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# MAGAZINE

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Towards **Novel Bioactive Antiperspirants**  
for Cosmetic Applications

Predicting One`s **Future Hair Condition**

**New Innovative Sunscreen** for Protection  
of Skin Types IV-V

Marketing Personalized Skincare Products in  
Accordance with the **EC Cosmetic Regulation**

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# Towards Novel Bioactive Antiperspirants for Cosmetic Applications

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**Keywords:** Sweat gland, TMEM16A, antiperspirants, deodorants, aluminum

This work received the top award in the Applied Research category at the 30<sup>th</sup> IFSCC Congress in Munich, Germany, September 18-21, 2018.

## INTRODUCTION

Sweating is an important physiological process and evolved in humans as a way to regulate body temperature. In today's modern society, however, extensive sweating is mainly perceived as a cosmetic issue, but it can also be linked to the chronic sweating disorder hyperhidrosis. Extensive sweating is often associated with psychosocial pressure and can negatively impact the quality of life [1-3]. Common antiperspirants are based on aluminum salts as major active ingredients. Aluminum salts simply physically block the sweat gland pore and hence only prevent the release of sweat to the skin surface but they do not prevent sweat production (**Figure 1**). Their use is highly under debate due to potential health risks [4], but so far no alternatives exist.

Sweat is formed in the eccrine sweat glands, which can be divided functionally into the secretory coil, where the primary fluid is formed, and the connecting duct, where ion reabsorption occurs while the fluid is transported to the skin surface [5, 6]. Sweat formation in secretory cells is triggered mainly by cholinergic neurotransmitters, but adrenergic as well as purinergic signals can also regulate the secretory activity [5, 7-9]. First, Ca<sup>2+</sup> is released from intracellular stores, which leads to Ca<sup>2+</sup> influx into secretory cells

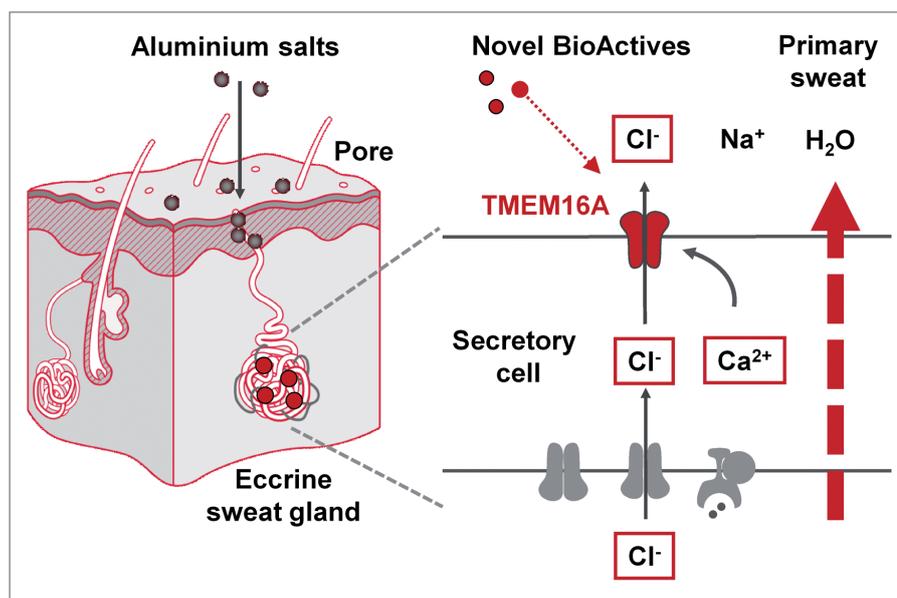
from the surrounding interstitium [5, 7, 10-12]. The increase in cytoplasmic Ca<sup>2+</sup> then stimulates Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels (CaCCs), which mediate efflux of Cl<sup>-</sup> through the apical membrane of secretory cells into the sweat gland lumen [5, 13] (**Figure 1**). The resulting electrochemical gradient drags Na<sup>+</sup> into the sweat gland lumen, leading to elevated luminal Na<sup>+</sup> and Cl<sup>-</sup> concentrations. This in turn establishes an osmotic gradient

that provides the driving force to move water into the sweat gland lumen, leading to isotonic primary sweat [5, 14, 15] (**Figure 1**). CaCCs can be considered key players of sweat formation since they mediate transepithelial Cl<sup>-</sup> transport, triggering primary fluid formation. Modulating the activity of CaCCs by small-molecule compounds may therefore be a promising new approach to reduce fluid formation (**Figure 1**).

## Abstract

*Sweating is a fundamental process required for human thermoregulation. In today's modern society, however, extensive sweating is rather considered unpleasant or embarrassing, or can even cause severe psychosocial pressure. Sweat reduction by antiperspirants is therefore of huge cosmetic interest. Currently, the global use of aluminum salts as antiperspirants is controversial, but no alternatives exist so far. We developed a new concept for sweat reduction which is based on directly targeting primary fluid secretion in human sweat glands. We identified a long searched for key player in human sweat glands - the ion channel TMEM16A, also known as ANO1. We extensively characterized TMEM16A and its function in native*

*human sweat glands and sweat gland tissue culture cells by using a wide variety of different techniques such as immunohistological staining, chloride flux assays, automated patch clamping as well as state-of-the-art CRISPR/Cas9 genome editing technology. We generated a proprietary cell-based assay to emulate TMEM16A function in a cellular sweat gland environment. We combined this cell-based assay with our cherry-picked compound libraries and performed high-throughput screening campaigns which uncovered small-molecule modulators of TMEM16A. In silico and in vitro toxicological assessments as well as stability and formulation tests were performed and yielded compounds that are currently being tested for their sweat reduction efficacy in vivo.*



**Figure 1** Mode-of-action of novel bioactives targeting TMEM16A in human eccrine sweat glands to reduce sweat formation. Schematic drawing of the human skin (left). Simplified molecular mechanism of sweat formation in secretory sweat gland cells (right).

Aluminum salts simply block the sweat gland pore but do not reduce primary fluid formation in the secretory coil of the eccrine sweat gland. TMEM16A is the long searched for CaCC. TMEM16A is activated by intracellular  $\text{Ca}^{2+}$  and permits the efflux of  $\text{Cl}^-$  into the sweat gland lumen. This establishes an electrochemical and osmotic gradient which provides the driving force to move water into the sweat gland lumen. Small molecules that act as antagonists of TMEM16A reduce the activity of TMEM16A, leading to reduced  $\text{Cl}^-$  secretion and less primary fluid secretion.

$\text{Ca}^{2+}$ -activated chloride channels mediate transepithelial chloride secretion in various tissues [13, 14, 16] but their molecular identity has long remained elusive. Transmembrane protein 16A (TMEM16A), also known as anoctamin 1 (ANO1), was identified as the first CaCC [17-19]. TMEM16A is composed of eight transmembrane segments flanked by N- and C-terminal intracellular domains [20-22]. Alternative splicing gives rise to multiple TMEM16A splice variants [17, 23] which differ in their ion channel properties, such as  $\text{Ca}^{2+}$  sensitivity, voltage dependence and activation/deactivation kinetics [23-25]. Expression of TMEM16A has been reported for various cell types and tissues. We were the first to describe that TMEM16A is also expressed in human eccrine sweat glands and in the sweat gland model cell line NCL-SG3 and that several TMEM16A splice variants mediate  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  secretion in NCL-SG3 cells [26].

This study was performed to further corroborate the importance of TMEM16A as the major CaCC in human eccrine sweat

glands by immunohistochemistry and electrophysiological analysis as well as CRISPR/Cas9-based knock-out studies. Moreover, the study was designed to identify small-molecule compounds that act as TMEM16A antagonists in a high-throughput screening. In addition, the evaluation of their suitability as novel active ingredients for a new type of antiperspirant was started.

## EXPERIMENTAL

### Cell culture studies

General cell culture conditions, the generation of stable NCL-SG3 cell lines and the cell-based assays to measure TMEM16A ion channel activity (fluorescence-based  $\text{I}^-/\text{Cl}^-$  flux assays using the halide-sensitive YFP as well as high-throughput gigaseal patch clamping using the SyncroPatch 384PE from Nanion Technologies, Munich, Germany) were described earlier [26].

### Immunohistochemistry

Biopsies from apparently healthy skin of volunteers were taken at the Department of Dermatology and Allergology of the

Ludwig-Maximilians-University Munich (Germany) according to the Declaration of Helsinki. Ethical committee permission was granted and patients gave their written informed consent. Paraffin-embedded skin sections were deparaffinized using Roticlear® (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) followed by subsequent washing steps in ethanol (AppliChem, Darmstadt, Germany). To unmask antigens, samples were boiled in EDTA solution pH 8 (AppliChem, Darmstadt, Germany). After blocking with nonfat dry milk (Santa Cruz Biotechnology, Dallas, TX, USA), primary anti-TMEM16A antibody was applied (ab53212, Abcam, Berlin, Germany) and antibody binding was visualized using the ChemMate™ DAKO EnVision™ Detection Kit (DakoCytomation, Glostrup, Denmark).

### Genome engineering using CRISPR/Cas9

General genome-editing using CRISPR-Cas9 and subsequent preparation of genomic DNA and PCR analysis were described earlier [27]. Single-guide RNAs targeting different regions of the TMEM16A gene were designed and the corresponding coding DNA sequence was cloned into proprietary sgRNA/Cas9 expression vectors containing CMV and U6 promoters to drive Cas9 and sgRNA expression, respectively. After electroporation of NCL-SG3 cells, the cleavage efficiency of the Cas9 nuclease was determined by Sanger sequencing and subsequent TIDE analysis [28].

### Toxicological and safety assessments

Cytotoxicity of the hit compounds was determined by using Neutral Red solution (Merck, Darmstadt, Germany) according to the manufacturer's instructions. In brief, 3,000 BALB/3T3 fibroblasts (clone A31) were seeded in 96-well plates. After 24 h, cells were treated with hit compounds in different concentrations ranging from 10 to 250  $\mu\text{M}$  for an additional 48 h. Then the Neutral Red solution was applied for 3 h and the absorbance at 540 nm was measured.

*In silico* toxicological analysis of the hit compounds was performed using the commercial tools DEREK and SARAH (Lhasa Ltd., Leeds, UK). The software reveals concerns according to structure,

substructure(s) and/or fragment(s) by correlation with available toxicological data and/or published studies.

All available toxicological data and compound information was subjected to toxicological assessment by an independent expert toxicologist, who evaluated the existing data and determined the maximal applicable amount of the hit compound in a given cosmetic formulation to be used in sweat reduction tests with human volunteers.

### Chemical and physical stability tests

To investigate long-term stability, the hit compounds as well as the o/w emulsions were stored at different temperatures (4°C, 40°C, 50°C and RT) and the parameters color, odor, homogeneity of the emulsion, pH and viscosity monitored over several weeks. During the observation period no phase separation, compound precipitation or any other noticeable changes were observed for the two development candidates.

