Aus der Klinik für Dermatologie, Venerologie und Allergologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

The Future of Sunscreen Efficacy Evaluation - a Comparative Study of Universal Sun Protection Factor, Radical Formation Ratio and Sun Protection Factor Assessing the Protective Value of Sunscreen Formulations Containing Chemical-, and/or Physical Filters as well as Antioxidant Additives

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List of Abbreviations

AO	Antioxidants
AUC	Area under curve
AST	Average sum transmission
c-UVF	Chemical UV Filters
EPR	Electron paramagnetic resonance
IR	Infrared
MED	Minimal Erythemal Dose
NIR	Near Infrared
PF	Physical Filter
RF	Radical formation ratio
RPF	Radical Protection Factor
SPF	Sun Protection Factor
SSPF	Spectroscopic Sun Protection Factor
T	Transmittance
USPF	Universal Sun Protection Factor
UV	Ultraviolet
Vis	Visible

Abstracts

English

The sun protection factor (SPF) has been the internationally accepted standard characterizing sunscreen efficacy for the past decades. This factor is based solely on prevention of erythema, principally induced by UVB irradiation. However, UVA and even visible and infrared radiation have been equally implicated to contribute towards sun induced skin damages, thereby highlighting limitations implied with the use of the SPF as single indicator. Alternative efficacy indicators, providing a more comprehensive approach for characterization, have been proposed.

The aim of this study was to determine the protective efficacy of sunscreen utilizing two of these alternative indicators, the spectroscopic universal sun protection factor -USPF- and the radical formation ratio -RF-, calculated based on electron paramagnetic resonance measurements. By comparing these results to SPF values, provided by an outside institution, conclusions regarding capabilities and limitations of both efficacy indicators could be drawn.

Five specially developed formulations, containing commonly utilized active ingredients - chemical filters, physical filters and antioxidants, which exhibit different mechanisms of action, were evaluated in changing composition.
As expected, chemical filters were shown to provide a protective effect measurable by each method utilized. When physical filters were investigated as single active ingredient, USPF and SPF values increased. Due to the relatively low amount (2%) of physical filter contained in the formulations and a possible interaction of physical filters with antioxidants, leading to a decrease in antioxidant capacity, no clear conclusion could be drawn when physical filters were utilized in combination.
Antioxidants were shown to significantly increase SPF values. As anticipated, this effect failed to appear in the solely spectroscopically based USPF values. However, there was also no effect observed for RF values, possibly attributable to the high radiation intensity used in the ex vivo setting, obliterating antioxidants early on.

A comparison of determined USPF values with previous results from sunscreen formulations containing similar compositions of active ingredients confirmed the expected linear correlation for USPF and SPF values.
The values obtained verify the significance of USPF values for objective evaluation of sunscreen efficacy over the entire UV spectrum, independent of biological responses. In combination with the RF for infrared and visible ranges, these indicators could lead to a more comprehensive sunscreen characterization.

The results of this study provide important information regarding effectiveness and capabilities of the investigated efficacy indicators, but also highlight the need for further research to eventually implement altered, more comprehensive efficacy indicators in international sunscreen evaluation standards.

Deutsch

Der Lichtschutzfaktor SPF ist aktuell die anerkannte Größe zur Kennzeichnung der Wirksamkeit von Sonnenschutzmitteln. Dieser Faktor beruht allein auf der Basis der vorrangig durch UVB-Strahlung ausgelösten individuellen Erythembildung. Indessen sind jedoch auch UVA-Strahlung sowie sichtbares und infrarotes Licht für ihre hautschädigende Wirkung bekannt. Diese Erkenntnisse waren Grundlage dafür, neue Messgrößen vorzuschlagen, die das vollständige Gefahrenpotential der Sonneneinstrahlung berücksichtigen.

Zielstellung der vorliegenden Arbeit war es, zwei dieser neuen Messgrößen, den spektroskopisch bestimmten Universellen Lichtschutzfaktor - USPF - und einen über die Messung der paramagnetischen Elektronenresonanz zugänglichen Radikalbildungsquotienten - RF- zu bestimmen. Durch Vergleich mit dem klassischen SPF-Wert, der durch einen Partner gemessen wurde, konnten Hinweise über die Leistungsfähigkeit und die Grenzen beider Kenngrößen erhalten werden.

Für diese Untersuchungen waren fünf spezielle Formulierungen verfügbar, die typische Inhaltstoffe der Sonnenschutzmittel mit unterschiedlichen Wirkmechanismen in wechselnder Zusammensetzung enthielten: Chemische Filter, physikalische Filter und Antioxidantien. Erwartungsgemäß zeigten in allen Fällen die chemischen UV-Filter den stärksten Schutzeffekt. Bei Einsatz der physikalischen Filter als Einzelkomponente konnten nur Einflüsse auf die USPF- und SPF-Werte nachgewiesen werden. Bedingt durch den relativ geringen Gehalt von 2% physikalischen Filtern in den verfügbaren Proben und durch eine mögliche Wechselwirkung der Partikel mit Antioxidantien, ergaben sich für USPF und RF bei kombiniertem Einsatz keine eindeutigen Ergebnisse. Die Wirkung der Antioxidantien war erwartungsgemäß beim Vergleich zwischen USPF und SPF besonders deutlich ausgeprägt. Der SPF zeigte gegenüber dem USPF erhöhte Werte. Bei den Radikalbildungsquotienten konnte, wahrscheinlich bedingt durch die erforderlichen hohen Bestrahlungsintensitäten, kein Einfluss der Antioxidantien nachgewiesen werden.

Der Vergleich der gemessenen USPF-Werte mit in der Arbeitsgruppe vorliegenden Werten anderer Sonnenschutzformulierungen ähnlicher Zusammensetzung, bestätigt

den erwarteten linearen Zusammenhang zwischen USPF und SPF. Die Beeinflussung der SPF-Werte durch die spezifischen, die Erythembildung beeinflussenden, Antioxidantien wird auch bei dieser Interpretation deutlich.
Die erhaltenen Ergebnisse bestätigen die Bedeutung der USPF-Werte zur objektiven Beurteilung der Effizienz von Sonnenschutzmitteln im gesamten UV-Bereich. Sie sind unabhängig von einer bestimmten biologischen Schädigung und erfassen die Erniedrigung der Strahlungsintensität im UVA- und UVB-Bereich.
Der eindeutige Zusammenhang zwischen USPF- und RF- Werten wird durch den bestimmenden Einfluss der chemischen Filter erklärt.

Die durchgeführten Untersuchungen geben wichtige Hinweise auf die Effektivität und die Einsatzmöglichkeiten der neuen Lichtschutzfaktoren, dem USPF und dem Radikalbildungsquotienten. Sie unterstreichen die Notwendigkeit erweiterter Studien, um ergänzende Aussagen mit dem Ziel zu erhalten, eine Akzeptanz geänderter Lichtschutzfaktoren im Rahmen der internationalen Standardisierung zu erreichen.

1 Introduction

As skin cancer rates continue to increase [1, 2] sunscreens are gaining more and more in importance. While consumer awareness is rising and the public is being broadly educated by media and health care providers, misconceptions concerning the sun protection factor (SPF) indicating the efficacy of a sunscreen, are still widespread.

Historically, the SPF of a product is determined by its ability to prevent reddening of the skin, also called erythema formation. It is a measure of how many times longer a person protected with sunscreen can stay in the sun without having to fear the consequences of sunburn. Erythema formation is predominantly induced by UVB irradiation, radiation of other wavelengths contribute only marginally to its development [3]. Few are aware that the radiation that causes the skin to redden and burn is not the single malefactor responsible for the detrimental effects of the sun such as skin cancer formation, premature aging and immunosuppression [4]. These consequences are to a large extent caused by radiation of other wavelengths, especially in the UVA, but also the visible and infrared spectrum of the light, unaccounted for in the calculation of the SPF of a product.
Hence, consumers may have a false sense of security evaluating a sunscreens' protective effectiveness solely based on a high SPF label.
In order to ensure safe sun protection, in 2006, Cosmetics Europe adjusted their guidelines for sun protection products to include and label at least 30 percent of the SPF as UVA protection [5].
While the cosmetics industry has quickly adapted, now offering broad-spectrum sunscreens and adding filters and additives, which are in part effective even in the visible and near infrared ranges, the traditional method of characterizing a product's protective abilities using the SPF still lags behind, denying the consumer the opportunity to easily deduce the comprehensive protective efficacy of a product from a simple label.

Recent scientific findings have led to different proposals for alternative evaluation methods and resulting measurands to evaluate the efficacy of sunscreen products [6-8]. In the present study two of these efficacy indicators, i.e., the spectroscopic protection

factor, called universal sun protection factor (USPF) and the radical formation ratio (RF), determined by electron spin resonance spectroscopy, will be compared with each other and with the sun protection factor (SPF). Resulting values will be analyzed for influences of active ingredients, correlations between efficacy indicators and assessment of capabilities to evaluate their use in future sunscreen protection evaluation.

2 Background

2.1 The skin - measures of innate sun protection

Measuring on average 1.5-2 m^2, the skin is the largest organ of the human body. It serves to protect from trauma, changes in temperature, toxins, bacteria and last but not least solar irradiation. Two layers constitute the skin: the epidermis and the dermis. They sit on a fatty layer of connective tissue covering fascia, muscles and bones, the subcutis. Depicted in Figure 1 is a schematic illustration of the skin structure and epidermal skin layers. The epidermis forms the uppermost layer of the skin. It does not contain any blood vessels and relies entirely on the lower part of the skin, the dermis, for its nutrients and waste transport. It is primarily made up of corneocytes sloughing off over time and can be divided into four or five layers depending on location. They are categorized by the degree of differentiation of the corneocytes contained. While the stratum basale, forming the basis of the epidermis, primarily proliferates corneocytes, the cells become more differentiated and eventually lose their nuclei and slough off the closer they approach the skin surface. As pictured below in Figure 1, these layers are from bottom to top: the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum (only present on the soles of the foot and palms) and the stratum corneum.
In regard to UV radiation, two cell types found within the epidermis play an important role: melanocytes and Langerhans cells. Melanocytes are the cells that produce a tan by developing melanin, a pigment functioning to protect the cell from harmful rays by shielding the DNA from radiation via absorption, thereby preventing UV-induced damage. Langerhans cells, on the other hand, are part of the immune system, which may be depleted due to apoptosis prompted by solar irradiation, ultimately leading to sun induced immunosuppression, which is discussed in further detail in section 2.3.2.

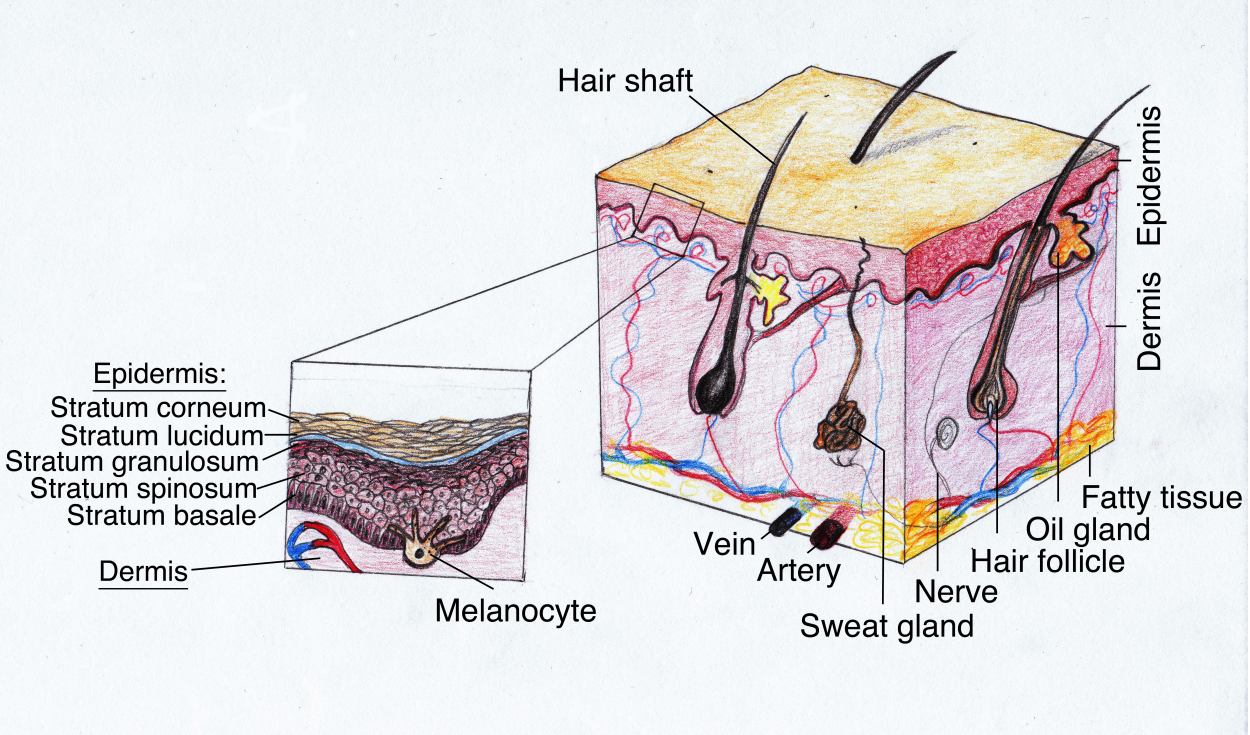

Figure 1: Schematic drawing of the skin structure and epidermal skin layers.

