 6 μm compared to 14 μm) showed a considerably smaller Sa value than the heat-separated skin layer. These results indicate that the thickness of the skin sample affects its roughness.

Two of the PMMA plates (Helioplate HD6 and Schönberg) exhibited an Sa value of approximately 6 μm. This value is smaller than the one of full-thickness skin but larger than those of the 2 skin preparations. By comparison, the SPF Master PA-01 PMMA plates, which were developed to mimic the topography of human skin [37], had an Sa value of roughly 22 μm, which was comparable to that of full-thickness skin. The Sa measures of all PMMA plates were in line with the data provided by the suppliers for the used batches.

Finally, to obtain a UV-transparent skin surface substrate having the Sa of full-thickness skin, trypsin-separated SC was laid on the SPF Master PA-01 PMMA plates. The measured Sa of this combined substrate was approximately 19 μm (table 4).

The effect of the different substrates and their Sa values on SPF in vitro is discussed below.

Measurement of SPF

Three types of skin preparations were used for SPF measurements, i.e. heat-separated epidermal membrane on quartz plates, trypsin-separated SC on quartz plates and trypsin-separated SC on PMMA plates (SPF Master PA-01). Directly after the preparation procedure, the skin samples looked translucent and became transparent during storage under controlled temperature and humidity conditions. The time required to reach sufficient transparency for UV transmittance measurements with trypsin-separated SC and heat-separated epidermal membrane samples was 24 h and 4 days, respectively, after preparation. This is related to thickness of the obtained tissue layer.

The impact of the size of the plate on SPF in vitro was firstly determined with the 3 types of PMMA plates using sunscreen O/W No. 1. The measurements were carried out by 2 operators. The SPF in vitro values of 5.0 × 5.0 cm (n = 3) plates were not found to be significantly different from SPF in vitro values of 2.0 × 2.0 cm (n = 3) plates independently of plate type and operator (Mann-Whitney, p > 0.05; results not shown). As a result, a sample area of 2.0 × 2.0 cm was used for further studies.
SPF in vitro data measured on PMMA plates and on skin preparations were compared to the SPF in vivo. The results for each substrate with sunscreens O/W No. 1 and O/W No. 2 are shown in figures 5 and 6, respectively. The relative standard deviation for O/W No. 1 and operator 1 was 32–38% for PMMA plates and 38% for skin substrate; for operator 2, it was 21–43% for PMMA plates and 32–57% for skin substrate. The relative standard deviation for O/W No. 2 and operator 1 was similar to that of O/W No. 1. A statistical analysis of the difference between SPF in vivo and SPF in vitro for each substrate is summarized in table 5.

The reproducibility was assessed using sunscreen O/W No. 1. It is worth pointing out that SPF in vitro obtained with PMMA plates may depend among other factors on the operator [9]. In the present study, there was a statistically significant difference between SPF in vitro values measured by the two operators for all types of PMMA plates (Mann-Whitney, p < 0.05) but not for the heat-separated epidermal membrane (Mann-Whitney, p > 0.05). This result might suggest that the biological substrate possibly provides more reproducible data than the habitually used synthetic plates even though standard deviations of the results by the 2 operators may vary.

For sunscreen O/W No. 1, trypsin-separated SC on PMMA SPF Master PA-01 plate and heat-separated epidermal membrane on quartz plate yielded SPF in vitro results that were not statistically significantly different from the SPF in vivo value. Of the skin-based substrates, only the result of trypsin-separated SC on quartz plate was significantly different from the SPF in vivo (fig. 5; table 5). These results demonstrate that synthetic plates were not adequate for testing the SPF of this sunscreen since none of the PMMA plates approached the SPF in vivo. With 2 out of the 3 skin
preparations, on the other hand, the SPF in vivo value was also obtained in vitro.

The SPF in vitro of sunscreen O/W No. 2 was evaluated by one operator using the 3 PMMA plate types and the heat-separated epidermal membrane on quartz. Also with this sunscreen, the skin preparation produced no statistically significant difference from the in vivo reference. Of the PMMA plates, the ones manufactured by mold injection (Helioplate HD6 and SPF Master PA-01) gave lower and the one manufactured by sand blasting (Schönberg) higher SPF in vitro values than the in vivo reference. This was different from the result obtained with sunscreen O/W No. 1. Nevertheless, the result of PMMA SPF Master PA-01 showed no statistically significant difference to the SPF in vivo while the other 2 PMMA plates did. Interestingly, the PMMA SPF Master PA-01 plate has the greater roughness (Sa = 22.23 μm) of the 2 mold-injected plates. Generally, an impact of substrate roughness on efficacy, reproducibility and repeatability of in vitro sunscreen measurements has also been reported by Fageon et al. [38] and Ferrero et al. [39].