## RESULTS AND DISCUSSION

### Expression and localization of TMEM16A in human eccrine sweat glands

We recently showed that the TMEM16A gene is transcribed in isolated human eccrine sweat glands as well as in NCL-SG3 cells [26]. To more precisely localize TMEM16A within the sweat gland substructures, we performed immunohistological staining in human skin biopsies. TMEM16A antibody staining was not detectable in eccrine sweat gland ducts, whereas secretory coils were heavily stained. Strikingly, TMEM16A was located predominantly in apical and not in basolateral membranes of secretory cells. A similar localization pattern of TMEM16A in human sweat glands has been reported by using a different TMEM16A antibody [29]. These results are in perfect agreement with our proposed function of TMEM16A as an ion channel that mediates transepithelial chloride secretion in human eccrine sweat glands.

### CRISPR/Cas9- and pharmacological inhibitor-based loss of function studies in sweat gland NCL-SG3 cells

To determine whether TMEM16A is indeed functionally important for Ca<sup>2+</sup>

-dependent Cl<sup>-</sup> secretion in sweat gland cells, we treated NCL-SG3 cells with various pharmacological CaCC and TMEM16A antagonists and performed I<sup>-</sup>/Cl<sup>-</sup> flux analysis using the halide-sensitive hsYFP. Strikingly, the TMEM16A inhibitor T16AlnhA01 as well as the CaCC inhibitor niflumic acid fully suppressed Ca<sup>2+</sup>-dependent Cl<sup>-</sup> secretion in a dose-dependent fashion [26]. These results strongly suggest that TMEM16A is directly involved in Ca<sup>2+</sup>-dependent Cl<sup>-</sup> secretion.

To further corroborate the role of TMEM16A as the CaCC in secretory sweat gland cells, we additionally performed genetic loss-of-function studies in NCL-SG3 using the CRISPR-Cas9 genome editing technology. We successfully generated heterozygous NCL-SG3 knock-out cells that carry one mutant TMEM16A allele (NCL-SG3-TMEM16A<sup>+/-</sup>). Strikingly, in NCL-SG3-TMEM16A<sup>+/-</sup> cells CaCC activity was reduced exactly by half compared with wild type cells. Similar results were obtained with an RNAi approach in NCL-SG3 cells [30]. Moreover, it was shown that CRAC channels, in particular ORAI1 and STIM1, are responsible for Ca<sup>2+</sup> influx, which is then a prerequisite for activation of TMEM16A [30]. Taken together, gene expression analysis and loss-of-function studies provide compelling evidence that TMEM16A is the long searched for CaCC in human eccrine sweat glands.

### Ion channel properties of TMEM16A splice variants

Our gene expression analysis revealed that several TMEM16A splice variants are expressed in isolated whole eccrine sweat glands as well as in NCL-SG3 cells [26]. We identified known variants and also one novel TMEM16A(acΔe3) splice variant which lacks the recently mapped dimerization domain encoded by exon e3 [31]. To determine whether TMEM16A splice variants differ in their ion channel properties, we generated NCL-SG3 cells which stably express either TMEM16A(acΔe3) or the canonical TMEM16A(ac), in addition to all endogenously expressed TMEM16A splice variants [26]. The fluorescence-based I<sup>-</sup>/Cl<sup>-</sup> flux assays as well as high-throughput gigaseal patch clamping revealed that TMEM16A mediates Ca<sup>2+</sup>

-activated Cl<sup>-</sup> conductance in NCL-SG3 cells. Interestingly, recombinant expression of TMEM16A splice variants showed that TMEM16A(acΔe3) forms a functional CaCC only in NCL-SG3 cells but not in HEK293 cells. Moreover, basal Cl<sup>-</sup> currents as well as Cl<sup>-</sup> currents induced by internally perfused Ca<sup>2+</sup> are modified in the novel TMEM16A(acΔe3) isoform compared with the canonical TMEM16A(ac). Taken together, these results suggest that eccrine sweat gland cells are characterized by cell type-specific transepithelial Cl<sup>-</sup> transport achieved by a complex interaction of different TMEM16A isoforms in conjunction with accessory, sweat gland-specific factors.

### Cell-based assay to identify TMEM16A antagonists in high-throughput screenings

Since we provided compelling evidence for a similar, sweat gland-specific composition and function of TMEM16A isoforms in human eccrine sweat glands and in NCL-SG3 cells, we used NCL-SG3 cells as a chassis cell line and developed a proprietary Sweat Gland ScreenLine<sup>®</sup> that permits measuring sweat gland-specific TMEM16A activity using the fluorescent halide-sensitive hsYFP in a high-throughput-compatible format [32]. In the next step we initiated a high-throughput screening including approx. 15,000 small molecules from our proprietary compound library (CompActives<sup>®</sup>). After primary and secondary screening, approx. 200 small molecule compounds were rated as hit compounds.

### In vitro efficacy, toxicological and safety assessment of hit compounds

A subsequent validation phase yielded approx. 30 hit compounds of which 16 passed *in vitro* cell-based toxicological assessment, i.e. these compounds showed no cytotoxicity in Neutral Red assay. These 16 development candidates were structurally diverse and showed TMEM16A inhibition in a dose-dependent fashion with IC<sub>50</sub> values in the low μM range. Based on the outcome of our *in silico* toxicological analysis, two promising development candidates were further subjected to and, importantly, passed the toxicological safety assessment by an expert toxicologist,

allowing us to use these compounds in future sweat reduction tests with human volunteers.

### Formulation development and preparation phase for *in vivo* studies

Next the two development candidates were further assessed with respect to their usability in cosmetic formulations. First, a deodorant formulation was developed to allow compound application in *in vivo* application studies. Although there is a huge variety of deodorant formulations such as sprays, aerosols and sticks, we chose a common oil-in-water emulsion (o/w emulsion) suitable for roll-on application since it is suited for a wide variety of active ingredients regardless of their lipophilic or hydrophilic preferences. Appropriate solvents which are commonly used in the cosmetic field were evaluated for their suitability to dissolve the compounds. Complete solubility of the compounds in the cosmetic solvent with no precipitation was essential, also after long-term storage. Finally, the dissolved compounds were formulated into the o/w-emulsion and the chemical and physical long-term stability was proven.

### CONCLUSION

To develop aluminum-free antiperspirants, we have taken a novel approach that is based on targeting fluid secretion mediated by CaCCs in the secretory coil of eccrine sweat glands. Our research and subsequent work of others undoubtedly show that TMEM16A is the long searched for CaCC in human eccrine sweat glands. We generated and patented a proprietary cell-based assay to emulate TMEM16A function in a cellular sweat gland environment [32] and performed an initial high-throughput screening of our proprietary CompActives® library. Bioactives that act as antagonists of TMEM16A were identified and toxicologically assessed. Currently, first promising bioactives are being analyzed in sweat reduction tests to prove their *in vivo* efficacy.

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# 25<sup>TH</sup> IFSCC CONFERENCE

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30 SEPTEMBER 1-2 OCTOBER



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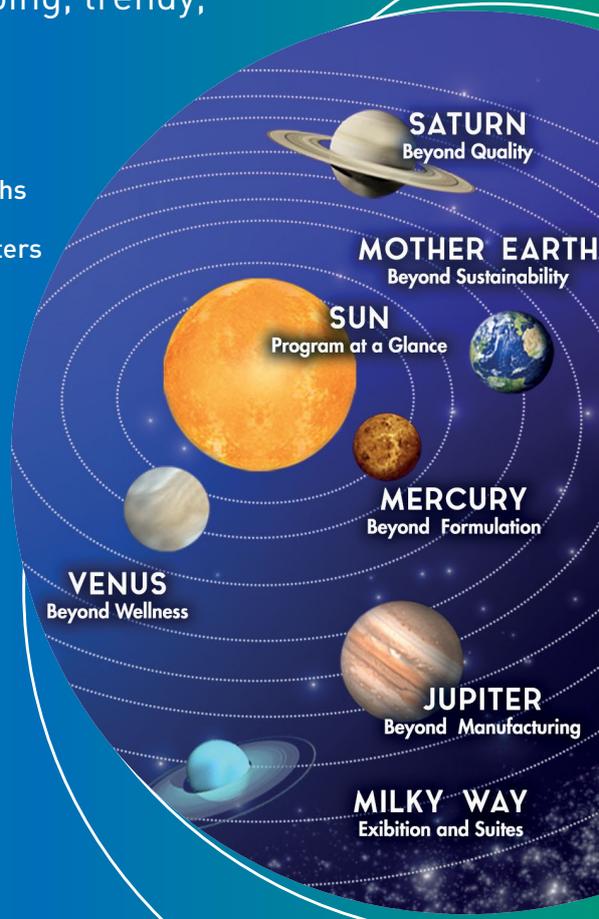
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9 Asteroids for a total of 45 booths facing 9 Gourmet stations and allocating the scientific posters

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# Predicting One's Future Hair Condition

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**Keywords:** Hair, scalp, prediction, grey hair, aging

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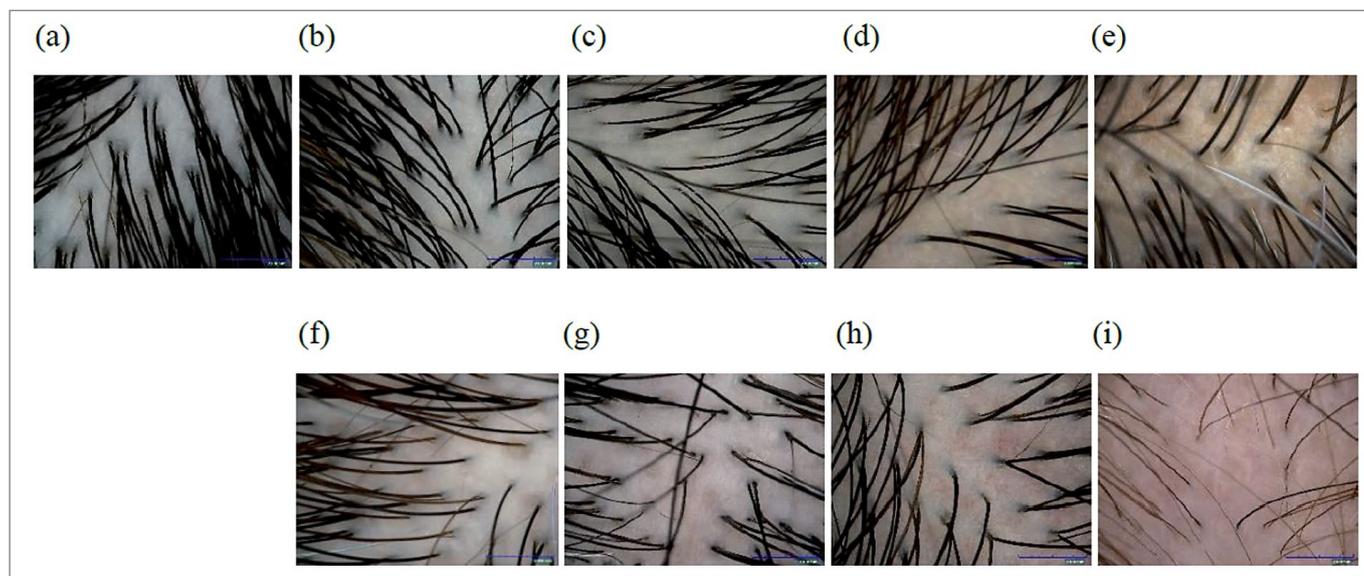
## INTRODUCTION

In recent years as women's lifestyles have globally diversified, there has been an increasing need to customize products to individual consumers, including a demand for customized cosmetics. To support this trend, the use of genetic testing has increased annually in Japan. Such testing reflects the consumer demand for effective services based on scientific evidence, and it can be anticipated that in the future consumers will have a greater objective knowledge of their own specific needs. In the field of facial skin care, techniques are being developed that will allow the skin type to be evaluated based on the palpation of skin elasticity

## Abstract

We undertook a comprehensive study of the hair and scalp to develop a system of personalized hair analysis, measuring in thousands of Japanese women a range of skin properties, including sebum and moisture content, and conducting image analyses of the scalp. In addition, expert panels evaluated 20 characteristics of hair, including their elasticity, smoothness and gloss. Correlations of each characteristic were evaluated, and parameters that could be an indicator of the hair

analysis technique were examined. The key findings were as follows: (1) good correlation of the area of black hair with age, hair volume, hair thinning, hair graying and hair loss, (2) association of yellowing of the scalp with the progression of age-induced changes in hair shape that in turn produces a reduction in hair gloss, and (3) association of the redness value of the scalp with accelerated hair graying. Based on these findings, we developed a technique to analyze the hair age and to predict future changes in hair gloss and gray hair ratio from scalp images.