The dermis is the thickest part of the skin, the part that contains blood- and lymphatic vessels, connective tissues, collagen and elastic fibers. It builds the basic structure and support for the skin. UV radiation can lead to a loss of these elastic fibers and can result in premature aging as a consequence.

Armed against harmful effects of sunlight, the skin is equipped with several natural protective mechanisms lessening the consequences of solar irradiation. These mechanisms comprise an increase in pigmentation, the formation of light calluses, enhancing the scattering and reflection of irradiation in the skin and the radical scavenging activity of antioxidants present in the skin layers.

Both UVA and -B radiation can lead to the induction of pigmentation. However, they differ in their effectiveness and type of provoked pigmentation. While UVA causes an almost immediate tanning effect by stimulating a redistribution and oxidation of existing melanin, UVB rays evoke a synthesis of new melanin to protect the DNA from further damage. Subsequently, the UVB tan takes longer to develop than a tan from UVA radiation, yet it is of some protective value for the skin [9].

Genetic predisposition is responsible for an inclination towards rapid pigmentation or burning of an individual's skin. A commonly utilized classification system by Fitzpatrick uses those attributes to categorize different skin types in respect to their frequency to tan and burn (Table 1) [10].

Skin type	Skin Color	Features
I	White or freckled skin	Always burns, never tans
II	White skin	Burns easily, tans poorly
III	Olive skin	Mild burn, gradually tans
IV	Light brown skin	Burns minimally, tans easily
V	Dark brown skin	Rarely burns, tans easily
VI	Black skin	Never burns, always tans

Table 1: The Fitzpatrick Scale defining different skin types adapted from [10].

Apart from increased pigmentation, the epidermis exhibits further protective mechanisms shielding the skin from radiation.
Urocanic acid has been identified as an important endogenous UV protective factor [11], acting as a chromophore, by absorbing radiation in the epidermis [12]. Also, induced by UV radiation, proliferation of the basal cells increases, which in turn leads to a so-called light callus, further enhancing the light reflective properties of the stratum corneum, inhibiting radiation from penetration into deeper skin layers.

In order to appreciate the protective properties of antioxidants in the skin during sunlight irradiation, it is important to have a basic grasp of free radical formation.

Free radicals are substantially involved in many vital biological processes. Acting as a main defence mechanism against intruding bacteria and viruses, these chemically highly reactive molecules have also been implicated to play a role in cell signaling processes [13]. While function is fundamental, higher levels of free radicals can lead to considerable damage of the entire system. In particular regard to the skin and UV radiation, free radical formation and reactive oxygen species, radicals produced by oxygen metabolism, have been identified as a principal player in the formation of skin tumors, skin wrinkling and skin aging [14].

UV radiation, in particular of the UVA range, has been suggested as a major inductor for free radical formation. In contrast to UVB radiation, which has been shown to directly cause DNA damage, the longer wavelength of UVA rays enable a deeper skin penetration causing the formation of free radicals and resulting in indirect cell damage [15] .

However, Zastrow et al. found in 2009 that not only UV radiation but also near infrared and visible light contributes a major part to the total free radical formation as depicted in Figure 2. They discovered that "50% of the total skin oxidative burden was generated by visible light " and also concluded that excess free radicals were evidenced by near IR-radiation [16]. Darvin et al. confirmed these findings in 2010, employing in vivo resonance Raman spectroscopy as well as EPR ex vivo [17].

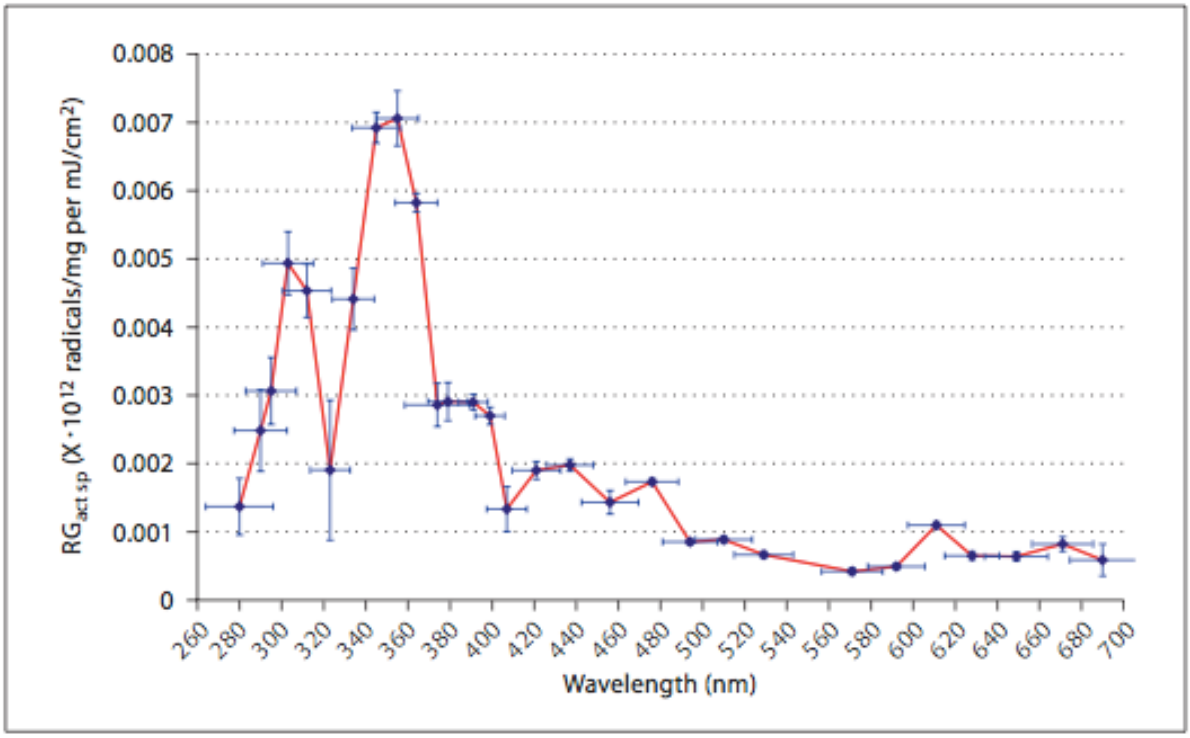

Figure 2: Action spectrum wavelength dependence for free radical generation. Horizontal bars represent the spectral bandpass value of pairs of adjacent cut-off filters, vertical bars represent the standard error associated with radical generation (RG) measurement. Taken from [16], with friendly permission of S. Karger AG.

Accounting for possible detrimental consequences, the body employs protective measures to guard the organism from substantial free radical mediated damage. Antioxidants such as vitamins A, C and E, next to other defence mechanisms, act as natural antagonists of radical formation. They act by protecting lipids from oxidation,

donating an electron, breaking up a chain reaction and consequently prevent further cell damage [18].

These protective effects have also been reported using topical antioxidants [19, 20], as well as dietary products [21-24] and therefore are a useful tool in the development of sun protection products [25].

2.2 The radiation spectrum of sunlight

Solar radiation ranges from gamma- to x-ray, to ultraviolet, visible and infrared radiation. Of these rays only part will reach the surface of the earth after passing through the atmosphere and ozone layer. Solar irradiation reaching our skin is therefore primarily made up of ultraviolet, visible and infrared radiation (Figure 3).

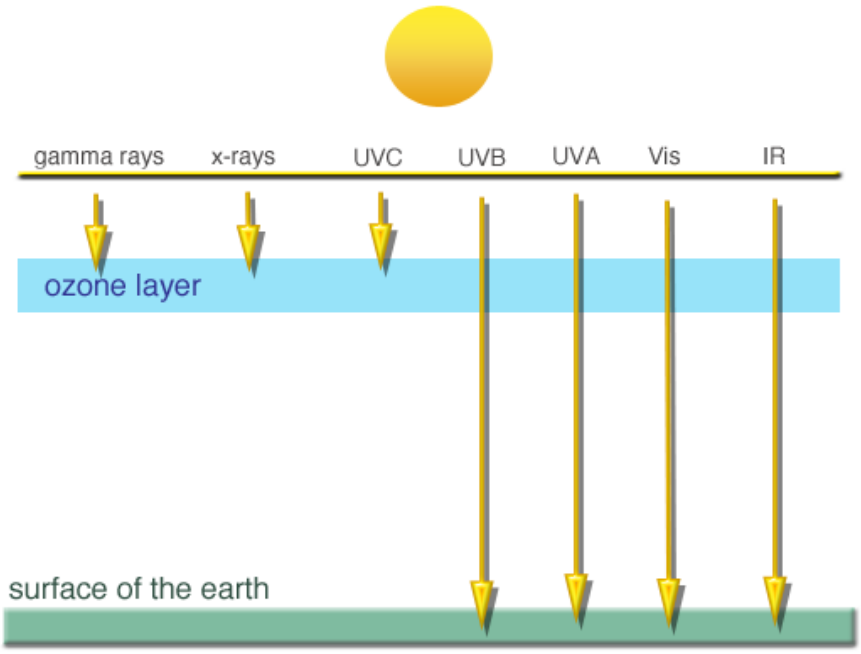

Figure 3: Solar irradiation reaching the surface of the earth adapted from [26].
UV = Ultraviolet, Vis = Visible, IR = Infrared.

2.2.1 Ultraviolet radiation

Essentially, there are three different types of ultraviolet radiation (UVR) with wavelengths ranging from 100 through 400 nm all of which are invisible to the human

eye. UVC radiation is of the shortest wavelength (100 – 280 nm) in the UV spectrum and can be neglected in regard to skin damage as it is filtered virtually in total by the ozone layer [3]. UVB radiation has a wavelength of 280 to 320 nm and UVA radiation is of the longest wavelength ranging from 320 to 400 nm.

UVA and UVB radiation differs in its effects on the skin. While UVA radiation is responsible for photo aging, the immediate tanning effect by redistributing and oxidizing the existing melanin and excess free radical formation, UVB radiation is accountable for erythema formation, the reddening of the skin, for sunburns, direct breaks in DNA strands, for the biosynthesis of Vitamin D_3 and also for the formation of a tan that takes longer to appear but will be more intense and last longer [27]. Both UVA and UVB radiation contributes to the development of skin cancer through various mechanisms [28-30]. The different properties of radiation can be explained by its wavelengths and depth of skin penetration, illustrated in Figure 4. Battie and Verschoore report that 70% of UVB radiation is absorbed by the stratum corneum, while about 20% reaches the viable epidermis and only 10% of rays penetrate the upper part of the dermis. In contrast, they state that 20-30% of UVA radiation reaches the dermis providing an explanation for processes taking place in the deeper layers of the skin such as photo aging [3] .

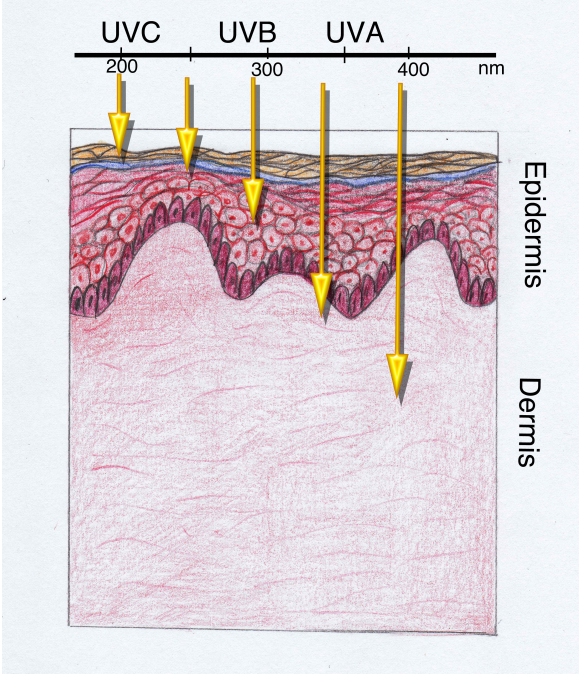

Figure 4: Schematic drawing of the penetration depth of different wavelengths into human skin. UV = Ultraviolet.

It is important to note that while wavelength determines the penetration depths of rays into the skin, energy of radiation is inversely related to the wavelength in agreement with the Planck relation, therefore UVA radiation reaches deeper skin layers, yet is much less energetic than UVB radiation. However, next to wavelength and energy the occurrence frequency of radiation is also of importance. Overall UVA radiation is much more ubiquitous than UVB radiation as it can pass through clouds and most glass; hence its consequences should not be underestimated in spite of its lower energetic value.

2.2.2 Visible light and infrared radiation

Visible light (Vis) is the only electromagnetic light we can see. It depicts the colors of the rainbow and ranges from approximately 400 through 760 nm in wavelength. Infrared radiation has an even longer wavelength (approximately 760 nm through 1 mm) and can be further divided into near-, mid- and far-infrared radiation; it includes most of the thermal radiation. While UV radiation has been the main focus of interest in terms of sun damage for the past decades, Zastrow et al point out that: “The visible and infrared (IR) parts of the sun spectrum have received little attention concerning their possible contribution to skin damage.” Yet, they state that 50% of total skin oxidative damage has been shown to be generated by visible light [16], revolutionizing the idea of current sun protection and provoking new categories of sun protective products.