The outcome of the SPF in vitro assessment with 2 different O/W sunscreens showed that the used biological substrate yielded results reaching in most cases the SPF in vivo value while the PMMA plates generally did not. Roughness differed considerably between the skin-based preparations (Sa 2.56 vs. 19.2); however, this did not seem to affect the determined SPF in vitro. Only trypsin-separated SC on quartz having the smallest roughness (Sa 1.26) did not reach the reference SPF value. This preparation may therefore not be suitable for SPF in vitro testing suggesting that a minimal roughness of the substrate may be required. Conversely, none of the synthetic substrates having different roughness characteristics achieved a satisfactory SPF in vitro with the 2 O/W sunscreens. Even the SPF Master PA-01 plate which has an Sa value and a surface pattern similar to that of human skin did not always yield accurate results. This implies that roughness may not be the sole critical surface characteristic of the substrate to be considered for achieving accurate SPF in vitro measurements.

Besides surface topography, the affinity of the sunscreen for the substrate seems to be equally important. Affinity refers to the propensity of the sunscreen to be distributed and to adhere to the substrate upon spreading. In vitro measurements carried out on skin-based substrates are assumed to better simulate the product-to-substrate affinity that applies to the in vivo situation. This may explain why these substrates, including the heat-separated epidermal membrane on quartz and the trypsin-separated SC on PMMA SPF Master PA-01 plates, resulted in more accurate SPF in vitro values compared to the PMMA plates.

It should be pointed out that the present data were collected with O/W formulations. An a priori transfer of the results to other types of formulations cannot be made at this point. For example, water-in-oil or single phase formulations might exhibit a different affinity and/or spreading behavior. Therefore, additional investigations are required for generalizing these observations.

The affinity aspect was further invoked in connection with the poor SPF in vitro value obtained with highly hydrophobic mold-injected plates that caused a lack of adherence of the product and consequently a nonuniform

### Table 5. Difference between SPF in vitro and SPF in vivo

<table>
<thead>
<tr>
<th>Sunscreen Substrate</th>
<th>Statistically significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O/W No. 1 Trypsin-separated SC on quartz</td>
<td>yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>Trypsin-separated SC on PMMA SPF Master PA-01</td>
<td>no (p &gt; 0.05)</td>
</tr>
<tr>
<td>Heat-separated epidermal membrane on quartz</td>
<td>no (p &gt; 0.05)</td>
</tr>
<tr>
<td>PMMA Helioplate HD6</td>
<td>yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>PMMA Schönberg</td>
<td>yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>PMMA SPF Master PA-01</td>
<td>yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>O/W No. 2 Heat-separated epidermal membrane on quartz</td>
<td>no (p &gt; 0.05)</td>
</tr>
<tr>
<td>PMMA Helioplate HD6</td>
<td>yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>PMMA Schönberg</td>
<td>yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>PMMA SPF Master PA-01</td>
<td>no (p &gt; 0.05)</td>
</tr>
</tbody>
</table>

Statistical significance between SPF in vitro and SPF in vivo at the 5% confidence level (Mann-Whitney test).
In addition to roughness and affinity, the influence of other experimental factors on SPF in vitro has already been examined such as the application process [38], the spectrum of the lamp source [42] or the amount of product applied [12, 22, 37]. Other possible influence factors such as the impact of pressure or the formation of the film during product application have not been fully explored yet constituting a still open area of research in the field.

The approach introduced in this study provided interesting insights into the in vitro methodology for predicting SPF in vivo. It could be useful in the final stage of product development for determining absolute SPF values prior to carrying out clinical studies. Also, use of the method may be recommended for sunscreen performance verification, for example, in case of change of raw material, vehicle composition or manufacturing process. Since, however, the method is rather laborious, it may not be appropriate for screening or large-scale product comparison tests.
Porcine Skin for in vitro Testing of Sunscreens

DOI: 10.1159/000358273