**Figure 1** Scalp color level scale: (a) healthy scalp color level 1, (b) yellow level 2, (c) yellow level 3, (d) yellow level 4, (e) yellow level 5, (f) red level 2, (g) red level 3, (h) red level 4 and (i) red level 5.

[1] or analysis of the surface morphology in microscope-based images of skin [2]. Such technologies are used to support in-store product recommendations and to select appropriate treatments at aesthetic salons.

In the field of hair cosmetics several methods for diagnosing patients with alopecia have been developed [3]. However, there currently are limited hair analysis techniques aimed at cosmetics and few studies on the hair and scalp of healthy women have been reported. Therefore, to support the development of personalized hair analysis we undertook a comprehensive study of the hair and scalp of thousands of healthy Japanese women living in western Japan. We measured a range of skin properties, including sebum and moisture content, and conducted image analyses of the scalp. In addition, expert panels evaluated 20 characteristics of hair, including their elasticity, smoothness and gloss. The correlations of each characteristic were evaluated, and parameters that could be an indicator of hair analysis technique were examined.

## EXPERIMENTAL METHODS

### Subjects

Two thousand four hundred and sixty-one Japanese females ranging in age from 20 to 79 and living in western Japan participated in this study (UMIN ID: UMIN000030179).

### Analysis of microscopic images

The parietal region of each scalp was photographed with a digital microscope (KH – 8700, HIROX Co.,Ltd., Tokyo, Japan) and a Smart Skin Care® microscope equipped with a moisture meter (S2C-1, IT Access Co.,Ltd., Yokohama-shi, Japan). Each image was divided into a scalp area and a hair area according to the brightness threshold value and analyzed as follows. Using image analysis software (WinROOF 2015), the average b \* value of the scalp area was calculated and set as the index value of yellowness. The hue, saturation, and lightness values were analyzed and the index value of redness was calculated using equation:

**Table I** Correlation between sensory evaluation results and age

		Mean	SD	Correlations
1	Hair softness <sup>1</sup>	2.970	1.016	-0.171
2	Hair type (coarse or fine) <sup>1</sup>	2.464	0.940	-0.239 *
3	Hair amount <sup>1</sup>	2.882	0.867	-0.293 *
4	Hair volume at the crown <sup>1</sup>	2.984	1.459	-0.193
5	Hair length <sup>1</sup>	7.925	3.592	-0.434 *
6	Hair brightness (tip) <sup>2</sup>	2.099	1.046	-0.302 *
7	Hair brightness (All head) <sup>1</sup>	2.914	1.090	-0.330 *
8	Hair gloss <sup>1</sup>	3.858	1.066	0.232 *
9	Curly Hair (crown) <sup>1</sup>	3.961	0.957	-0.243 *
10	Curly Hair (hair line) <sup>1</sup>	3.934	1.016	-0.252 *
11	Curly Hair (arch) <sup>1</sup>	2.148	1.389	-0.323 *
12	Grey hair ratio <sup>1</sup>	1.875	0.982	0.780 *
13	Age-specific hairs <sup>1</sup>	3.691	1.020	0.431 *
14	Hair smoothness <sup>1</sup>	2.283	0.926	-0.070
15	Hair damage level <sup>3</sup>	2.872	1.384	0.178
16	Hair collectability <sup>1</sup>	2.881	1.196	-0.151
17	Scalp skin color (Yellowness) <sup>1</sup>	3.201	1.163	0.378 *
18	Scalp skin color (Redness) <sup>1</sup>	7.944	3.065	0.419 *
19	Scalp skin mobility (forehead)	11.592	3.338	-0.190
20	Scalp skin mobility (temporal)	3.093	1.227	-0.212 *

Note: \* $P < 0.01$ , N=2461

Measured on: <sup>1</sup> a five-point scale, <sup>2</sup> a eighteen-point scale, <sup>3</sup> a four-point scale, <sup>4</sup> a twenty-four-point scale.

**Table II** Correlation between instrument measurement results and age

		Mean (arbitrary unit)	SD	Correlations
1	Scalp skin brightness	58.185	3.209	0.207 *
2	Scalp skin yellowness	9.609	1.953	0.463 *
3	Scalp skin redness	2760	2868	0.539 *
4	Black hair area	29.438	7.056	-0.567 *
5	Number of hairs	45.325	16.101	-0.306 *
6	Grey hair ratio	0.110	0.148	0.574 *
7	Hair diameter	18.695	2.980	-0.099
8	Scalp moisture content	1.344	0.702	0.047
9	Scalp sebum content	9.180	5.725	-0.319 *
10	Scalp pH	5.085	0.495	0.007

Note: \* $P < 0.01$ , N=2461

**Table III** Correlation between the black hair area and evaluation parameters of each hair

		Mean	SD	Correlations
1	Hair softness <sup>1</sup>	3.533	0.978	0.121
2	Hair type (coarse or fine) <sup>1</sup>	2.970	1.016	0.224 *
3	Hair amount <sup>1</sup>	2.464	0.940	0.306 *
4	Hair volume at the crown <sup>1</sup>	2.882	0.867	0.299 *
5	Grey hair ratio <sup>1</sup>	2.148	1.389	-0.689 *

Note: \* $P < 0.01$ , N=2461

<sup>1</sup>Measured on a five-point scale.

Index value of redness =  
Average saturation of red ×  
Ratio of the area occupied by red

The area of the black hair region within the visual field of the image was also calculated.

### Sebum content

The sebum content at the top of the scalp was measured with a sebumeter (SM 815 MP, Courage + Khazaka Co., Cologne, Germany) and a Smart Skin Care® microscope (S2C-1, IT Access Co.,Ltd.).

### Moisture content

The moisture content of the parietal region of each scalp and forehead was measured with a horny layer thickness/moisture content meter (ASA-M2, ASA-HIBIOMED Co., Ltd., Yokohama, Japan) and a Smart Skin Care® microscope (S2C-1, IT Access Co.,Ltd.).

### Scalp and hair panel evaluation

Indicators were set for 20 parameters, including color of the scalp, elasticity of hair, smoothness and gloss, and sensory evaluation, and assessed by two full-time evaluators. Shown in *Figure 1* as an example is the scalp color level scale.

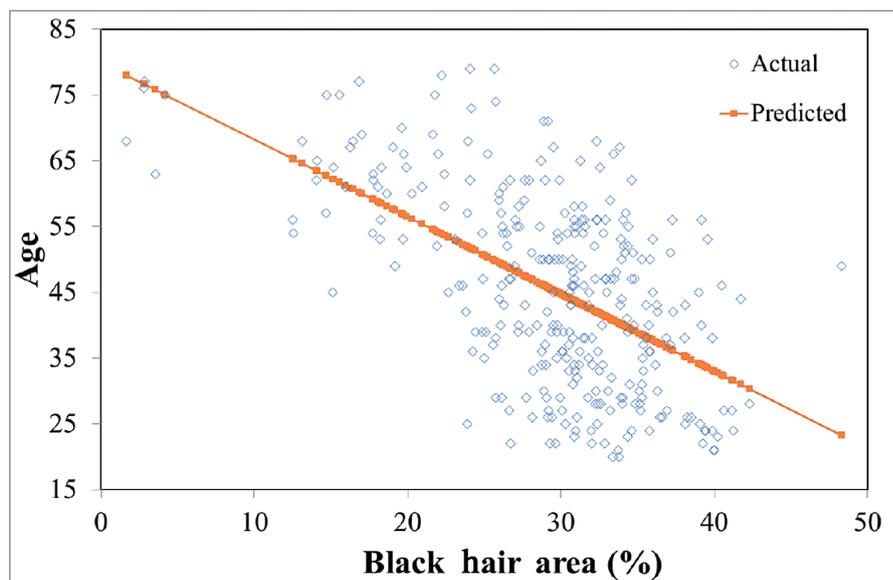
## RESULTS AND DISCUSSION

Correlations between the age and the organoleptic evaluation results and the instrument measured values are compiled in *Table I* and *Table II*, respectively. Parameters in these tables with a correlation coefficient *r* of 0.2 or more are listed with an asterisk. As is generally known, in sensory evaluations softening of the hair quality accompanies aging along with decreases in the amount of hair and hair gloss and increases in the degree of stiffness and age-specific hairs with an uneven shape. In the instrument measured values, correlations were found between the analysis results of the scalp images and the sebum content with age. In facial skin, changes in sebum content and moisture content with aging have been reported [4] but in the scalp, although the sebum content similarly decreased as in the case of facial skin, there was no significant change in the moisture content. Unlike the face, the scalp is covered with hair and is an environment with a large amount of sebum. As the scalp environment changes with aging it is thought to affect the moisture content.