2.3 Short- and long-term implications of solar irradiation

2.3.1 Short-term implications

Shortly after solar irradiation several changes in the skin can be observed. In addition to first signs of macroscopically visible skin damage such as erythema and sunburn formation, desirable effects of the sun manifest similarly. This includes improved vitamin D synthesis [31-33], recently credited with a protective effect for an array of diseases, including cancer [34, 35] as well as an increase in serotonin levels leading to a sense of well-being [36, 37]. Additionally, a decrease in stress levels and even pain reduction has been noted [38]. Not least, the often anticipated tanning of the skin occurs. For the purpose of this study erythema formation, building the basis for SPF

calculations will be illuminated in more detail in the following.

Following UV irradiation overexposure, the first change to be noted is an increase in redness of sun exposed skin. This alteration is called an erythemal reaction. It is a sign of vasodilatation of cutaneous blood vessels and is elicited for the most part by UVB radiation [3]. Battie and Verschoore describe that an increase in wavelength considerably decreases the erythemal effectiveness of a ray. Thus, UVC radiation would be extremely dangerous in terms of potential erythemal effectiveness, due to their short wavelength. However, it can be neglected in consequence of the filtering properties of the ozone layer. The authors add that in spite of the long wavelength, "UVA contributes to at least 15% of the sun induced erythema".
Continuous irradiation of the skin induces further changes, as mast cell and cytokine release heighten the inflammatory response [39]. Within two hours of exposure, DNA damage can be observed. Histologically, epidermal keratinocytes with a pyknotic nucleus and eosinophilic cytoplasm, the so-called sunburn cells [40], stand out. Furthermore, Langerhans cells undergoing apoptotic changes [4] can be noted. Dermatitis solaris, also known as sunburn, is the result. The height of symptoms occurs after 12-24 hours and manifests itself as an itching, burning sensation. Depending on skin type and amount of radiation, solar dermatitis can produce blistering and is classified as first-degree, and in case of blistering, a second-degree burn.

2.3.2 Long-term implications

When sunlight irradiation persists over an extended period of time, additional changes in the skin become evident.
Since the 1970s ultraviolet radiation (UVR) has been known for its immunosuppressive properties [41]. Today these qualities are frequently used for their therapeutic value in the treatment of psoriasis [42], atopic dermatitis [43] and vitiligo [44]. However, a restricted immune system also limits its protective properties. A significant correlation between immunosuppression and skin cancer development has been described [30].
Overall, the link between skin cancer development and UVR exposure has been well established [45, 46]. An in-depth review on skin cancer and solar ultraviolet radiation in 2009 by C. Young concludes, "There is a clear positive association between solar UVR

and all types of skin cancer [47].” UVB-induced DNA damage, immunosuppression caused by UVR, mutations in the p53 tumor oncogene [28] and formation of reactive oxygen species [29] have all been listed as contributing factors.

And finally, not as detrimental, yet a feared consequence of prolonged sunlight exposure is photo aging [48]. This term is used to describe extrinsic aging caused by long-term UV exposure exacerbating the effects of intrinsic aging.

Both UVA and -B radiation contribute in different ways to this process. While UVA rays trigger the formation of reactive oxygen species, which in turn prompt a cascade of events leading eventually to collagen breakdown, UVB rays cause direct damage to DNA strands. However, both processes ultimately lead to premature signs of skin aging such as wrinkles, dyspigmentation and telangiectasia [49].

2.4 Sunscreens - mechanisms of action and main range of protection

Generally, sunscreen filters can be divided into two main categories: inorganic (physical) and organic (chemical) filters. While both absorb high intensity UV rays to some extent [50], physical filters operate primarily as blockers by reflecting and scattering rays depending on their particle size and shape [51]. Titanium dioxide and zinc oxide constitute for physical filters, they are considered broad-spectrum agents as they block radiation over the entire light spectrum. Organic filters, on the other hand, exert their protective properties by absorption, exciting UV rays to a higher energy state while de-excitation may occur by fluorescence and thermal energy, amongst others [52]. The range of protection differs from compound to compound. Hence, filter substances such as avobenzone provide protection primarily in the UVA spectrum. Padimate O, on the other hand, exerts effects primarily in the UVB range. However, broad-spectrum agents such as oxybenzone absorb radiation over the entire UV spectrum.

Together, physical and chemical filters have been shown to work synergistically increasing the sun protection factor of a product [53].

2.5 The sun protection factor

2.5.1 History of the sun protection factor

In 1934 Friedrich Elling was the first to evaluate sunscreens for their protective abilities. He used the Minimal Erythemal Dose (MED) of the skin to determine the protective properties of a product and calculated a coefficient [54].

More than 20 years later, in 1956, Rudolf Schulze developed a method to calculate a protection factor applicable for every product [55]. The Schulze method was non-standardized, yet it is equivalent to the calculation that is still in use, he named the resulting coefficient the Schulze factor.

The factor was renamed in 1962 by Greiter, which from then on became the sun protection factor (SPF) [56]. A standardization of the method followed.

In 2003, Colipa, now known as Cosmetics Europe, developed in cooperation with Japan and South Africa the first international sun protection factor guidelines. They were revised in 2006 when the project was joined by the US and published under the international sun protection factor method [57].

First published in 2007, and in a revised version again in 2011, Cosmetics Europe supplemented their recommendations with an additional guideline concerning an in-vitro method for the determination of the UVA protection factor and 'critical wavelength' values of sunscreen products [58].

Given the historically rooted sun protection factor evaluation method it stands to reason that advances in research over the past decades have led to few additions and changes. It can therefore be assumed that limitations, further discussed in section 2.6.4, may become apparent and the need for a revision of the factor reflecting the current state of research should be considered in the near future.

2.5.2 Definition and practical implementation of the sun protection factor

The sun protection factor (SPF) of a product is defined as "the numerical ratio between the Minimal Erythemal Dose (MED) of sunscreen-protected skin, applied in the amount of 2 mg/cm^2 and the Minimal Erythemal Dose of unprotected skin [54]." Testing involves a group of 10 to 20 volunteers, in which each volunteer is partly treated with sunscreen (2 mg/cm^2), and partly left unprotected. Following a 15 to 30-minute waiting period, allowing the sunscreen to penetrate, each volunteer is subjected to radiation emitted by

a xenon arc lamp solar stimulator. After 16 to 24 hours both protected and unprotected areas are inspected for redness and the Minimal Erythemal Dose is calculated. Cosmetics Europe defines the Minimal Erythemal Dose as “the lowest ultraviolet (UV) dose that produces the first perceptible unambiguous erythema with defined borders appearing over most of the field of UV exposure 16 to 24 hours after UV exposure [59].“ The SPF is then calculated by dividing the minimum amount of light required to cause redness in protected skin (MED) by the dose of light required to cause redness in unprotected skin (MED_U), the dose of light being defined as the light intensity multiplied by time.

$$SPF = \frac{Minimal\ \ Erythemal\ Dose\ of\ protected\ Skin}{Minimal\ Erythemal\ Dose\ of\ unprotected\ Skin}$$

2.5.3 Current sun protection product labeling

Cosmetics Europe states on their website the following recommendations regarding the labeling of a sun protective product [5] :

1. The SPF is the main indicator of sun protection.
2. The following labeling categories are listed in Table 2: ‘Low protection’, which corresponds to an SPF of 6 and 10, ‘Medium protection’, an SPF level of 15, 20, and 25, ‘High protection’, SPF values of 30 and 50 and finally ‘Very high protection’, an SPF of 50+.

European Commission System for SPF Labeling	
Labeled category	**Labeled sun protection factor (SPF)**
Low protection	6 10
Medium protection	15 20 25
High protection	30 50
Very high protection	50+

Table 2: European Commission System for SPF Labeling

3. „ UVA protection of a product should be in relation to its SPF „ Cosmetics Europe proposed that the „UVA protection should be at least 1/3 ratio of its SPF“ and further states that „Manufacturers will show that their products meet the SPF/UVAPF ratio by displaying the letters “UVA” inside a circle whose diameter should not exceed the height of the SPF number. „

2.5.4 Limitations of the sun protection factor

While several limitations of the SPF have been pointed out by different authors, including the artificial source of radiation utilized for testing [54] and reduced efficacy of sunscreens due to individual variation in application methods [60], for the purpose of this study the focus will lie primarily on limitations due to erythema formation as single efficacy indicator illuminated further in the following.

The main indicator used to determine a product's protective capacity is the individual erythema. The reddening of the skin is a biological marker. Therefore, response time depends on many variables such as skin type, individual skin structure and age [61]. Hence, by using only 10-20 different volunteers, variability of the mean SPF value may increase considerably. Even when volunteers are preselected by photo skin type I-III, as required by Colipa standards, variability may occur due to individual differences in skin structure and age of volunteers. This provides a possible explanation for SPF value discrepancies of up to 40% between different laboratories seen in a study conducted in 2002 [62]. Variability was shown to be larger the higher the SPF value.
Aware of this problem, Colipa revised their guidelines in 2006, adopting a more standardized SPF testing approach and now recommend using add-ons, stating whether a product is in the low, medium, high, or very high protective range (Table 2), thereby reducing the use of specific numbers and minimizing individual laboratory variance.

Furthermore, using erythema formation as the only indicator of sun damage poses the risk of underestimating effects of additives such as antioxidants and anti-inflammatory components of commercially available sunscreens [63]. The SPF may be artificially increased due to a lack or delay of biological response.

Last but not least, the development of erythema is induced predominantly by UVB radiation. While many detrimental effects of the sun are at least in part caused by UVA radiation [47], this aspect stays unaccounted for using the SPF as a label. In 2003, Haywood et al demonstrated a large disparity between SPF values, principally representing UVB radiation, and UVA-related radical formation measurements for the same products [64]. They suggest that the use of sunscreens could increase the risk of UVA-induced radical damage owing to prolonged sunbathing, as consumers feel protected by sunscreen products. Responding to this problem, Colipa revised their guidelines in 2006 and now recommend including at least 30 percent of the total SPF value as UVA protection [59], which protects the consumer. However, the exact amount of UVA protection cannot be inferred easily from the SPF label. Also, Zastrow's findings that visible light and infrared radiation produce 50% of total reactive oxygen species [16] cannot be accounted for. The consumer feels protected by a product, owing to the lack

of macroscopically visible damage, namely the erythema formation, yet is oblivious to any underlying damage produced by UVA, Vis or near infrared radiation.

Finally, limitations are posed by the invasiveness of the current SPF testing method. Each time a new product is tested, test persons are subject to radiation and therefore exposed to possibly harmful consequences.

2.6 Efficacy evaluation approaches alternative to the sun protection factor

Several new approaches to quantifying sun protection efficacy have been suggested.

2.6.1 The universal sun protection factor

In 2007, a spectroscopic factor to determine sunscreen protective efficacy over the entire UV range was proposed - the universal sun protection factor [6].
The basis for this method is the process of tape stripping, in which layers of the stratum corneum are removed using an adhesive film from previously sunscreen treated skin areas. The removed samples reflect the individual in vivo distribution of sunscreen formulation. Repeated removal of tape strips from an identical skin side transfers the uppermost part of the stratum corneum, containing sunscreen formulation, to a stack of individual tape strips. These strips are subsequently measured over the entire UV range (280 - 400 nm). The measurements obtained are the basis for forming sum transmission spectra allowing the calculation of the universal sun protection factor.
Advantages of this method are owed to the non-invasive procedure and the objective evaluation of protective properties of a sunscreen over the entire UV spectrum independent of biological responses.
However, its limitations may lie in the lack of the possibility to determine protective capabilities within the visible and near infrared ranges. Here, biological damages and protective properties such as radical scavenging activity prevail, which are not measurable using this method. Yet, in light of recent research developments, these spectral ranges may be of importance when considering measures of sun protection in the future [16, 25].

2.6.2 The radical formation ratio

A correlation between free radical formation and UV dose [7] suggests additional potential for determining a product's protective efficacy. Using electron paramagnetic resonance spectroscopy, free radical formation can be detected [65, 66]. Nitroxide spin probes can be utilized as free radical traps. A reaction of free radicals with the spin probe results in the loss of the probe-emitted EPR signal, indicating the amount of radicals formed [8]. Measurements are recorded prior to, during and after irradiation. Several systems to categorize radical formation, determined via EPR spectroscopy, have been described, such as the free radical protection factor [64], the Radical Sun protection Factor (RSF) [8] and the integrated sun protection factor [7]. For the purpose of this study, EPR signal intensity will be normed and a ratio will be established by forming a quotient of signal intensity before and after irradiation, prospectively termed radical formation ratio (RF). Considerable advantages of working with radical formation involve the possibility to quantify protective efficacy over the entire ultraviolet, visible and infrared spectrum. Protection provided by additions of antioxidant can be detected reliably [65]. However, the limitation of utilizing free radical formation to determine sunscreen efficacy is the invasive nature of the technique, although human skin samples may be substituted with porcine ear skin, as demonstrated by Haag et al in 2010 [67] and adopted in this study. Finally, a further limitation is the confinement to an underlying yet single biophysical answer to solar irradiation.