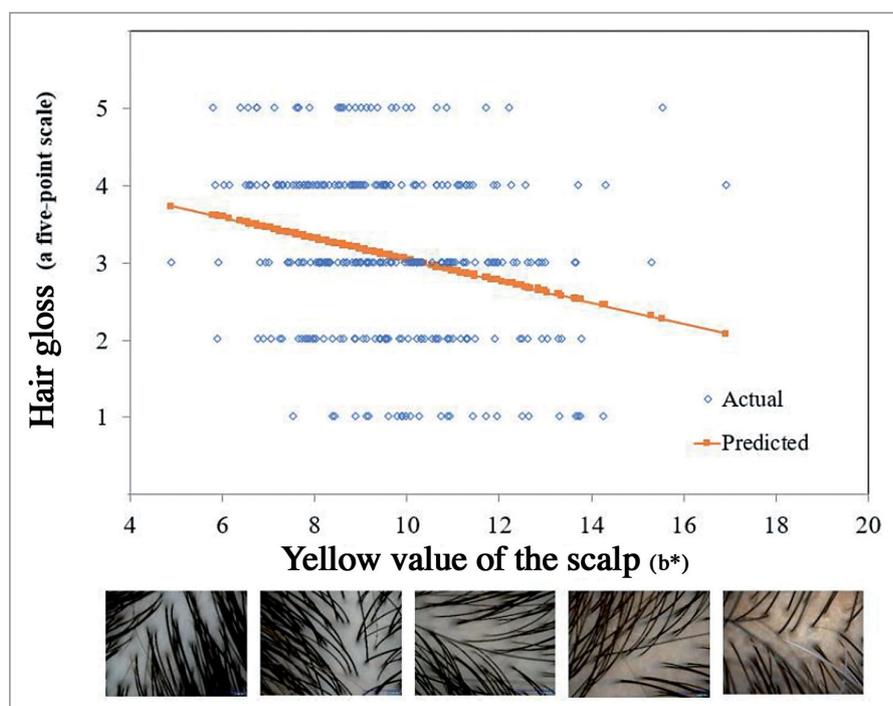
The parameter in *Table II* with the highest correlation coefficient *r* with age was the area of black hair in the visual field of

the scalp image. Therefore, we examined the correlation between the area of black hair and the sensory evaluation parameters. Many changes in the hair, such as the decrease in hair volume, hair thinning, hair graying and hair loss, occurred with age, but all those indicators correlated well with the area of black hair (*Table III*). Although this study was limited to Asian black hair, the area of black hair will be the optimal index of hair age (*Figure 2*).

Next, correlations between the scalp and the hair were investigated for their ability to predict the characteristics of the hair from the scalp. Yellowing of the scalp was associated with the progression of age-induced changes in hair shape that in turn produced a reduction in hair gloss (*Figure 3*). As in the case of facial skin [5], carbonylation of proteins in the scalp produces a yellowish tinge [6]. The accumulation of reactive oxygen species may also



*Figure 2* Regression of age on black hair area.  $y = -1.174x + 79.960$  ( $r > 0.56$ ,  $P < 0.01$ ).



*Figure 3* Regression of hair gloss on yellow value of the scalp. Gloss 1 is low and gloss 5 is high.  $y = -0.137x + 4.405$  ( $r > 0.24$ ,  $P < 0.01$ ).

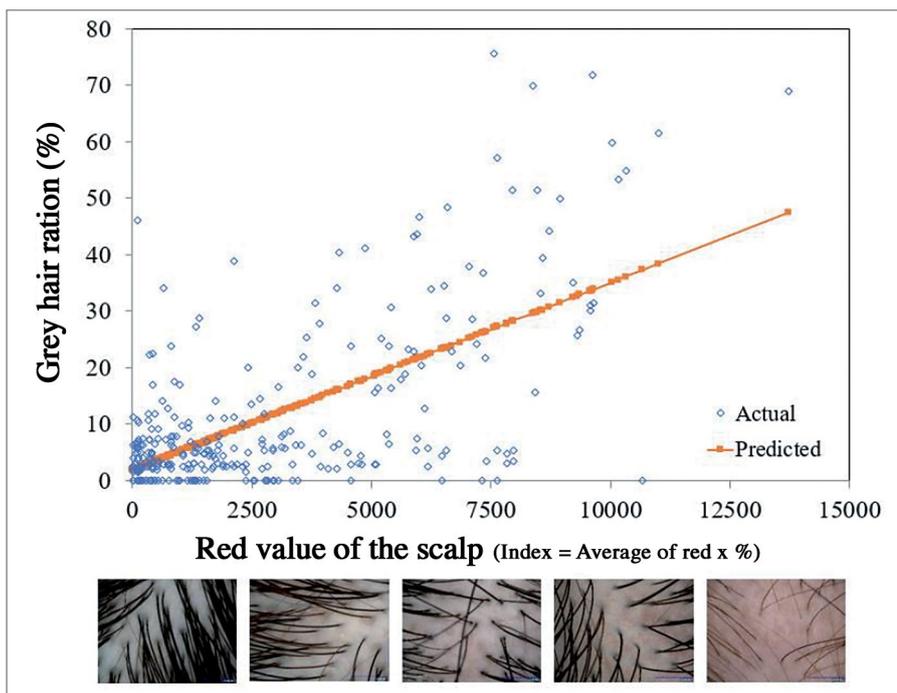


Figure 4 Regression of grey hair ratio on red value of the scalp.  
 $y = 0.00332x + 4.405$  ( $r > 0.64$ ,  $P < 0.01$ ).

introduce protein carbonylation, which alters hair shape by denaturing keratin proteins.

An increase in the redness value of the scalp was associated with accelerated hair graying (Figure 4). It is assumed that redness reflects inflammation, which may damage melanocytes similar to the case of vitiligo. Furthermore, it has been reported that the balance of bacterial flora affects the redness of the scalp [7,8] and is expected to be useful for the early detection of hair graying.

The results of this study thus suggest that it is possible to develop a method to predict hair age, gloss conditions and the gray hair ratio by analyzing a single scalp image.

The techniques of calculating hair age and predicting risks were applied to results obtained with the S2C-1 Smart Skin Care® microscope equipped with a moisture meter (Figure 5). To reveal regional differences in scalp hair conditions from place to place, scalp hair images of women living in different areas in Japan were investigated using the device (Figure 6). Women in the Kinki area showed the highest red and yellow values of the scalp, although there was no significant difference in the black hair area (data not shown). These results may have been affected by the fact that the investigation in the Kinki area was conducted in a city, while the investigations in the Chubu and Chugoku areas were performed in the countryside. People in cities are subjected to more stress and pollution.



Figure 5 Smart Skin Care® microscope (IT Access Co., Ltd.).

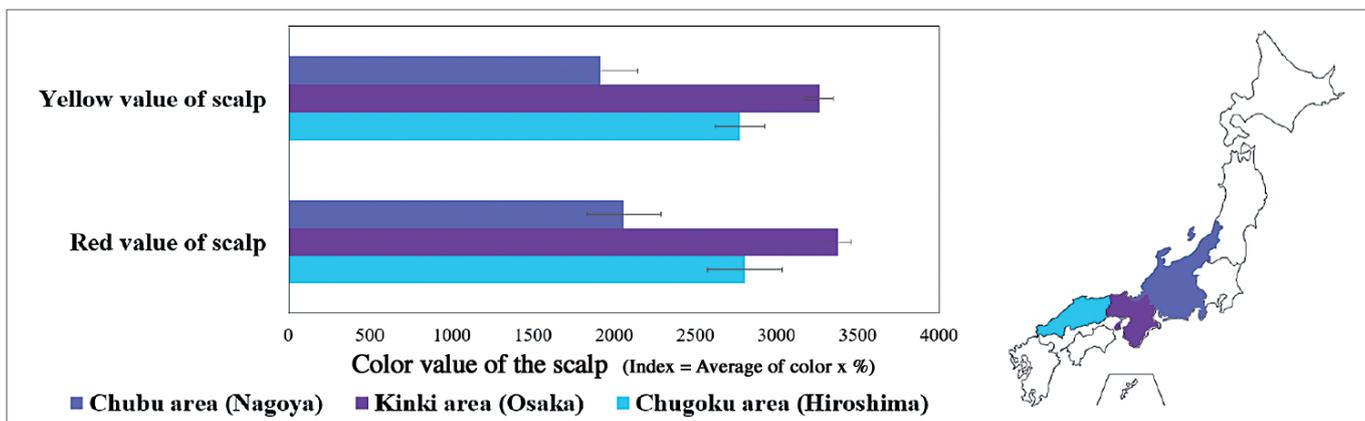


Figure 6 Regional differences in scalp hair condition in Japan: Chubu (N=76), Kinki (N=888) and Chugoku (N=191) area. All images were taken from april to august 2018. The average age in each region was 37-38. \* $P < 0.01$ .

This study will be expanded to include subjects of various ages, nationalities and hair colors in order to build a practical hair analysis technique.

## CONCLUSIONS

A comprehensive survey of the hair and scalp was performed to develop a hair analysis technique. We found three parameters that can be indicators:

- the area of black hair within the microscope image of the scalp for hair age,
- the yellowness value of the scalp for the gloss conditions of hair and
- the redness value of the scalp for the gray hair ratio. Using a method for hair age calculation and to predict hair gloss and the gray hair ratio, every consumer will be able to determine effective ways to achieve beautiful hair based on scientific measurement results.

## Acknowledgments

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# New Innovative Sunscreen for Protection of Skin Types IV - V

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**Keywords:** Electron paramagnetic resonance spectroscopy, resonance Raman spectroscopy, radical protection factor, scattering properties, radical formation

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## Abstract

In view of the globally increasing incidence of skin cancer, sun exposure and the application of sunscreens are steadily gaining in importance. Today it is known that skin damage is caused not only by UV-induced erythema but also by increased amounts of free radicals that are formed by solar radiation in the visible and near infrared (NIR) spectral regions and that sun protec-

tion could be different for different skin types. The antioxidant capacity and scattering properties of formulations were determined to be important protection not just against UV light. Radical protection in the visible and NIR range was determined *ex vivo* using EPR spectroscopy. The cooling effect of the formulation on Asian skin *in vivo* was analyzed and the NIR protection determined by measuring the cutaneous carotenoids before and after NIR radiation. Pechoin was the

first company to develop a patented sunscreen for skin types III–VI. This formulation protects both well from UV and effectively from infrared solar radiation. The formulation consists of a well-balanced selection of titanium dioxide pigments, highly efficient antioxidants as used in traditional Chinese medicine, and coolants. Thanks to all these ingredients the concentration of radicals can be clearly reduced, especially in darker skin types.

## INTRODUCTION

Life on earth would not be possible without solar radiation. It is responsible for the formation of vitamin D in human skin and for our wellbeing (1). However, solar radiation can also damage the human organism if its dose is higher than a critical value. Possible consequences include sunburn, immunosuppression and even skin cancer (2, 3) (Figure 1).

Today, it is known that 50% of the free radicals produced in the human skin by solar radiation are formed by visible (VIS) and infrared (IR) light (4, 5). Therefore, effective protection is needed also for these spectral regions. The right application and

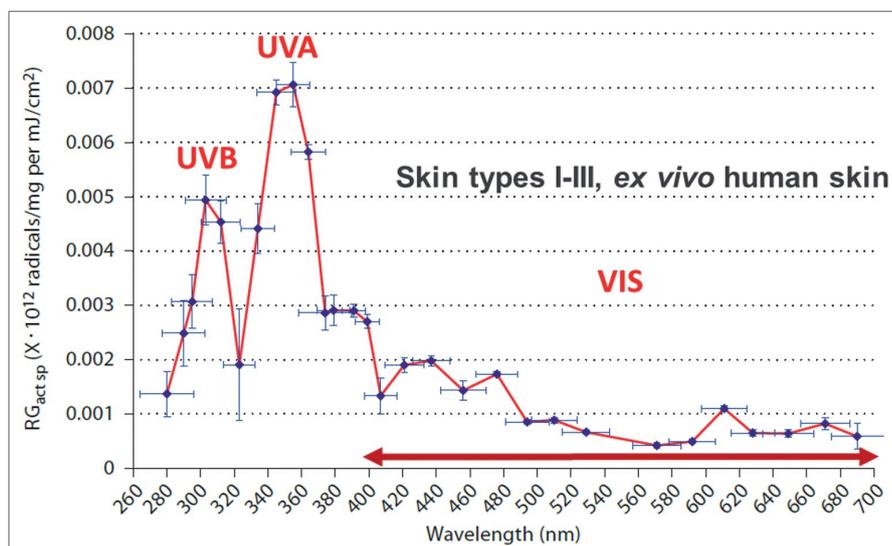


Figure 1 Ex vivo action spectrum of free radical formation.

the right protection against solar radiation have great importance especially for people on holidays or outdoor workers.

In general, sunscreens have been developed for Caucasian people with skin type I-III according to the Fitzpatrick scale (6). But also skin of people with skin type IV-VI will be damaged by high doses of solar radiation (7). For these people no optimal sunscreen is available. When developing a sunscreen product for them it has to be taken into consideration that they have much better protection in the UV spectral range than people with skin type I-III because of the higher melanin concentration in their skin. On the other hand, solar radiation in the visible and especially in the infrared spectral range is absorbed better by people with skin type IV-VI than by those with skin type I-III (7, 8).

The use of chemical filter substances in the visible and infrared spectral range is not possible, because in this case the sunscreen would be colored. Therefore, different strategies for sun protection are needed. For instance, TiO<sub>2</sub> pigments can be modified in such a way that they have strong reflection and scattering properties in the visible and infrared spectral range. Additionally, use of antioxidants with high radical protection factors in sunscreens is preferable. Because of enzymatic processes in human skin, radicals will also be produced by visible and infrared light due to the strong skin absorption (9, 10).

An innovative sunscreen product will be described in this paper that protects against sun damage in people with higher skin types (skin type IV-VI) like those usually occurring in the Asian regions. This sunscreen provides effective protection in the UV spectral range. Additionally, it has a high protection efficacy also in the visible and infrared spectral range of solar radiation. This advanced sun protection is obtained by using special TiO<sub>2</sub> pigments that reflect and scatter light effectively in the visible and infrared spectral region. Above all, this innovative sunscreen contains plant extracts well known from traditional Chinese medicine providing high antioxidative protection. Additionally, a cooling substance is used in the sunscreen

to reduce the skin temperature during exposure to solar radiation and all processes associated with high skin temperatures.

To characterize the efficacy of the new sunscreen, different investigations were carried out. The radical protection factor (RPF) was determined by EPR spectroscopy *in vitro* to analyze the antioxidant capacity of the cream. The scattering properties of the cream provided by a mixture of TiO<sub>2</sub> pigments of different size for protection in the VIS and IR spectral range were analyzed by spectroscopic measurements (*in vitro*). The cream formulations were investigated by EPR spectroscopy *ex vivo* for their capacity to protect the skin against the formation of free radicals induced by VIS/NIR irradiation. The cooling effect of the sunscreen was tested on untreated and with sunscreen treated skin areas by temperature measurements before and after NIR irradiation *in vivo*. For the *in vivo* investigations volunteers with skin type IV-V (according to the Fitzpatrick scale; Asian skin type) were examined. To analyze the NIR-protection characteristics of the sunscreen *in vivo*, the carotenoid concentration was determined in volunteers (skin type III) before and after NIR irradiation of untreated and treated skin areas. Finally, for protection in the UV range the SPF was determined *in vivo* by the gold standard and a new method based on reflectance measurements on volunteers with skin type I-III as recommended by the guidelines.

## EXPERIMENTAL

### Subjects

For optimal characterization of individual sunscreen formulations, *in vitro*, *ex vivo* and *in vivo* studies were performed. For the *in vivo* studies an ethical approval statement was drafted by the Charité-Universitätsmedizin Berlin (EA1/237/17). For *ex vivo* and *in vivo* testing of the cream formulation, the formulation was applied in a concentration of 2.0 mg/cm<sup>2</sup> and evenly distributed on the skin according to the COLIPA standard procedure.

### Sunscreen

The sunscreen was a w/o emulsion. As a physical filter, selected Titanium Diox-

ide And Zinc Oxides were used. As an organic UV filter, combinations of Ethylhexyl Methoxycinnamate, Diethylamino Hydroxybenzoyl Hexyl Benzoate, Bis-Ethylhexyloxyphenol Methoxyphenyl Triazine, Octocrylene and Phenylbenzimidazole Sulfonic Acid were selected.

Antioxidants well known from traditional Chinese medicine with strong radical protection properties were used. These antioxidants were Green Tea Extract, Scutellaria Baicalensis Extract, Saussurea Involucrata Extract, Leucosium Aestivum Bulb Extract, Astragalus Membranaceus Root Extract, Saposhnikovia Divaricata Root Extract, Calendula Officinalis Flower Extract, Albizia Julibrissin Flower Extract and Gastrodia Elata Root Extract. Alcohol (Ethanol) and Menthol were used to cool the skin during exposure to solar radiation.

### Determination of the radical protection factor (RPF) of the sunscreen samples

The principle of the RPF technology is the determination of the radical scavenging activity of a substance/product which contains antioxidants *in vitro* (11).

This test was performed by electron paramagnetic resonance (EPR) spectroscopy (X-Band EPR: MS5000, Freiberg Instruments GmbH, Freiberg, Germany) using the test radical 2,2-diphenyl-1-picrylhydrazyl DPPH (Sigma-Aldrich, Steinheim, Germany), which is reduced by the antioxidative system. The number of reduced test radicals represents the radical scavenging activity that is normalized to 1 mg input of the antioxidant substance/product. The RPF is expressed by a positive number N with the measuring unit 10<sup>14</sup> radicals/mg, which means: RPF = N · [10<sup>14</sup> radicals/mg] (12-14).

For the measurements, samples were prepared as follows: 500 mg of the formulation was solubilized in 10 mL ethanol followed by a dilution (1: 1) with a 1 mM DPPH ethanolic solution. The samples were kept in the dark under room temperature by constant panning. The measurements were taken directly after sample preparation (0 hours) and 23 to 28 hours after sample incubation, until stabilization of the DPPH signal.

### Determination of optical properties of sunscreen samples

The double integrating sphere technique combined with inverse Monte Carlo simulation (iMCS) has been shown to be useful for determination of the optical parameters of turbid media (15).

The total reflectance  $R_d$  and the total transmittance  $T_t$  of the 100  $\mu\text{m}$  thick sunscreen sample were measured in the wavelength range 600 to 1800 nm using an integrating sphere spectrometer (Lambda 1050, PerkinElmer, Rodgau-Jügesheim, Germany) according to *Meinke et al.* (16). The Lambda 1050 is a two-beam spectrometer with a double monochromator system. The light source used consisted of a tungsten halogen lamp for the visible/ NIR range. The light intensity was adapted to the absorption behavior of the sample to maintain the intensity of the signal in the optimal range of the detectors. The cuvette could be fixed in a defined position at a constant distance to the sphere aperture, in front of or behind the integrating sphere, in order to measure the transmittance or reflectance spectra. To measure  $T_t$ , the reflectance port was closed with a diffuse reflecting Spectralon® standard (Lambda 650, PerkinElmer, Rodgau-Jügesheim, Germany).  $R_t$  was measured relative to the reflectance standard by replacing the special Spectralon® standard by the sample, which was inclined at an angle of 8° to the incoming light. This experimental set-up allowed measurement of the macroscopic radiation distribution with an extremely reduced error potential. The accuracy of repetitive measurements of the reflectance and transmission was below 2 because of minor inhomogeneities in the optical properties within the samples.

The optical parameters  $\mu_a$  and  $\mu_s'$  were calculated by inverse Monte Carlo simulation (iMCS) (15). In total, the cream was prepared two times and measure three times. The spectra for each sample were averaged and dependently simulated.

### Prevention of radical formation in the VIS/NIR range *ex vivo*

Porcine ear skin, which is a suitable model for human skin (17), was used to analyze the radical protection effect of the sunscreen formulation by EPR technol-

ogy, which enables measurements of free radicals within the skin induced by exogenous stressors like irradiation using the spin marker PCA (3-(carboxy)-2,2,5,5-tetramethylpyrrolidin-1-oxyl) (14). The EPR investigations were performed 30 min after cream application with the x-Band MS5000 (Freiberg Instruments GmbH, Freiberg, Germany). Skin biopsies were treated as previously described (14) followed by EPR investigations and calculation by the software "ESR studio" (14). Each experiment was repeated in duplicate on 6 porcine ears.

### Cooling effect *in vivo*

To analyze the cooling effect of the sunscreen formulation, 10 female volunteers (skin type IV to V according to the according to Fitzpatrick scale (6)) were investigated. Two areas were marked on the inner forearm and one was treated with the sunscreen. Directly after application of the sunscreen the skin temperature was measured on both areas in time intervals of 5-10 min 30 min before and 30 min during NIR irradiation (60 mW/cm<sup>2</sup>) in time intervals of 5-15 min *via* a noncontact infrared thermometer (62 mini IR Thermometer, Fluke, Everett, Washington, USA).

### SPF determination *in vivo*

The sun protection factor (SPF) of the cream formulation was investigated in two ways. It was determined noninvasively *in vivo* and by the invasive reference method according to ISO 24444. For the noninvasive method, the sensors used relied on a spatially resolved backscatter measurement with ultraviolet radiation far below the dose inducing sunburn (18, 19).

For the noninvasive method the cream formulation was applied to the backs of 6 (4 male and 2 female) volunteers (skin type I to III according to the Fitzpatrick scale (6)) and the reflectance of the application areas measured after 30 min 30 times per area by diffuse reflectance spectroscopy (prototype by Laser-Medizin Technologie Berlin, Germany) (18). The reflectance was measured with and without sunscreen. The SPF was calculated as previously described (18, 19). For the second method the reference methodology according to ISO 24444 was used.

### NIR protection *in vivo*

To analyze the NIR protection of the sunscreen formulation, the cutaneous carotenoid concentration was determined as a marker for free radical formation by resonance Raman spectroscopy (prototype by the Charité-Universitätsmedizin Berlin) on the inner forearm (20, 21) of 6 female volunteers (skin type IV to V according to the Fitzpatrick scale (6)). Two areas were marked and one area was treated with the sunscreen. The carotenoid levels were determined after 30 min penetration time before irradiation and 60 min after NIR irradiation. The irradiance was 60 mW/cm<sup>2</sup> and applied for 30 min.

### Statistical analysis

Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). To evaluate the significance over the entire measurement period, the generalized estimating equation (GEE) was used, where  $p \leq 0.05$  was considered significant. Data evaluation was based on the Mann-Whitney U-test for nonrelated samples and Wilcoxon for related data, with  $p \leq 0.05$  being considered significant.