3 Aims of the Study

In line with the preceding information, the overall aim of this study was to compare two alternative sunscreen efficacy indicators, the USPF and RF, to the sun protection factor and to assess their value with a view to developing a comprehensive, non-invasive sun protection evaluation method and efficacy indicator in the future.

In order to reach this goal, initially the USPF and radical formation ratio for five different specifically developed sun protective products from Merck KGaA containing known active ingredients were determined. The USPF was determined spectroscopically employing the tape stripping method and the radical formation ratio was calculated for each formulation based on electron paramagnetic resonance measurements. SPF measurements for four of the products were conducted according to standards of Cosmetics Europe by proDERM GmbH.

To help evaluate whether the addition of different active ingredients, in this case chemical and physical filters as well as antioxidants, have a similar effect on USPF and radical formation values as on the broadly utilized sun protection factor, the influences were assessed statistically. Consequently, it could be deduced whether additives influence radical formation and attenuation of radiation to a similar degree as they influence erythema formation assessed to determine the in vivo sun protection factor.

Finally, measurement results were analyzed for correlations between methods.

> ***Specific aim 1:*** To determine USPF, RF and SPF values for the provided formulations.
>
> ***Specific aim 2*****:** To assess the influence of physical filters, chemical UV filters and antioxidants on USPF, RF and SPF values.
>
> ***Specific aim 3:*** To determine correlations between USPF and RF values, USPF and SPF values, and SPF and RF values.

4 Materials and Methods

The aim of this study was to compare three different efficacy indicators. Hence, this thesis comprises three separate methods: the determination of the universal sun protection factor; the assessment of radical formation; and completive the in vivo sun protection factor determination, here carried out by proDERM GmbH to obtain secured reference values within the framework of this study.

4.1 Universal sun protection factor determination using spectroscopy

4.1.1 Volunteers

For the spectroscopic measurements 30 healthy volunteers, (22 female and 8 male test persons) aged 21 through 36 (mean 25.5 years) with skin type II or III on the Fitzpatrick skin type scale (Table 1) were selected. Measurements were conducted on untanned skin, no skin diseases were reported and no scars or visible damages to the skin were observed.

The study was conducted in compliance with the declaration of Helsinki [68]. Informed consent had been given by of each of the volunteers tested and permission from the ethical review committee of Charité Universitätsmedizin Berlin had been obtained.

4.1.2 Sunscreen formulations

For this study, five different formulations specifically prepared for the purpose of this study by Merck KGaA were used. For simplification purposes the formulations were labeled with numbers 1, 2, 3, 4, and 5. For each formulation the same base (Aqua, Butylene Glycol Dicaprylate/dicaprate, Glycerin, Dioctylcyclohexane, Polyglyceryl-2, Dipolyhydroxystearate, Glyceryl Stearate, PEG-100 Stearate, Cetearyl Alcohol, Cetyl Palmitate, Magnesium aluminium Silicate, Xanthan Gum, Disodium EDTA and preservative) was used. The products differed only by their active ingredients. Antioxidants (1%), in this case bis-ethylhexyl hydroxydimethoxy benzylmalonate (Merck

KGaA RonaCare© AP), chemical ultraviolet filters (10%), a combination of butyl methoxydibenzoylmethan (Merck KGaA Eusolex© 9020) and octocrylene (Merck KGaA Eusolex© OCR) and physical filters (2%) consisting of titanium dioxide, alumina and manganese dioxide (Merck KGaA Eusolex© T-Pro).

As summarized in Table 3, the active ingredients of the formulations are the following: Cream 1 consists of a base formulation and antioxidants. Formulation 2 contains in addition to the base, physical filters as the active ingredient. The active ingredients in formulation 3 are chemical ultraviolet filters. Cream 4 contains the base and two active ingredients, chemical ultraviolet filters and antioxidants. Whereas, cream 5 consists of the base, chemical ultraviolet filters, physical filters and antioxidants.

Formulation	Active Ingredients	Abbreviation
Base	--	
Cream 1	Antioxidants (1%) (bis-ethylhexyl hydroxydimethoxy benzylmalonate)	AO
Cream 2	Physical Filters (2%) (titanium dioxide, alumina and manganese dioxide)	PF
Cream 3	Chemical UV Filters (10%) (butyl methoxydibenzoylmethan and octocrylene)	c-UVF
Cream 4	Chemical UV Filters and Antioxidants	c-UVF, AO
Cream 5	Chemical UV Filters, Physical Filters and Antioxidants	c-UVF, PF, AO

Table 3: Summary of formulation number, active ingredients contained and abbreviations used.

4.1.3 Methods - USPF

4.1.3.1 Preparation of the skin and application of sunscreen

To ensure similar conditions, volunteers had been previously instructed to avoid using cosmetic products on their forearms for at least 24 hours previous to commencement of the study. Upon arrival, one forearm was cleaned using cold running water and dried carefully with a paper towel (step 1 in Figure 5). Next, an 8 by 10 cm rectangle was drawn on the preferably hair-free forearm using a skin marker (step 2 in Figure 5).

Subsequently, in line with the Cosmetics Europe Association guidelines, 160 mg ($2mg/cm^2$) [5] formulation was evenly distributed within the markings using a saturated

gloved finger (step 3 in Figure 5). During the 60 min product penetration time interval, volunteers were asked to rest and avoid contact with the treated region.

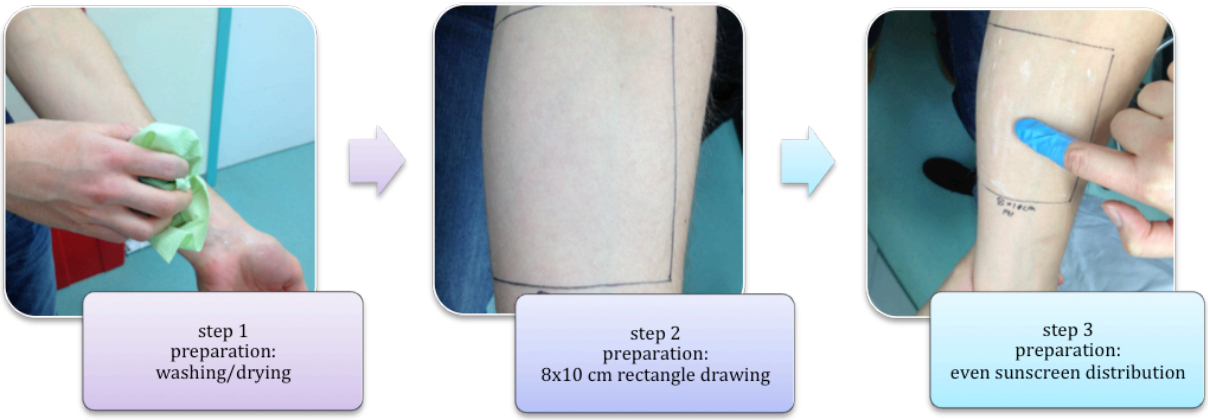

Figure 5: Steps 1 through 3 - preparation process before tape stripping.

4.1.3.2 Tape stripping procedure

After 60 minutes of sunscreen penetration the tape stripping method was performed. A preferably hairless area in the treated rectangle was chosen to apply a 19-mm-wide, approximately 6-cm-long adhesive tape strip (tesa-Film No. 5529 Beiersdorf, Hamburg, Germany). To ensure the same location for the succeeding tape, markings were applied (step 4 Figure 6). Next, the tape was fixated onto the forearm by applying evenly distributed pressure (14.5 kp/cm^2) over the entire area using a weighted stamp for three seconds (step 5 figure 6).

Figure 6: Steps 4 through 6 - the tape stripping procedure.

The tape was then quickly removed and secured on a rectangular, plastic sample holder measuring 4.5 by 4 cm (step 6 Figure 6) and measured against an empty tape in the 240-500 nm UV range of a Perkin Elmer Lambda 650 S UV/Vis spectrometer in front of the integrated sphere, measurement area was set to 4 nm.

Steps 4 through 6 - the application, pressure and removal of a tape strip were repeated for a total of 10 times from the same location. Previous studies have shown that complete removal of UV filters is achieved by removing 10 or fewer layers of corneocytes, as UV filter substances are present only in the upper cell layers of the stratum corneum [6].
Additionally, a similar field of untreated hairless skin on the same forearm was marked. The same procedure of marking, applying pressure and removal of a tape strip was repeated another 10 times to obtain reference values for skin layers without formulation.

4.1.3.3 Spectroscopic measurements

Spectroscopic measurements were carried out in front of the integrated sphere of the Perkin Elmer Lambda 650 S UV/Vis spectrometer (PerkinElmer LAS GmbH, Rodgau, Germany) to account for all transmitted radiation including diffused scatter radiation. Measurements were conducted within 15 seconds of tape removal to ensure characteristic skin distribution of the active ingredients, as previous studies have shown that diffusion processes may occur if the time period between removal and measurement increases [69, 70].
The measurement area was set to 4 nm and measurements were conducted in the 240-500 nm range, analysis occurred for measurements from 280 through 400 nm.

4.1.3.4 Determination of the average sum transmission and universal sun protection factor calculations

Once measurements for tape strips reflecting the in vivo distribution of sunscreen and for tape strips with untreated corneocytes were conducted, subsequently, in order to obtain absorbance capacity for each individual formulation, the influence of the corneocytes on the spectra was corrected. This was achieved by subtracting the spectrum of the untreated skin tape from the sunscreen-treated skin tape spectrum

consecutively (treated skin tape 1 - untreated skin tape 1, etc.), using the UV Winlab program (UV Winlab version 6.0.3.0730 (Perkin Elmer, Frankfurt/Main, Germany) and UV Winlab Data Processor and Viewer (Perkin Elmer 2009 version 1.00.00.0010).

The resulting spectrum exclusively depicted the impact of the applied UV filter, eliminating any corneocyte influence on the spectrum. To diminish minor flaws of the spectra, a smoothing degree of 8 was applied.

The individually corrected spectra were then summed up one by one (the 1st with the 2nd tape spectrum, the corresponding sum of both with the 3rd, the sum of all three with the 4th and so on) (Figure 7) to determine the sum transmission spectra. Calculations were carried out in the absorbance scale, as working with this scale ensured not only a linear correlation between the spectra and the concentration of the absorbers, but also warranted that sum spectra were obtainable by simple addition. The resulting spectra were changed to transmission scale later (Figure 8).

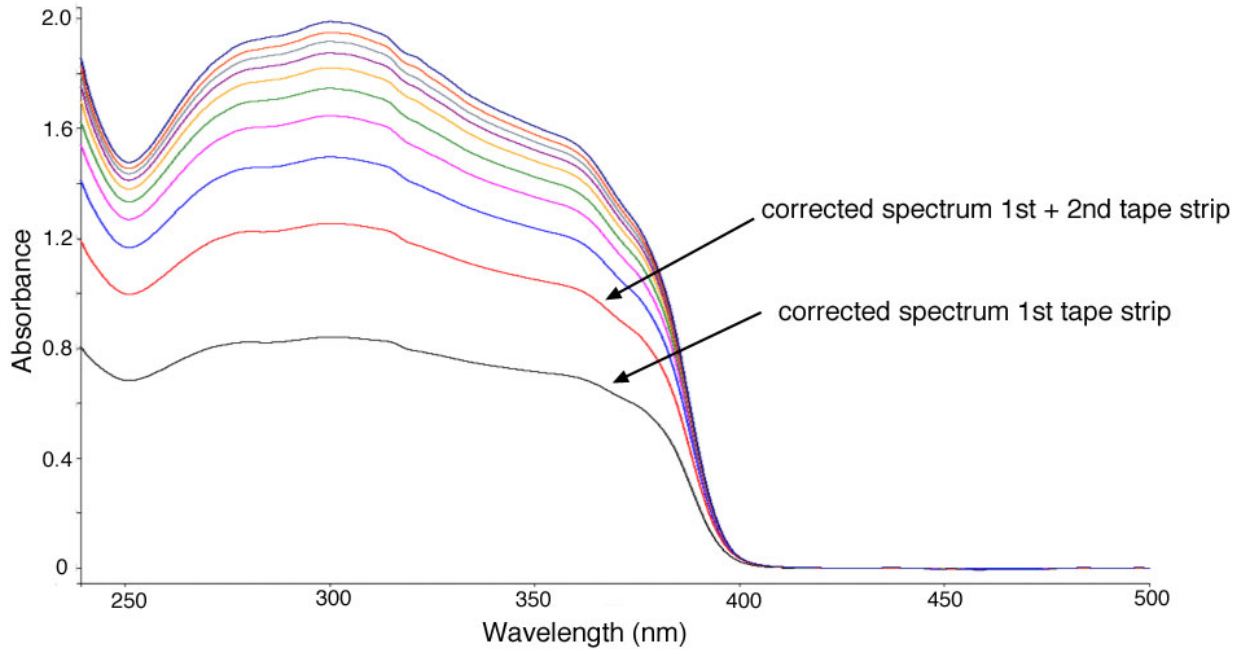

Figure 7: Example of sum absorbance spectrum determination. Each corrected spectrum is added consecutively.