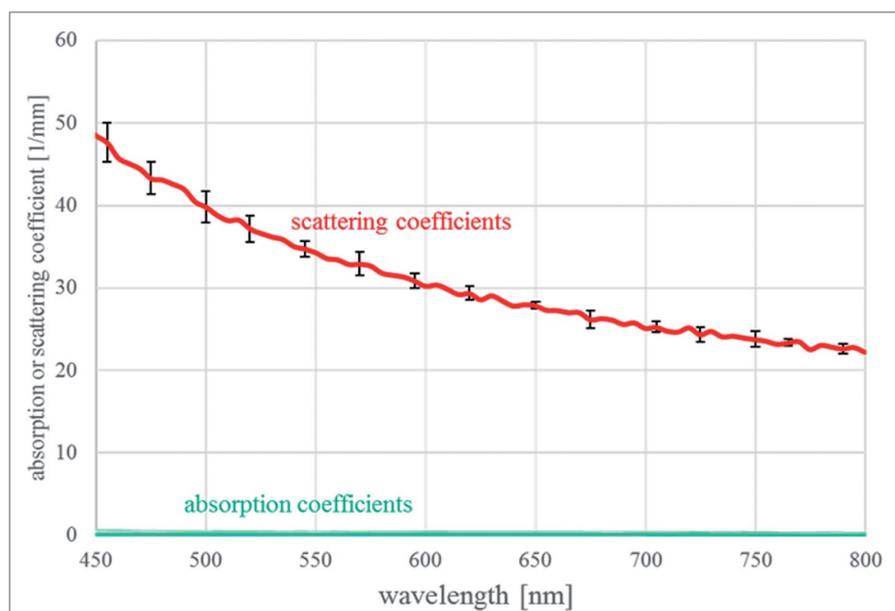
## RESULTS AND DISCUSSION

To protect against solar radiation, different mechanism can be used: absorption of UV photons with chemical filter substances, reflection of photons, and scattering effects with physical filters. Neutralization of free radicals can also be achieved with antioxidants (22).

The overall concept of this study was to characterize an optimal sunscreen formulation for skin type IV to V with the focus on Asian skin. The sunscreen, enriched with antioxidants, should mainly protect against radical formation and antioxidant degradation in the VIS/NIR spectral range. Due to the enrichment with antioxidants and pigments the cream formulations should provide good protection in the VIS/ NIR spectral range. The RPF of the sunscreen is in the medium range if we compare the results with those of a previous investigation by *Meinke et al.* (12) on commercial sunscreens (*Table I*). In the previous publication Cream 4 and Cream 3 showed the best radical protec-

**Table 1** RPF and scattering coefficients  $\mu_s'$  at 800 nm of the investigated formulation and previously investigated sunscreens (12).

Formulation	RPF in $10^{14}$ radicals/mg	$\mu_s'$ at 800 nm in 1/mm
New innovative sunscreen	33	23
Colipa standard	22	6
Cream 4	40	20
Cream 3	119	12



**Figure 2** Absorption and scattering coefficients of the new innovative sunscreen as a function of wavelength.

tion on skin in the NIR spectral range. The RPF of the new sunscreen is in the range of Cream 4. The advantage of Cream 4 was the high scattering properties of the cream.

Absorption and scattering effects influence both the transmittance and the reflectance spectra. Only for the optical properties is there a clear separation of absorption and scattering effects. Therefore, the absorption ( $\mu_a$ ) and effective scattering ( $\mu_s'$ ) coefficients were calculated for the new sunscreen (**Figure 2**) using inverse Monte Carlo simulations.

The new innovative sunscreen shows high scattering parameters that decrease with increasing wavelength. The absorption in the investigated spectral region can be neglected. In **Table 1** the RPF values and the scattering properties at 800 nm for the new

sunscreen and some literature values are summarized. The  $\mu_s'$  value of the new sunscreen is very high and promises a high radical protection in the skin like that shown by cream 4 in the Meinke publication (12).

The effect of radical protection in the VIS and NIR spectral region can be investigated *ex vivo* using EPR spectroscopy (14). The radical protection effect of the new sunscreen and a control sample without cream application (untreated, control) during VIS/NIR irradiation was investigated on six different porcine ear skins in duplicate.

The results for the cumulative radical formation during 10 min of irradiation are shown in **Figure 3**. The skin treated with the new sunscreen shows a significant reduction in radical formation compared with the untreated skin ( $p \leq 0.001$ ).

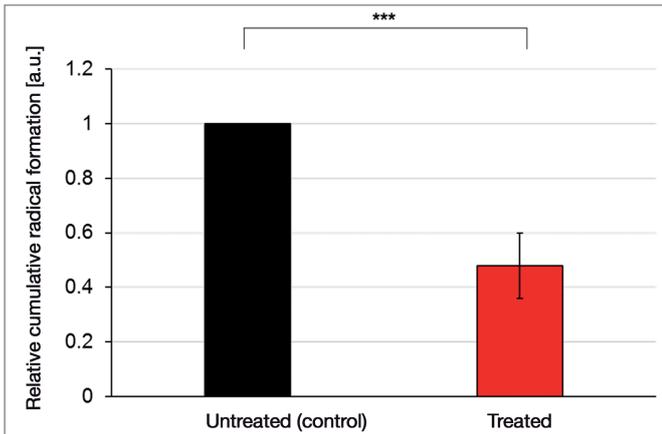
The results of the carotenoid concentration studies with resonance Raman spectroscopy show that the new sunscreen reduces radical formation in treated skin by 53 % compared with untreated skin (**Figure 4**). The treated skin shows high protection *versus* the unprotected skin ( $p \leq 0.001$ ) and a small spread of the results (**Figure 4**), indicating homogeneous distribution of the sunscreen. These results confirm that high scattering properties as well as antioxidants are responsible for the protective effect in the VIS/NIR range (12, 14). Thus the new sunscreen displays very good sun protection in the VIS/NIR spectral range.

The main reason for radical formation in the NIR spectral range is the increase in temperature (23, 24). A reduction in temperature could reduce radical formation. Thus, the addition of cooling substances could reduce the temperature of the skin and contribute to radical protection. Furthermore, the cream formulation should provide a cooling effect for a pleasant feeling on the skin. The new sunscreen therefore contained menthol (1 % menthol) as a cooling agent.

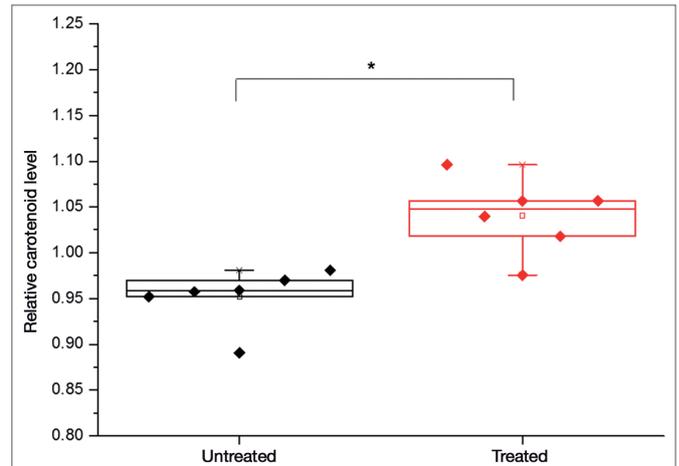
The cooling effect was investigated *in vivo* on the inner forearm. The cooling effect starts already after application and remains effective during the radiation period of 30 min (**Figure 5**).

The cooling effect of the new sunscreen reduces the skin temperature on average by 0.44 °C but the difference is not significant. However, menthol is an effective coolant for Asian skin type and more and more cosmetic products for the summer contain cooling substances. They bind to the skin's cold receptors to give relief (25). Cosmetics companies are looking for coolants that are odorless and can even be mixed into creams. Many substances are found in nature, including the essential oil in menthol (25, 26).

The protection provided by the new sunscreen in the VIS + NIR spectral range could be clearly demonstrated. Nevertheless, sun protection in the UV spectral range is important as well. Therefore, the SPF, a measure of how well a sunscreen will



**Figure 3** Cumulative radical formation in the skin (treated) after irradiation (71 mW/cm<sup>2</sup>) in the VIS/NIR spectral regions relative to the control (untreated) measured by EPR spectroscopy. Mean +/- SEM, \*\*\*p ≤ 0.001, n=6.



**Figure 4** Box plot of the relative carotenoid concentration in the skin before and after NIR irradiation (60mW/cm<sup>2</sup>) for untreated and sunscreen-treated skin (2 mg/cm<sup>2</sup>, n=6).

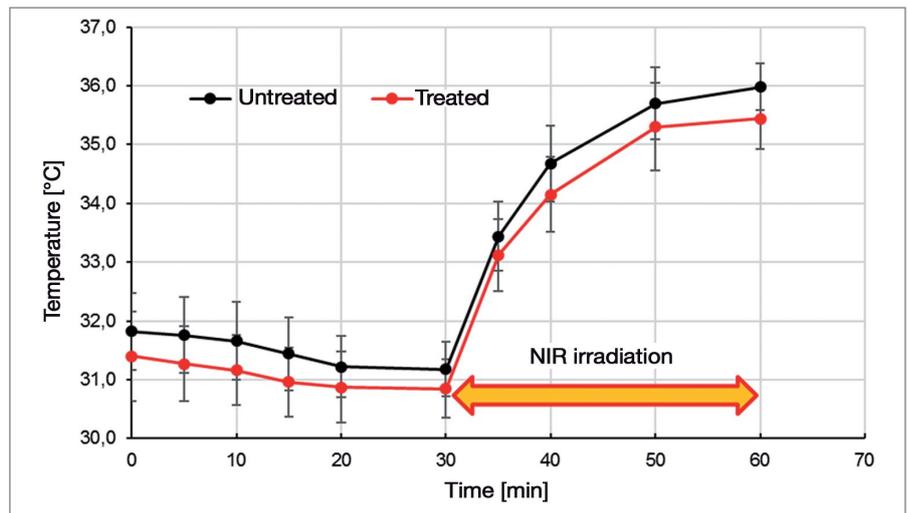
protect skin from UVB radiation, was determined. An SPF value of 41 was determined. These results correlate very well with the results from test institutes, which gave an SPF of 44.

The *ex vivo* results for the protection in the VIS+NIR region were supported by the *in vivo* investigation on volunteers with skin type IV to V using cutaneous carotenoids as a marker substance of the antioxidative network of the human skin (27, 28). Using resonance Raman spectroscopy the influence of NIR irradiation on the antioxidative network of the skin was analyzed on untreated and sunscreen-treated skin. In untreated skin the carotenoid level decreased after NIR irradiation. When the new sunscreen was applied, the values not only remain stable, they increase. (Figure 6).

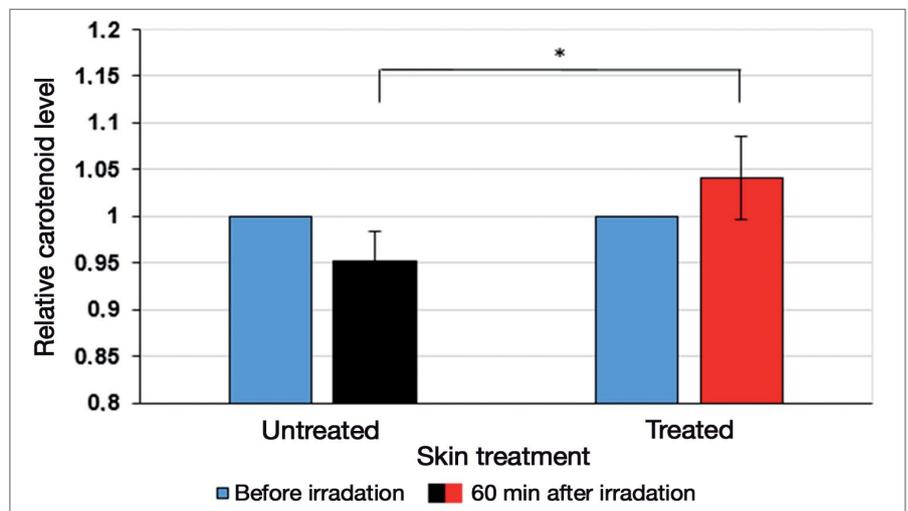
This is a strong indicator that also *in vivo* the sunscreen provides very good radical protection in the NIR spectral range and that the antioxidants in the new sunscreen support the antioxidative network of the skin. It is known that NIR irradiation reduces carotenoids in the skin (20) and that antioxidants in the skin can be enhanced by topical application (29).

## CONCLUSION

The investigated formulation is an innovative sunscreen especially developed for the Asian and dark skin type that effec-



**Figure 5** Change in the temperature of sunscreen-treated (red line) and untreated (blue line) skin over time before and during NIR irradiation.



**Figure 6** Relative carotenoid levels in sunscreen-treated and untreated skin of 6 females before and 60 min after NIR irradiation.

tively protects against radical formation in the VIS/NIR spectral range. The high scattering properties due to pigments and radical scavenging properties due to the selected antioxidants complete the product, which contains as well selected chemical UV filters to ensure adequate protection in the UV spectral range. Together with the coolants, the cream provides a pleasant feeling on the skin and provides protection in the whole solar spectral spectrum.

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# Marketing Personalized Skincare Products in Accordance with the EC Cosmetic Regulation

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## INTRODUCTION

### The evolution of personalized skincare

For many years most cosmetic products have been categorized by three to five classical skin types defined by the skin's level of dryness and oiliness. Additional basic parameters are the skin's sensitivity, pigmentation, and wrinkle levels [1].

From time to time cosmetic brands launch new cosmetics ranges to meet consumer concerns like age spots, volume loss, deep wrinkles and fine line for-

mation, skin sagging, uneven pigmentation, and reduction in glow and flexibility [2]. Modern cosmetics consumers express dynamic changes in their skin concerns and consequently request changes in product activity claims. During the short period from 2009 to 2015 there were worldwide significant increases in the number of activity claims requested of cosmetic products, such as »long-lasting« (+6.2%), »brightening« (+5.0%), »immediate effect« (+6.3%), »seasonal treat« (+4.9%), demonstrating the need for manufacturers to modify the characteristics of their products according to

the dynamic requirements of consumers [3]. Personalization of cosmetic products has been rising lately in skincare markets. Today, more customers wish to purchase skincare products specially created for their special skin needs, which are believed to be more effective and supported by a scientific understanding of the vast diversity of individuals [4].

New noninvasive techniques enable much better and quicker skin profiling [5-7]. The involvement of many elements in skin characterization drives the cosmetic industry to propose tailor-made personalized products, i.e. unique cosmetic items designed for each customer based on analysis of his or her singular physical skin characteristics.

### The regulatory challenge for personalized skincare

The market call for personalized skincare has pushed the industry to try different solutions, but the arrangement of modern manufacturing technologies and personalized cosmetic products is not simple. One of the toughest challenges to overcome before marketing a tailor-made formula is compliance with cosmetics regulations. It is the competent authorities' task to control skincare products in order to ensure that the cosmetic products presented on the markets are in line with the regulations, and personalized cosmetic products are no exception.

### Abstract

One of the biggest challenges of personalized cosmetics marketing is to meet the tough requirement to comply with cosmetic regulations. The Integrated Personalized System (IPS) is a new skincare concept targeting the manufacture and marketing of customized cosmetics and offering a solution for alignment with regulatory restrictions. According to IPS the manufacturing process is divided into two different stages, the later stage being performed in a mini-factory equipped to modify a limited number of pre-prepared bases and create an infinite number of unique formulas.

To comply with the EC Cosmetics Regulation, all processes should meet several requirements listed in the different articles of the EC Cosmetics Regulation, in particular, those on safety, the responsible person, good manufacturing practices, safety assessment, the product information file, notification and labeling. Each cosmetic product has its own unique formula; therefore, notification uses a concentration range, as "range" is a legal option for recording the composition of a cosmetic product. How IPS is an original way to market personalized cosmetics and fulfill the EC Cosmetics Regulation requirements is described in detail.

Regulation EC 1223/2009 is currently in effect in the European Community [8], developed as an anticipated evolution of the previous European Directive 76/768 of 1976 on skincare products. The fundamental principles remained unchanged but were rationalized in order to implement product safety and optimize registration procedures [9].

### **IPS – a new proposed solution for personalized skincare realization**

The Integrated Personalized Cosmetic System [IPS] is a new concept targeting the manufacture and marketing of personalized cosmetics that were clinically tested during the SuperFlex Research Project [10, 11]. According to the IPS the manufacturing production process of personalized skincare products is separated into two different stages. The later stage is performed in mini-factories located near the point of sale, enabling modification of a limited number of pre-prepared concentrated bases manufactured in a modern cosmetic industrial plant to create an infinite number of different cosmetic final products; each one is a unique personalized item. In this work we present for the first time a legal solution, developed especially for IPS personalized cosmetics, to enable coping with the various tough demands of the EC Cosmetic Regulation and the cosmetic products desired by the market.

## **RESULTS AND DISCUSSION**

### **Relevant EC regulatory requirements for personalized skincare**

To comply with the same standards and guidelines as those required by the EC Cosmetics Regulation, the entire process from production to marketing of personalized products has to meet various requirements as listed in the different articles of the European Cosmetics Regulation [8]. Some of the Regulation articles are quite challenging for production in the small volumes and short-time processes from formulation to application that characterize the process for obtaining personalized products. Concentrated bases are manufactured in central “mother factories” using conventional methodologies and therefore can easily comply with the

standard cosmetics regulation. The final manufacturing stages aimed at the modification of pre-prepared bases to create unique personalized products in mini-factories have to cope with a number of challenging burdens in order to comply with the same cosmetic standards and guidelines. These demands, listed in the different articles of the EC Cosmetics Regulation, include the requirements on safety (Article 3) [8], the responsible person (Article 4 and 5) [8], good manufacturing practice, (Article 8) [8], safety assessment (Article 10) [8], the production information file (Article 11) [8], notification (Article 13) [8] and labeling (Article 19) [8]. In the case of the proposed IPS, all of these articles evaluated and solutions established to enable compliance with the existing EC Cosmetics Regulation. The IPS solutions making it possible to overcome these regulatory challenges, with all steps conforming to the EC Cosmetics Regulation, are described in this paper.

### **Placing cosmetic products on the market – regulatory framework and guidelines**

The fundamental concepts describing the EC Cosmetics Regulation are responsibility for legislation, no need for pre-market approval by external authorities, and post-launch market controls. The EC Cosmetics Regulation states that skincare products may be placed on the market without a pre-market approval process but following the implementation of all compulsory activities. Those who place the product on the market take upon themselves all responsibility and name a so-called “responsible person” identified by Regulation 1223/2009, Article 4. The competent authorities of each European country organize control activities in their markets to verify correct implementation of the legislation. This obligation, described in Article 4, applies to all cosmetic products. However, complying with Article 4 is no more complicated for personalized products than for other standard skincare products.

### **Product notification – how to make notification of an infinite number of formulas**

Notification of cosmetic products placed on the market is obligatory according to

Article 13 of the EC Cosmetics Regulation and includes online submission of well-defined product information to the CPNP, Cosmetic Products Notification Portal [12]. The main purpose of registering at the CPNP is to enable immediate access to information on the composition of products placed on the market that must be available to poison centers in case of an emergency.

Each personal product has its own unique formulas; therefore, information notification uses a concentration range. This solution is based on the fact that “range” is one of three options to record the composition of a cosmetic product stated in the official EC guidelines [11]. Consequently, each personalized product modified in a mini-factory has a common, well-defined base cosmetic product and a pool of active ingredients with concentrations in the formula that can be changed within a predefined range according to personal dermatological and cosmetic inputs.

### **Labeling cosmetic personal products**

Labeling of a product must comply with the criteria already described in Article 19 of Reg. 1223/2009. Some of the information that must be reported is easily pre-determined, such as the weight/volume (default) or name and address of the responsible person. This information may already be screen-printed or printed on any product packaging that will be made because this information will be shared by all products. Other information regarding the specific formula, the list of ingredients in descending order and the batch number will be printed on the individual product. We consider that in the special case of mini-factories the product batch consists of the single product made at that time.

In fact, each product batch (and the identification number belongs to the mandatory information) identifies all homogeneous products coming out of the same production run. In the case of mini-factories, the production batch is therefore a single piece. Thanks to the controls and the scheduled data recording, it is possible to achieve full traceability and record all the parameters pertaining to the single produced product.

## Product Information File [PIF] concerning safety assessment and packaging evaluation of personalized items

The Product Information File (PIF, 1223/2009 / EC art.11) is one of the most important tools for maintaining (and demonstrating to the competent authorities) control of the safety of products that are placed on the market by the responsible person. In particular, the most relevant document in the PIF is the Cosmetic Product Safety Report (CPSR, 1223/2009/EC, Annex I), which includes the CPSR Part A (relevant data) and the CPSR Part B (safety evaluation, reasoning and credentials of the safety assessor). The PIF Product Information File must be kept at the disposal of the authorities at the address of the responsible person indicated on the packaging and not at the production site.

The PIF therefore is a technical document that can be requested at any time by the competent authorities and must be representative of the cosmetic product placed on the market, bearing in mind that any cosmetic product will be subject to production variables. The safety assessor must take these into account, both in terms of the »specification range« for the acceptability of raw materials and the range of production parameters and parameters for the finished product. For this reason, the SCCS Notes of Guidance guidelines allow that the safety assessment to be carried out taking into consideration also the possible variants of the formulas provided that the assessment of the safety assessor is implemented by applying the »principle of prudence«.

Therefore, according to the SCCS guidelines the safety assessment must be performed by first doing a risk assessment of each ingredient, and then the consumer's exposure to the individual ingredients and to the product should be evaluated [13].