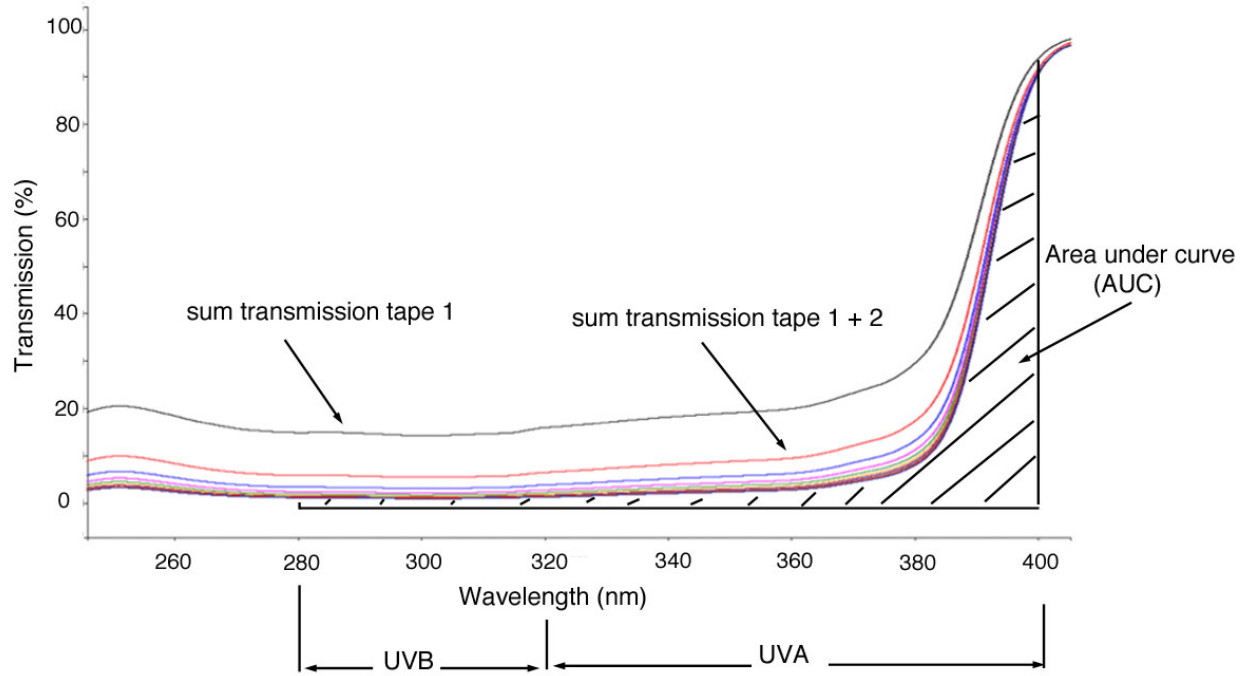

Figure 8: Example of sum transmission spectrum. Sum transmission tape 1 = Sum 0 in Table 4. Sum transmission tape 1 + 2 = Sum 1 in Table 4. The hatched area under the curve is the basis for USPF calculations.

The corresponding percent transmission values were read at 300 nm, the maximum of the UVB absorbance curve, for each of the 10 sum spectra and transferred to a table. In this table the first tape strip was set to 100% and the percent difference of each tape was assessed subsequently (Table 4). The first tape spectrum with less than 1% transmission difference to the previous tape spectrum transmission was selected for every ensuing calculation, as this spectrum would represent the last skin layer containing any formulation substances.

	% Transmittance (T) at 300 nm	Δ *T*	% Δ
Sum 0	21.14	-	100
Sum 1	8.32	12.82	61
Sum 2	4.02	4.30	20
Sum 3	2.34	1.68	8
Sum 4	2.00	0.34	2
Sum 5	1.83	0.17	1
Sum 6	***1.75***	***0.08***	***0***
Sum 7	1.68	0.07	0
Sum 8	1.62	0.06	0
Sum 9	1.53	0.09	0

Table 4: Example of percent transmission differences between sum spectra. Percent Transmission (T) is read at 300 nm. $\Delta T\ sum\ (n+1) = sum\ (n) - sum\ (n+1)$. Sum 0 is set to 100% difference. Spectrum representing the last skin layer containing formulation is printed in italics and underlined.

The average sum transmission describes the residual radiation reaching the skin after sunscreen application. This value is the foundation for the calculation of a spectroscopically defined sun protection factor, which similarly to the SPF shows how much longer a person can stay in the sun when using a sun protective product to avoid sun-induced consequences.

To determine the average sum transmission, the area under the curve (AUC) of the last spectrum containing formulation has to be computed. It is calculated using the following equations:

$$AUC_{\ UVB} = \int_{280\ nm}^{320\ nm} T_{sum}\ (\lambda) \cdot d\lambda$$

$$AUC_{\ UVA} = \int_{320\ nm}^{400\ nm} T_{sum}\ (\lambda) \cdot d\lambda$$

$$AUC_{\ UV} = \int_{280\ nm}^{400\ nm} T_{sum}\ (\lambda) \cdot d\lambda$$

The average sum transmission (AST) values for the UVA, UVB and the 280-400 nm range can now be generated.

$$AST_{UVB} = \frac{AUC_{UVB}}{40\ nm}$$

$$AST_{UVA} = \frac{AUC_{UVA}}{80\ nm}$$

$$AST_{UV} = \frac{AUC_{UV}}{120\ nm}$$

And finally, spectroscopic sun protection factors (SSPF) may be formed employing the following equations:

$$SSPF_{UVB} = \frac{100}{average\ UVB\ sum\ transmission}$$

$$SSPF_{UVA} = \frac{100}{average\ UVA\ sum\ transmission}$$

The resulting spectroscopic factor for both the UVA and UVB ranges is defined as the universal sun protection factor (USPF).

$$USPF = \frac{100}{average\ UV\ (UVA + UVB)\ sum\ transmission}$$

4.2 Radical formation ratio determination using electron paramagnetic spin resonance spectroscopy

4.2.1 Skin samples

For electron paramagnetic resonance spectroscopy measurements, porcine ear skin samples were used. In a study published in 2010, Haag et al demonstrated that porcine skin ear was the most suitable type of skin to simulate human skin in electron paramagnetic resonance-based detection of radicals [67].
Six (6) fresh porcine ears provided by a local butcher were utilized. Ethical approval to conduct these experiments had been obtained from the Veterinary Office, Dahme Spreewald.

4.2.2 Methods - RF

4.2.2.1 Preparation of skin and sunscreen application

First, the porcine ears were cleaned and carefully shaved under cold running water. The ears were then dried using paper towels. To allow for a better penetration of PCA, the first skin layer was removed employing the tape stripping procedure. This was only carried out once to remove the very first horny layer of the porcine ear skin.

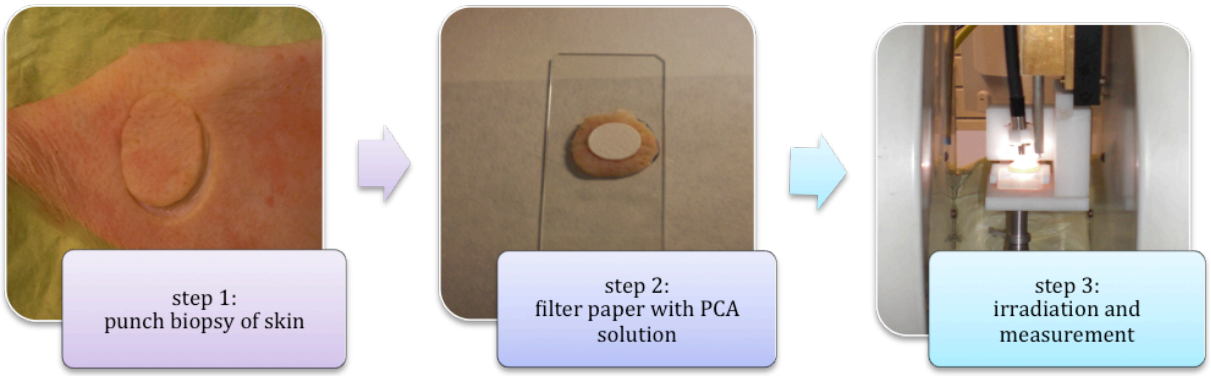

Figure 9: Preparation of porcine skin ear samples for electron spin resonance measurements

Next, a stamp, 19 mm in diameter, was used to remove an unscathed skin sample from the ear (Figure 9, step 1). The sample was placed on a previously prepared and marked object slide. To ensure an even concentration of marker, a filter paper disk (11 mm in

diameter) was placed on the biopsy, which was then treated with 50 µl of water: ethanol (1:1) 0.2 % PCA (3-Carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl, Sigma-Aldrich, Steinheim, Germany) solution (Figure 9, step 2). Subsequently, the biopsy was immediately covered by a light-impenetrable occlusive covering (Epitest Ltd Oy, Tuusula, Finland) to circumvent radical production by an outside light source. The penetration time was set to 20 minutes. Following the elapsed penetration time, 5.7 mg of formulation (2 mg/cm^2, in accordance with Cosmetics Europe Guidelines [5, 59]) was evenly distributed on the skin sample and stored in a light-protected container for 30 minutes.

For each experiment, punch biopsies were prepared in duplicate to allow for local variations in porcine ear skin samples. After both samples were measured before and after irradiation an arithmetic mean was generated. Also, a control biopsy using only base formulation without active ingredient was prepared for each experiment to investigate for possible complications with the porcine ear skin sample, the measurement was conducted under the same conditions.

Overall, the skin of six (6) different porcine ears was used, due to space limitations. At least six (6) mean values for each cream were determined in total.

4.2.2.2 Electron spin resonance spectroscopy measurements

Then, the slide was placed in the L-band electron spin spectrometer LBM MT 03 (Magnettech, Berlin, Germany). Measurements of the electron spin signal were taken continuously, each measurement lasting 15 seconds for 16 minutes total. Using the solar simulator (LS0104, LOT, Darmstadt, Germany) the ultraviolet/visible spectrum light irradiation was started and the measurements were repeated again for a total of 16 minutes (Figure 9, step 3).

For this study a solar simulator (LS0104, LOT, Darmstadt, Germany) containing a 150 W Xenon arc lamp for the ultraviolet and visible light irradiation was utilized. The light was coupled into the spectrometer using the liquid light guide LLG 113 (3 mm diameter, LOT, Darmstadt, Germany). The transmittance of the liquid light guide was set to 300 to 650 nm and the distance to the biopsy fixed at 1 cm. Irradiation intensity was measured at 90 mW/cm^2 determined with a radiant power meter LSZ011 (LOT, Darmstadt, Germany). The UVA portion of the intensity was 8.55 mW/cm^2, the UVB portion 1.75

mW/cm^2 and the VIS portion 79.9 mW/cm^2 assessed by the ILT 1400 Radiometer Photometer (Polytec, Waldbronn, Germany). Samples were measured for 16 min without irradiation and subsequently irradiated for another 16 min. The accumulated energy after 16 min yielded 86.4 J/cm^2.

4.2.2.3 Analysis of results

Mplot.exe was used to determine the EPR signal intensity for each formulation. As each measurement takes 15 seconds, the first 8 spectra substituting for the first 2-minute measurement were retrieved at once and an arithmetic mean was formed. Then a peak-to-peak measurement in the central line of the spectrum was performed to determine the EPR intensity (Figure 10). This was repeated for the subsequent measurements. To account for varying peak intensities of different ears, the data was normed. The first measurement was set to 1.

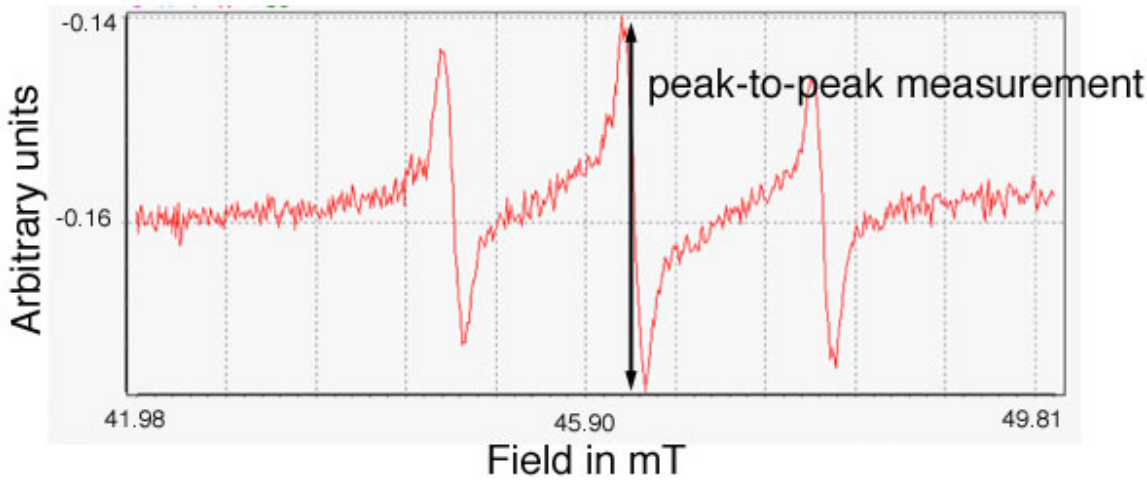

Figure 10: Arithmetic mean of first eight spectra. Peak-to-peak measurements were performed in the central line of the spectrum.

Ratios of radical formation were then determined by calculating the quotient of the normed EPR signal of a sample before irradiation with the normed EPR signal of the sample after UV/VIS irradiation, subsequently termed radical formation ratio (RF).

$$RF = \frac{EPR\ signal\ before\ irradiation}{EPR\ signal\ after\ irradiation}$$

4.2.2.4 Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics version 20 and Microsoft Excel for Mac 2011. $P \leq 0.05$ was found to be statistically significant. When $p \leq 0.1$ a trend could be observed. The Kruskall-Wallis and Mann-Whitney tests were used to establish significant differences between the independent mean values obtained.

4.3 Sun protection factor determination

The in vivo sun protection factor determination for four formulations (creams 2, 3, 4, and 5) was carried out by proDERM GmbH according to Colipa standards. The method isdescribed in detail in section 2.6.2 - Definition and practical implementation of the sun protection factor.

In summary, to determine the SPF, the back of each volunteer was partly treated with 2 mg/cm^2 formulation and partly left untreated. After penetration time volunteers underwent irradiation. Later on, both treated and untreated areas were inspected for redness and the Minimal Erythemal Dose (MED), the lowest ultraviolet dose that produces redness, was determined.

The SPF was then calculated by dividing the MED of the protected skin by the MED of the unprotected skin.

5 Results

5.1 Universal sun protection factor measurements

The spectroscopically determined universal sun protection factor values are summarized in Table 5. Measurements were carried out according to the protocol (section 4.1.3) with a total of 30 volunteers, resulting in 6 individual USPF values for each formulation. Active ingredient abbreviations for each cream are listed in the table. Statistically determined outliers are marked with [1] and are neglected in all subsequent calculations.

As shown in Table 5, formulations 1 and 2 exhibit the lowest USPF values, with a mean value of 1.05 and 1.24, respectively; the standard deviation is low in both cases. Measurement values for the other three formulations were clearly higher, ranging 11.11$\pm$ 0.38 with standard deviation values of 1.75 $\pm$ 0.49.

Formulation	**Cream 1** ***(AO)***	**Cream 2** ***(PF)***	**Cream 3** ***(c-UVF)***	**Cream 4** ***(c-UVF, AO)***	**Cream 5** ***(c-UVF, PF, AO)***
Individual USPF value	1.03	1.31	9.89	11.13	10.25
	(1.12)[1]	1.27	14.17	8.84	13.20
	1.03	1.41	11.21	8.63	12.90
	1.06	1.14	12.01	11.33	11.05
	1.06	1.14	13.45	11.99	10.38
	1.02	1.19	8.15	12.45	11.31
Mean USPF	**1.05**	**1.24**	**11.48**	**10.73**	**11.51**
STD	0.04	0.11	2.24	1.62	1.26

[1] outlier- not considered in all subsequent calculations

Table 5: Overview of individual USPF values, mean USPF and standard deviation for each tested formulation. AO = Antioxidants, PF = Physical filter, c-UVF= chemical UV filter, STD = standard deviation.

Statistical analysis of the creams revealed a significant difference between cream 1 and every other formulation. The same was true for cream 2. However, the values of creams 3, 4 and 5 did not show a statistically significant difference as demonstrated in Figure 11. Here, median USPF values (horizontal black line within the colored box), minimum and maximum (vertical lines outside the box), lower and upper quartiles (upper and lower margin of the colored box) as well as outliers (small circle) are graphically depicted. Differences are marked with an asterisk (*), indicating a p-value of less than 0.05 and again, active ingredient abbreviations are noted below each cream (explanations are applicable to each boxplot diagram in the results section). Calculations were carried out using the Mann-Whitey U test with the SPSS program.

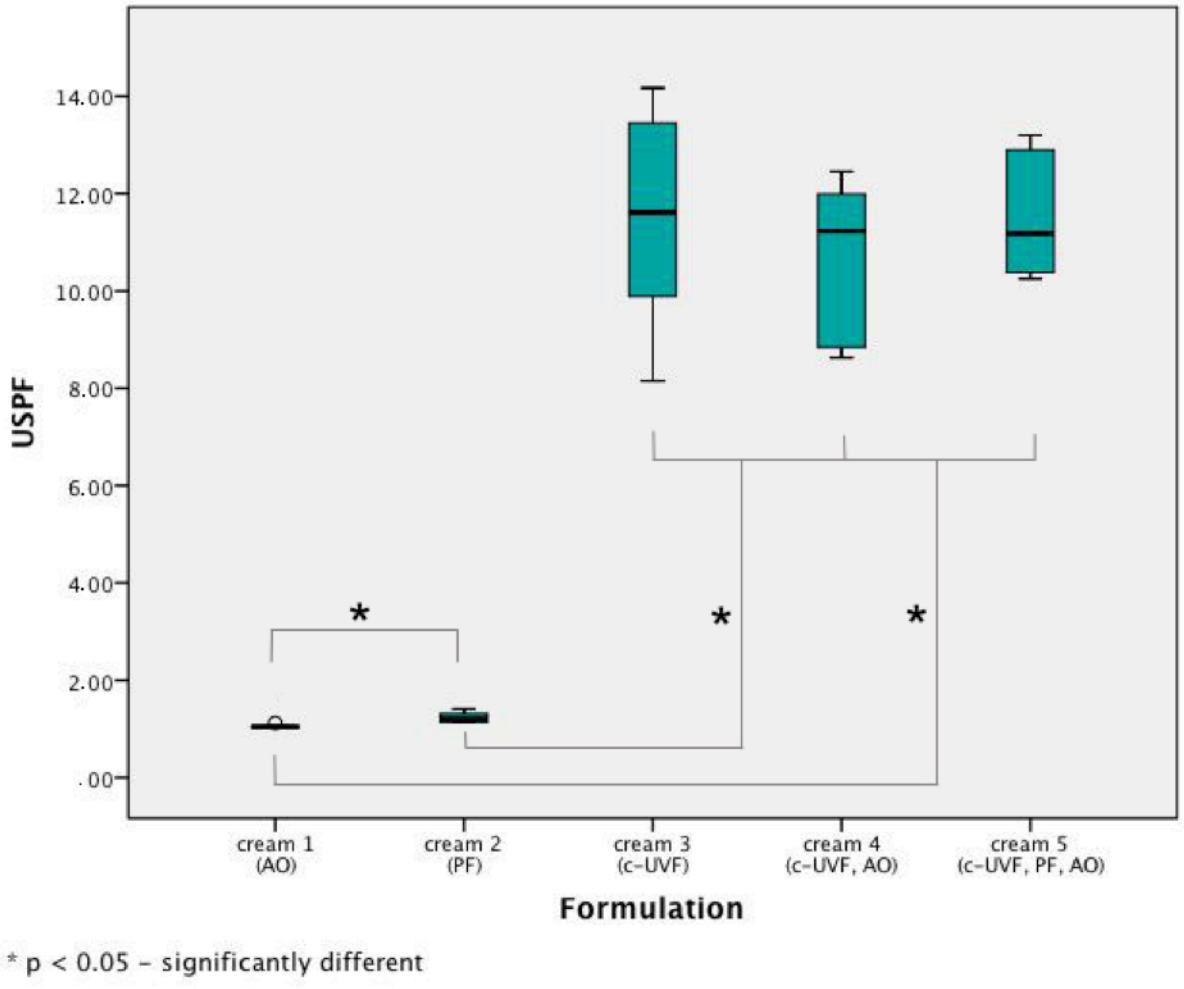

Figure 11: USPF value boxplot for each tested formulation. Significant differences between creams ($p < 0.05$) are marked with * ($n = 6$ for each cream). AO = Antioxidants, PF = Physical filter, c-UVF= chemical UV filter.

5.2 Electron paramagnetic resonance measurements

In agreement with the protocol (section 4.2.2), the intensity of the EPR signal for each cream was recorded. For each formulation, means were determined for eight 15-second measurement intervals, resulting in a 2-minute mean for each interval. Measurements were carried out for a total of 16 minutes without, and subsequently 16 minutes with UV/VIS irradiation, producing sixteen 2-minute means, eight without irradiation and eight with UV/VIS irradiation. The first 2-minute measurement interval for both the non-irradiated time and the irradiated time was standardized to 1. For a better understanding, Figure 12 graphically illustrates mean EPR signal intensity measurements for two creams before and after irradiation.

It can be observed that both formulations show little signal intensity loss during the 16-minute period without irradiation, the base formulation (dark blue line, Figure 12) losing 6% and formulation 5 (light blue line) 7% of their initial signal intensity. However, while during UV/Vis irradiation cream 5 (purple line) containing active ingredients, continues to show little signal loss, the base formulation with a lack of active ingredients shows a loss of EPR signal intensity from 0.94 after 16 minutes without irradiation to 0.57 after the 16-minute irradiation period, an intensity loss of 39%.

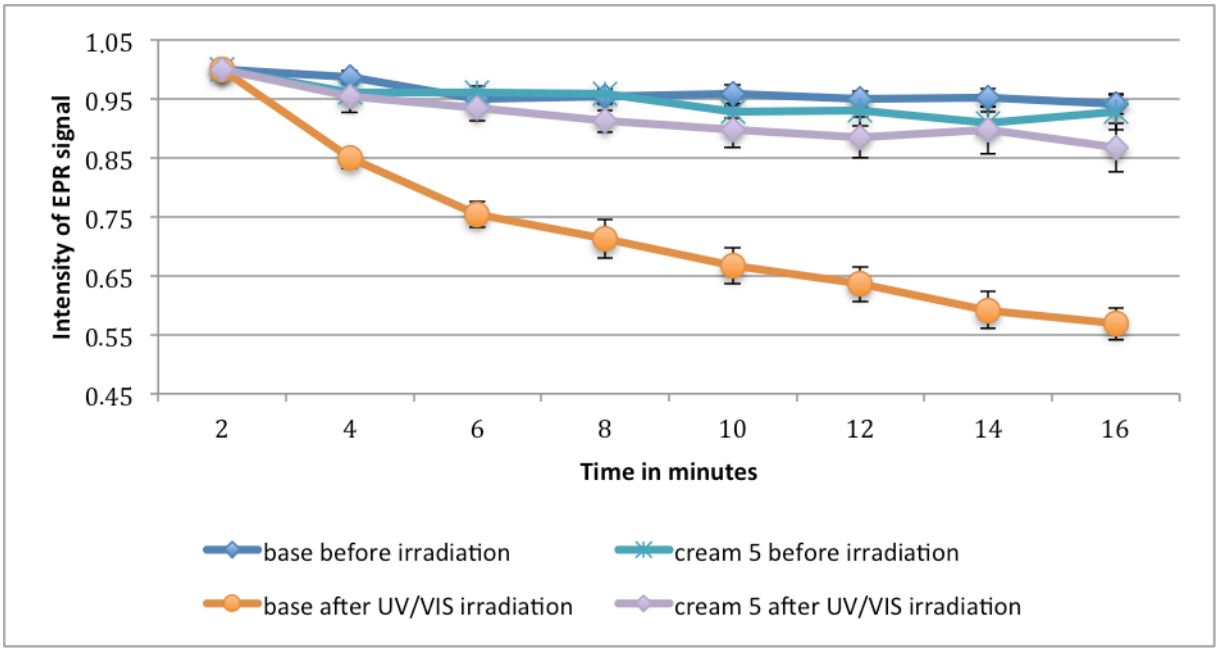

Figure 12: Mean EPR signal intensities ± standard error of base formulation and cream 5 before and after UV/VIS irradiation.

To facilitate comparisons between creams, ratios of EPR signal intensity without irradiation and EPR signal intensity of the UV/VIS irradiated sample were formed, exemplifying changes in radical formation. Thus, the mean 2-minute signal intensity value without irradiation was divided by the mean signal intensity after two minutes of irradiation. This was carried out for each non-irradiated - UV/VIS irradiated 2-minute pair. The mean results for each formulation are shown in Figure 13.

The changes in radical formation can be divided into two groups. Group A made up of the base formulation and formulations 1 and 2 showing an almost linear increase (dotted black trend line) in radical formation and group B with minor changes in radical formation comprising formulations 3, 4, and 5.

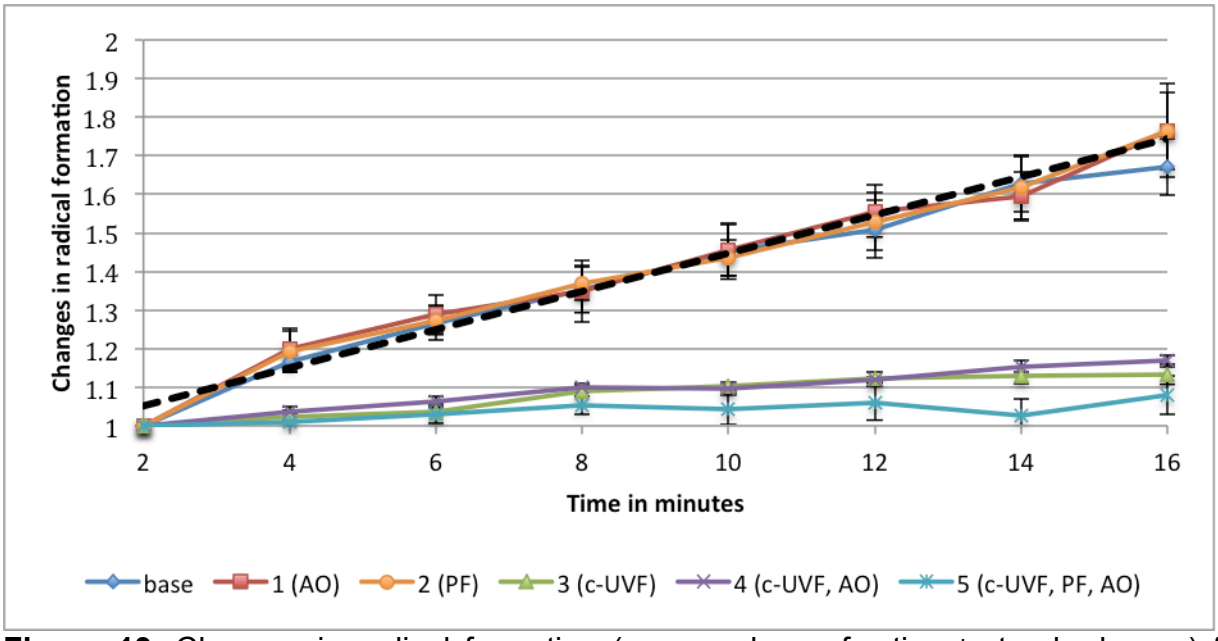

Figure 13: Changes in radical formation (mean values of ratios ± standard error) for each formulation and a dotted black linear trend line. (*n* = 6 for creams 1-4, *n* = 7 for cream 5). AO = Antioxidants, PF = Physical filter, c-UVF= chemical UV filter.