As with the CPNP portal registration, it is possible to consider the common base with additional evaluations of all possible ingredients in the prudently considered maximum concentrations that the mini-factory can use. This prudent evaluation,

according to the criteria recommended by The SCCS Note of Guidance, will be valid and applicable to any custom products manufactured by the mini-factory.

### Safety of personalized products – Article 3

A cosmetic product made available on the market shall be safe for human health when used under normal or reasonably foreseeable conditions of use, taking account, in particular, of the following:

- a) Presentation including conformity with Directive 87/357/EEC,
- b) Labeling,
- c) Instructions for use and disposal,
- d) Any other indication or information provided by the responsible person defined in Article 4.

The provision of warnings shall not exempt persons defined in Articles 2 and 4 from compliance with the other requirements laid down in this Regulation. Directive 87/357/EEC concerns the prohibition to put on the European market any article that emulates food products (so called "food-like" products).

In the case of mini-factories, product safety is therefore predetermined, because the major component of the product itself is constituted by the concentrated base, which is appropriately diluted during the production process of the single bottle, while only small quantities of specially calibrated active ingredients are modified. In an absolute sense, therefore, the variations in the formula are not very significant from the point of view of product safety. In fact, always following the approach dictated by the SCCS Notes of Guidance, it is sufficient that the safety assessor takes into account the maximum concentrations to which the consumer can be exposed, both in terms of the base and the active ingredients. This assessment will then be extended to all the different possible lots that can be made by the machinery. In this way, the safety of the product delivered to the consumer is guaranteed.

### Responsible person – Article 4

The role of the responsible person of the cosmetic product was introduced for the first time in Regulation 1223/2009

(Article 4). As in the previous Directive 76/768/EEC there was no clear and unequivocal definition of who assumed responsibility for compliance of a cosmetic product present in the European market. Every product introduced on the European market must refer to a responsible person, who is a natural or legal person with the sole requirement of residency in a country of the European Union. The responsible person is identified as the manufacturer and takes full responsibility for placing on the market the cosmetic product, listed in the following Art. 5.

The responsible person, in particular, must be indicated on the packaging of the cosmetic product, as defined in the subsequent Art. 19.

### Obligations of responsible persons – Article 5

Article 5 of Reg. 1223/2009 lists the responsibilities of the responsible person, referring to the relevant articles. In particular, the responsible person is responsible for the following:

- a) safety of the product placed on the market (Art. 3),
- b) compliance with GMP (Article 8),
- c) realization of a safety assessment (Article 10),
- d) the Product Information File (Art. 11),
- e) notification to the European CPNP portal (Article 13),
- f) proper labeling (Article 19) and
- g) market surveillance (Article 23, 24).

In addition, it should be kept in mind that the responsibility of the responsible person also extends to some aspects that are not directly indicated in the list of articles mentioned. For example, the responsible person must control the distribution chain as indicated in the following Art. 6. However, since this is not solely the of the responsible person according to Art. 6, it is not indicated in the list of Regulation articles for which the responsible person is held responsible. Therefore, the list of articles indicated in Article 5 must be understood as the obligations for which the responsible person is solely responsible, while there are other obligations for which the responsible person still assumes part of the responsibility.

Furthermore, it must be considered that the assumption of responsibility in relation to an article, which refers to a specific activity, implies the control of such action and the ability to demonstrate such control. A relevant consequence of this approach is that the responsible person, who in short is the entity that has decision-making power with regard to the production chain, must be able to check the listed obligations and be able to demonstrate this to the competent authorities, enabling them to verify in a quick and effective way the conformity of the production chain and of the cosmetic products placed on the market.

This basic concept of Reg. 1223/2009 is one of the fundamental aspects introduced with this legislation, since identification of a single interlocutor who takes responsibility for all the most important aspects allows quick identification of the manager and prevents a fragmentation of responsibilities, as was possible with the previous legislation.

In the case of realization of cosmetic products in mini-factories, this new and more rational approach greatly facilitates compliance with Reg. 1223/2009, as it does not identify separate entities as the developer and the manufacturer, each of which must have specific responsibilities (and it would have been very difficult to assign responsibility to an automated machine). According to the approach of Reg. 1223/2009, the verification and monitoring of production activities are carried out by the responsible person, who using the tools he deems suitable must check the conformity of production and be able to demonstrate to the authorities such control.

In this sense, Reg. 1223/2009 has made it possible to manufacture cosmetics using automated systems, provided that the responsible person ascertains that this realization can guarantee the production of safe and compliant cosmetic products.

### **Good manufacturing practice – Article 8**

According to the previous section, the responsible person must guarantee conformity of the products, which must be

produced according to GMP, as described in Article 8. The concept of good manufacturing practice is known, but in order to define in a more specific way what the requirements pertaining to production should be, Article 8 indicates that application of the harmonized standards published in the Official Journal of the European Union automatically implies compliance with the principles of good manufacturing practice. The Official Journal of the European Union, C123/3 of 21.4.2011, published the reference of harmonized standard: EN ISO 22716:2007 Cosmetics – Good Manufacturing Practices (GMP) – Guidelines on Good Manufacturing Practices (ISO 22716:2007).

Good manufacturing practice, therefore, is guaranteed if the guidelines EN ISO 22716:2007 are applied, which naturally were developed for »traditional« production plants. In reality, it is also possible to guarantee compliance with the requirements also in the case of mini-factories.

### **GMP-ISO 22716:2007**

The adaptation of good manufacturing practice according to EN ISO 22716:2007 for mini-factories required a thorough study due to the great differences existing between the proposed »mother factory – mini factory« system and the traditional factories.

In fact, it is incorrect to consider that the entire production process is carried out in the mini-factory because the concentrated product base is produced in the mother factory according to the developed and tested formulas. Therefore, the process takes place in two phases: the first phase follows the traditional path, and for this part it was easy to adopt the EN ISO 22716:2007 standard, as described in the GMP Manual. A second »manual« was developed for the mini-factory in which we had to adapt the »traditional« criteria to a miniaturized and automated factory. Below is a summary of the strategies adopted for each significant chapter provided by the standard.

### **Personnel**

According to the EN ISO standard, staff must be adequately trained in line with

their responsibilities. In the case of the mini-factory, the only human intervention involves refilling and maintenance, while the rest of the activities are handled by a processor.

### **Buildings**

Buildings must be adequate and fit: in the case of the mini-factory, the building is the mini-factory itself, which has been specially designed to have a compact but adequate size for the planned production (one piece at a time)

### **Quality Control Laboratory**

In the case of traditional products, the quality control laboratory is required to verify that the batch produced is comparable with the previous batches and the standard studied and evaluated by the safety assessor. In the case of the mini-factory, each batch is a single piece and (theoretically) unrepeatable. However, dosages are automated and predetermined, and there is no »human« intake as in traditional factories with the consequent possibility of making mistakes.

Therefore, in the case of the mini-factory, because of the very low probability of error and the impossibility of carrying out checks on the single product made, the concept of »validation« is applied, which is precisely within the ISO norms for those processes where there is a low probability of error and the inability to make checks on the product made. The quality control laboratory is then replaced by the process testing phase, where it is verified that mini-factory automation cannot produce products with serious production abnormalities due to both the simplicity of the final process and the total automation of the process itself.

### **Internal audit**

Internal audits can not be applied to a machine because they require interaction with people. Consequently, audits will be limited to the stages involving the assistance staff, while the mini-factory controls are supported by periodic maintenance checks and machine efficiency audits.

From the description of the requirements described above, it is clear that the respon-

sible person can guarantee GMP compliance also in the mini-factories part of the production chain, with the only difference being that some definitions must be appropriately adapted. For example, buildings, which in the case of the traditional cosmetic product are defined as the masonry structures within which the cosmetic product is made, in the case of the mini factories will be logically identified with the internal structure of the machine that makes the cosmetic, which is unlikely to be masonry. However, if we focus on the requirements of buildings, i.e. the need to guarantee hygiene and process flow control, we see that the same principles can also be guaranteed by mini-factories, since they are sealed, easily controllable and washable environments, inside which the handling of the ingredients takes place through pumping systems that prevent any cross-contamination.

From this point of view we can say that even if we have to adapt some terms, mini-factories can guarantee compliance with the principles of good manufacturing practice even better than the traditional cosmetics factory.

#### Restrictions on substances listed in the Annexes – Article 14

The cosmetic product can be made using any ingredient that can be considered safe for use by the safety assessor appointed by the responsible person, with the exception of preservatives, dyes, and UV filters that are listed in the respective annexes IV, V and VI of Reg. 1223/2009, constituting positive lists. Furthermore, substances listed in Annex II (list of prohibited substances) cannot be intentionally used, whereas the substances listed in Annex III can only be used in compliance with the limits described in this annex.

The decision of the Commission referred to in the first subparagraph, designed to amend nonessential elements of this Regulation, shall be adopted in accordance with the regulatory procedure with scrutiny referred to in Article 32(3).

The use of substances present in the annexes (under the intended conditions) and the exclusion of prohibited substances can

be guaranteed in the case of mini-factories in the same way as a »traditional« establishment, as the components and the respective impurities are approved by the safety assessor in all possible combinations, taking into consideration the maximum values for each ingredient that the mini-factory can use in the finished product.

#### Communication of serious undesirable effects – Article 23

The responsible person must take the necessary steps to ensure that undesirable effects (EU) and serious undesirable effects (SUE) are identified and classified, and that appropriate actions are taken to identify the causes of such effects and to verify possible correlation with the cosmetic placed on the market.

In the case of serious undesirable effects, the responsible person must promptly notify the health authorities of the country where the event occurred and cooperate to ensure consumer safety. To meet these obligations, control of the distribution chain is fundamental, which in the case of mini-factories can be considered superior to the traditional production chain, as it is possible to identify and trace the individual consumer who has received a specific cosmetic product of which the smallest details the composition are known. Production in mini-factories, in fact, concerns the creation of individual cosmetic products, each of which is a »batch«.

As traceability in this case is pushed to the extreme (i.e., it is possible to find the data of a single customer), management of reports of undesirable effects will be consequently actually facilitated.

#### CONCLUSIONS

IPS offers for the first time a tailor-made process for making personalized cosmetic products that takes into consideration the EC Cosmetics Regulation. IPS offers key solutions for obtaining personalized cosmetics conforming to the EC Cosmetic Regulation described in this paper.

We have been able to proceed with the project on mini-factories, as we were able

to previously verify that all the requirements contained in 1223/2009/EC and in the guidelines (EN ISO 22716: 2007 and SCCS Notes of Guidance) could be met by IPS as well.

Some concepts required only an adaptation, which does not affect compliance with the rules. On the contrary, some aspects, thanks to the peculiarities of micro-production, can bring important advantages and assure safety. For example, control of the composition of each lot is memorized and guaranteed, as well as the aspects concerning sanitization of the systems, which is done for each individual product package.

In addition, the absence of contact with operators (production takes place in a sealed environment) ensures a lower probability of contamination. Finally, mini-factories have the ability to track the data of the individual consumer for whom the IPS was created.

Therefore, IPS production with mini-factories can be carried out in compliance with Regulation 1223/2009/EC.

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