Summarized in Table 6 is the last radical formation ratio calculated (minute 16) for each formulation. Note that while for each formulation, six measurements were recorded; seven individual measurements were conducted for cream 5. A higher radical formation ratio was observed for creams 1 and 2 with a mean of 1.76, while mean values for creams 3, 4 and 5 resulted in 1.13 ± 0.05. The standard deviation was larger for creams 1 and 2 (0.24 and 0.30 respectively) than for the other creams measured (0.09 ± 0.05).

Formulation	**Cream 1** ***(AO)***	**Cream 2** ***(PF)***	**Cream 3** ***(c-UVF)***	**Cream 4** ***(c-UVF, AO)***	**Cream 5** ***(c-UVF, PF, AO)***
Individual radical formation ratio value	2.06	1.69	1.19	1.17	0.98
	1.14	1.65	1.18	1.13	0.95
	1.96	1.47	1.08	1.21	1.27
	1.88	2.23	1.16	1.19	1.01
	1.57	1.93	1.03	1.11	1.05
	1.67	1.57	1.16	1.19	1.03
					1.26
Mean radical formation ratio	**1.76**	**1.76**	**1.13**	**1.17**	**1.08**
STD	0.24	0.30	0.06	0.04	0.13

Table 6: Overview of individual 16-minute radical formation ratio values, mean radical formation ratio and standard deviation for each tested formulation. AO = Antioxidants, PF = Physical filter, c-UVF= chemical UV filter, STD = standard deviation.

A boxplot for the 16-minute radical formation ratio similar to the one described in section 5.1 is depicted in Figure 14. Significant differences between formulations, determined by the Mann-Whitey-U test with the SPSS program, are marked with an asterisk (*), indicating a p-value of less than 0.05.

Both formulations 1 and 2 are shown to be significantly different from creams 3, 4 and 5. Whereas no significant difference between either creams 1 and 2 or cream 3, 4 and 5 was evident.

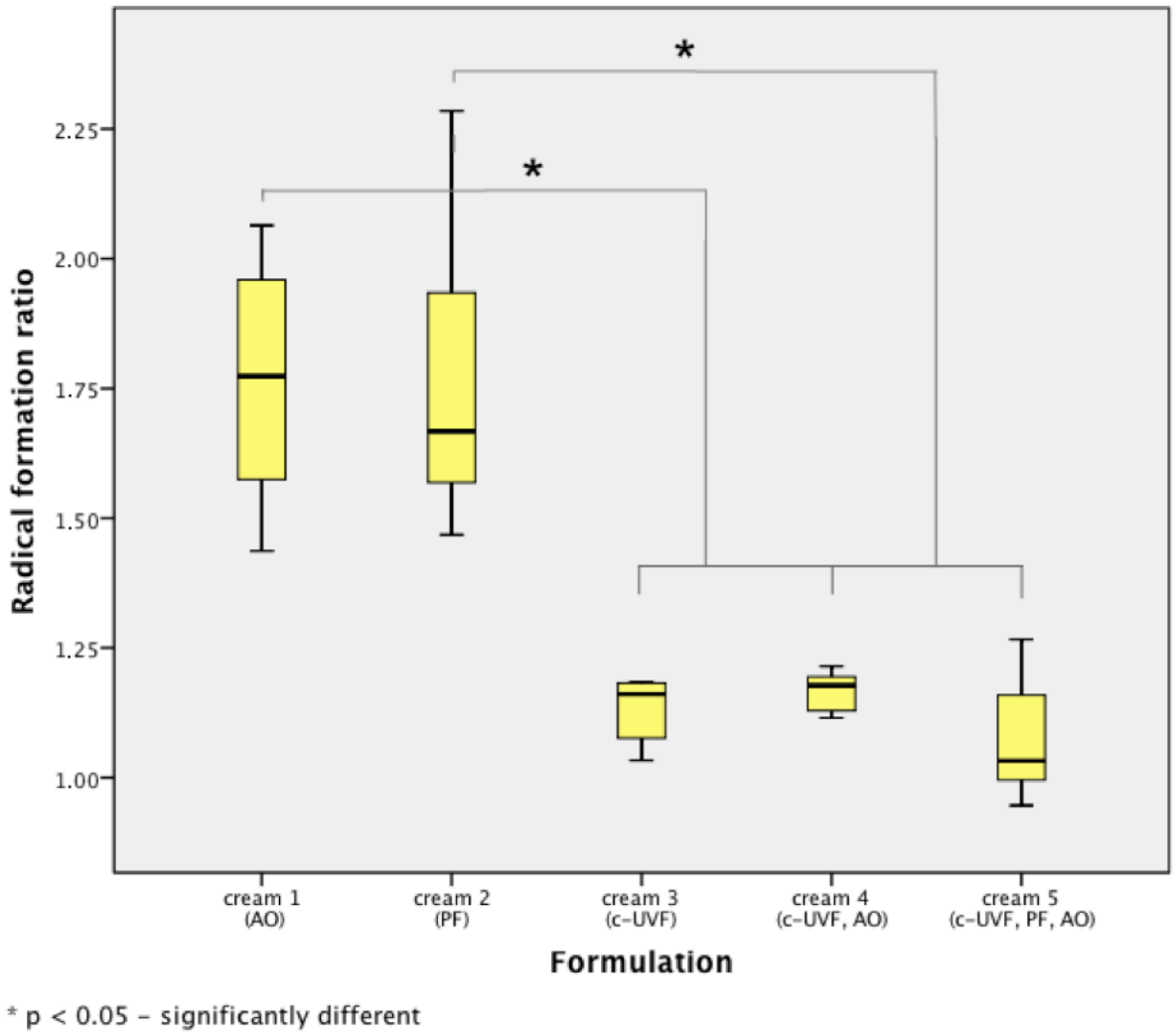

Figure 14: The radical formation ratio after 16 minutes for each formulation. Significant differences between creams ($p < 0.05$) are marked with *. ($n = 6$ for creams 1-4, $n = 7$ for cream 5). AO = Antioxidants, PF = Physical filter, c-UVF= chemical UV filter.

5.3 Sun protection factor measurements

The SPF testing was conducted by the proDERM GmbH according to Colipa standards [59]. The individual SPF values and their mean are presented in Table 7. Statistically determined outliers are marked with [1] and are neglected in mean value and all subsequent calculations. Cream 2 shows the lowest protective properties with a mean SPF value of 3.7. Cream 3 was measured to have a mean SPF value of 11.3, while creams 4 and 5 show the highest SPF values during testing. They exhibit a mean SPF value of 16.0 and 15.2, respectively.

Formulation	Cream 2 *(PF)*	Cream 3 *(c-UVF)*	Cream 4 *(c-UVF, AO)*	Cream 5 (*c-UVF, PF, AO)*
Individual SPF values	4.5	12.0	16.1	10.7
	5.0	11.2	18.0	15.0
	3.2	11.2	14.3	16.8
	2.8	10.0	16.1	15.0
	3.2	13.4	11.4	15.0
	3.6	10.7	12.8	10.7
	3.2	10.7	12.8	16.8
	4.0	13.4	22.6	18.8
	4.5	(18.8) [1]	20.2	21.1
	2.5	9.5	16.1	12.0
Mean SPF	**3.7**	**11.3**	**16.0**	**15.2**
STD	0.8	1.4	3.5	3.4

[1] outlier- not considered in any subsequent calculations

Table 7: Overview of individual SPF values, mean SPF and standard deviation for each tested formulation. AO = Antioxidants, PF = Physical filter, c-UVF = chemical UV filter, STD = standard deviation.

When creams were assessed for statistically significant differences in their SPF values, it was observed that creams 2 and 3 were significantly different (p-value of less than 0.05, marked with an asterisk (*)) from all other creams (Figure 15). However, no significant difference between creams 4 and 5 was ascertained.

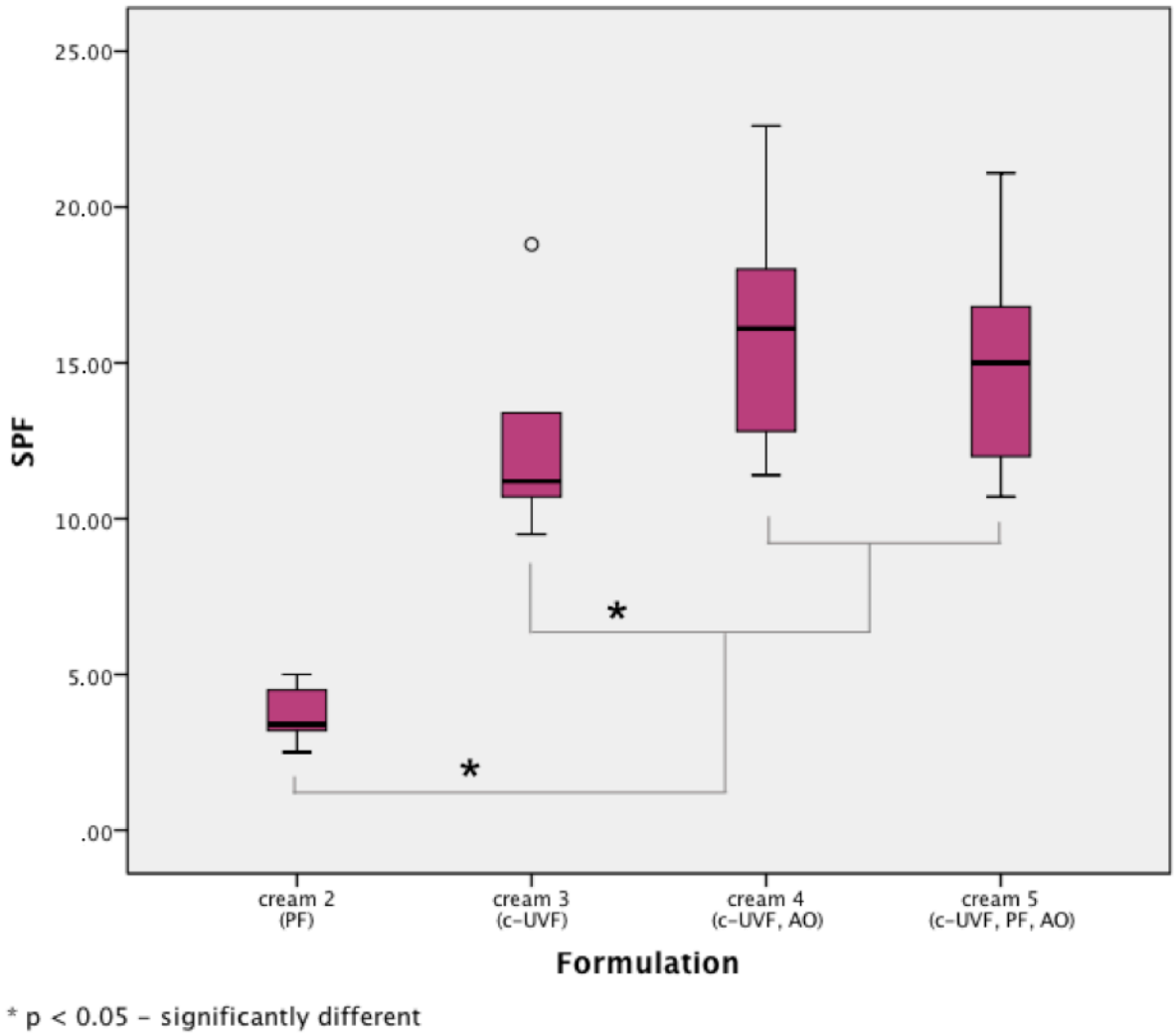

Figure 15: SPF value boxplot for each tested formulation. Significant differences between creams ($p < 0.05$) are marked with *. (*n* = 10 for each cream). AO = Antioxidants, PF = Physical filter, c-UVF= chemical UV filter.

5.4 Correlations between efficacy indicators

Table 8 summarizes the mean values for each cream and efficacy indicator previously described. For cream 1 no SPF value was determined.

Formulation	USPF	Radical formation (ratio)	SPF in vivo
Cream 1	1.04	1.76	[1]
Cream 2	1.24	1.76	3.7
Cream 3	11.48	1.13	11.3
Cream 4	10.73	1.17	16.0
Cream 5	11.51	1.08	15.2

[1] not determined

Table 8: Mean value overview of USPF, radical formation (ratio) and SPF for each formulation. *Cream 1* – Antioxidants; *Cream 2* – Physical Filters; *Cream 3* – Chemical UV Filters; *Cream 4* – Chemical UV Filters and Antioxidants; *Cream 5* – Chemical UV Filters, Physical Filters and Antioxidants.

In line with the aim of this study, testing values for each cream and method were assessed for possible correlations. For this purpose, mean values for each cream from any combination of two efficacy indicators were plotted in a graph and a linear trend line was added. In addition, in order to determine the Pearson Coefficient and significance level for each combination, statistical analyses using the SPSS program were carried out.

Figure 16 illustrates the correlation between radical formation and USPF values. Here, mean radical formation values for each formulation are plotted on the y-axis, and mean USPF values are plotted on the x-axis of the diagram. A linear trend line was added. The Pearson correlation was determined to be -0.998 in a two-tailed test, suggesting a very high correlation significant at the 0.01 level.

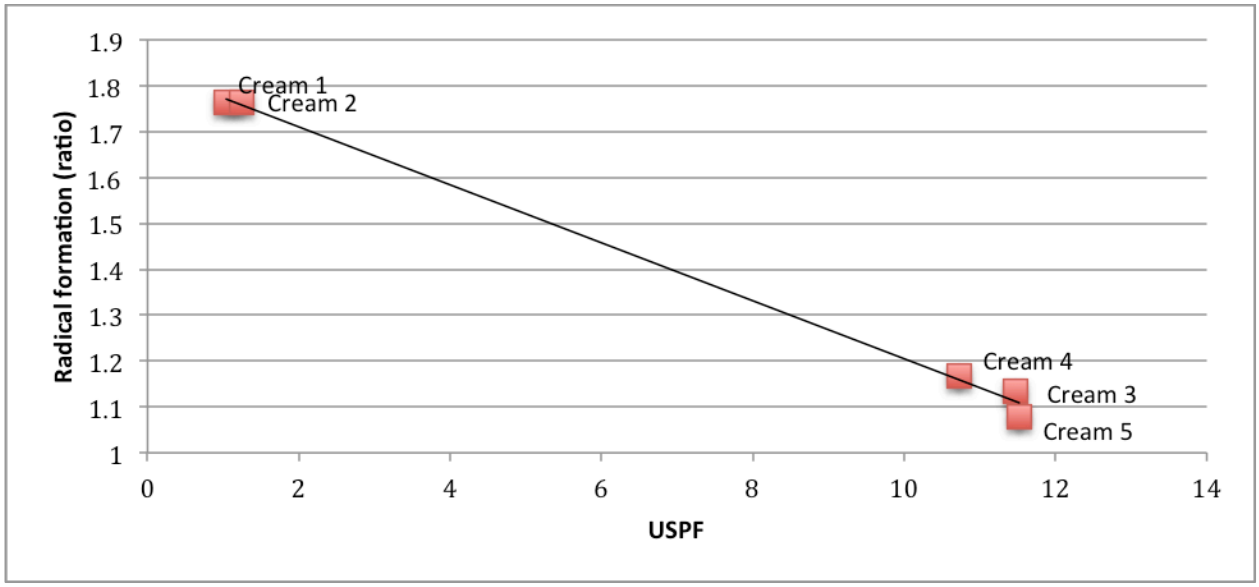

Figure 16: Correlation between mean radical formation ratio values and mean USPF values. Statistically determined: Pearson correlation -0.998. ** Correlation is significant at the 0.01 level. *Cream 1* – Antioxidants; *Cream 2* – Physical Filters; *Cream 3* – Chemical UV Filters; *Cream 4* – Chemical UV Filters and Antioxidants; *Cream 5* – Chemical UV Filters, Physical Filters and Antioxidants.

In Figure 17, SPF and USPF values are assessed for a possible correlation. Mean SPF values are shown on the y-axis, mean USPF are plotted on the x-axis. A trend line was added. The Pearson coefficient was calculated to be 0.913 in a two-tailed test, with a significance of 0.087. Therefore, no significant correlation was found, however a trend indicated by a p-value of less than 0.1 was observed.

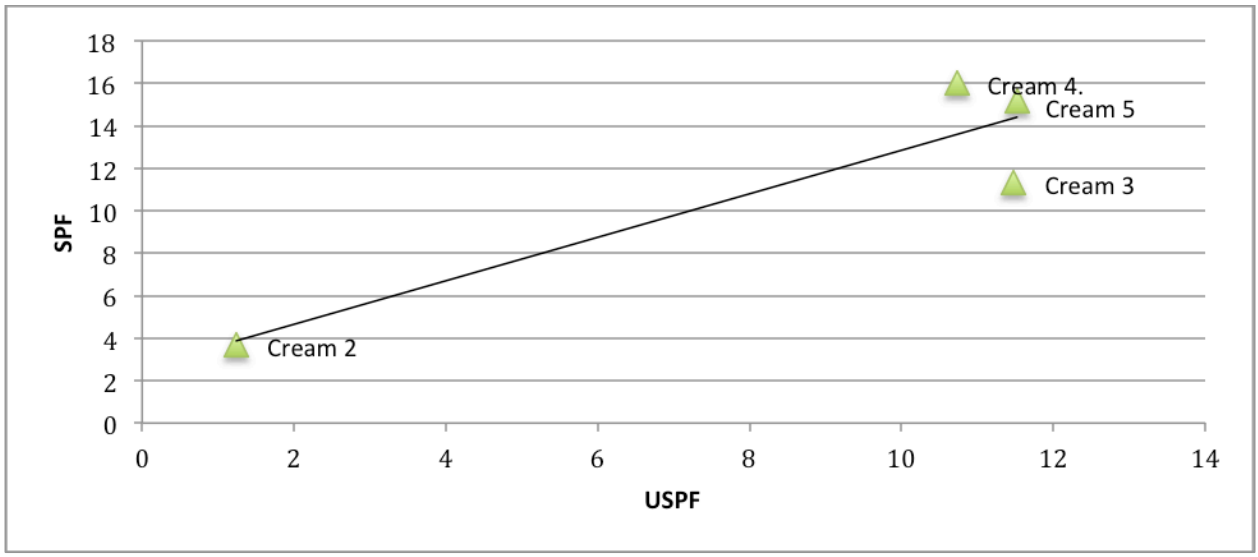

Figure 17: Correlation between mean SPF and mean USPF values. Statistically determined: Pearson correlation 0.913 (*) trend. *Cream 1* – Antioxidants; *Cream 2* – Physical Filters; *Cream 3* – Chemical UV Filters; *Cream 4* – Chemical UV Filters and Antioxidants; *Cream 5* – Chemical UV Filters, Physical Filters and Antioxidants.

Finally, the relationship of radical formation and SPF values was analyzed and evaluated for a possible correlation, illustrated in Figure 18. Mean radical formation values are plotted on the y-, SPF values on the x-axis, again, a trend line is added. Statistical analysis shows a Pearson correlation of -0.923 in the two-tailed testing and a significance of 0.077, suggesting a trend for a connection in the relationship between radical formation and SPF values.

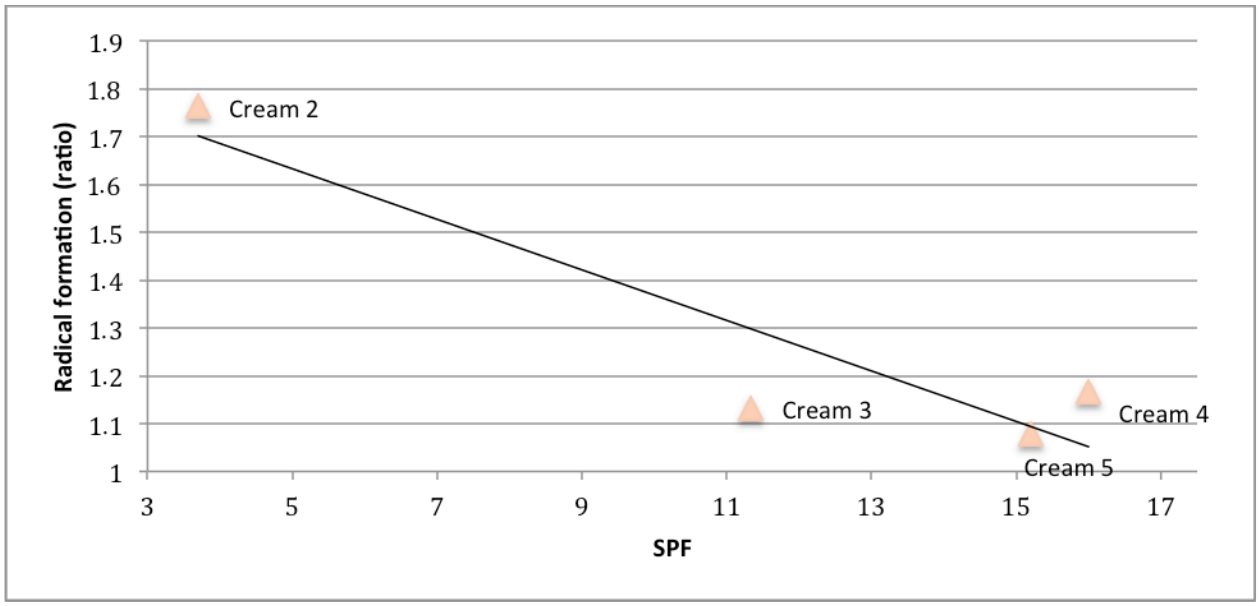

Figure 18: Correlation between mean radical formation ratio and mean SPF values. Statistically determined: Pearson correlation -0.923. (*) trend. *Cream 1* – Antioxidants; *Cream 2* – Physical Filters; *Cream 3* – Chemical UV Filters; *Cream 4* – Chemical UV Filters and Antioxidants; *Cream 5* – Chemical UV Filters, Physical Filters and Antioxidants.

6 Discussion

In the following, the results obtained and illustrated in the previous chapter are discussed and analyzed in regard to the overall aim of this study.

6.1 Preliminary Consideration

A short review of active ingredients and methods utilized is given in context to ensure a comprehensive grasp of the subject matter.

The protective properties of a sunscreen are similar to those of sun protective barriers in human skin. Scattering, reflection and absorption are the primary defense mechanisms against radiation. In the skin they are provided by a thickened stratum corneum, which scatters light, and an increased melanin production, which leads to an improved absorption of radiation. The second line of defense is represented by antioxidants; they lie in the upper and deeper skin layers and scavenge free radicals already produced. In sunscreens physical and chemical filters provide the first line of defense. Physical filters act primarily by scattering and reflection, although absorption properties have been equally identified in prior research [50], whereas chemical filters principally act by absorption. The secondary defense is similar to the antioxidants in the skin, here added in the formulations.

In order to judge the protective efficacy of a sunscreen formulation, several methods, resulting in characteristic protection indicators, can be employed. In this study the generally utilized SPF was compared to USPF and radical formation testing. Different in their approach, the SPF exclusively relies on the analysis of a biological signal - the erythema formation in the skin following irradiation - primarily elicited by UVB irradiation, while the USPF is a spectroscopic (physical) measure of a product's protective efficacy throughout the UVB and UVA ranges, and the radical formation ratio provides information concerning excess free radical formation (physical) in the UV, visible and near-infrared ranges.

6.2 The universal sun protection factor

The universal sun protection factor is based on the optical properties of a sunscreen. Light attenuation over the entire spectrum of the UVA and UVB ranges is considered and a factor similar to the SPF is determined based on calculations of sum transmission spectra of spectroscopic measuring values across the entire UV range.

6.2.1 Individual USPF values

Table 5 summarizes individual and mean USPF values for each investigated formulation. Results can be clearly categorized into two groups. Group A, exhibiting very low mean USPF values of around 1, comprised of creams 1 (antioxidants) and 2 (physical filters) and group B with mean values of around 11, made up of cream 3 (chemical filters), 4 (chemical and physical filters) and 5 (chemical-, physical filters and antioxidants). The differences seen in these groups can be largely attributed to the presence of chemical UV filters in group B, and the lack thereof in group A. The effectiveness of the chemical filters in regard to the achieved light attenuation can be explained by the absorbance properties of the filter. Individual variation between volunteers can be ascribed to volunteer-specific skin profile as demonstrated in a previous study [71].

6.2.2 Influences of active ingredients

Statistical analyses for differences between formulations yielded the expected outcome for creams of group A in regard to creams of group B. Values of creams containing chemical filters were shown to be statistically significantly higher than values for creams containing no chemical filters (Figure 11). However, a significant difference could also be established for cream 1 containing antioxidants and cream 2 made with physical filters. Cream 2 was shown to have a significantly higher USPF value than cream 1 (1.24 and 1.05, respectively), suggesting a protective effect of physical filters. This finding was not reproducible for creams containing physical filters in addition to chemical filters. Evidence that physical filters in the present concentration exert a protective effect demonstrable in spectroscopic measurements was predicted, as physical filters operate by means of reflection and absorption. Whereas the absence of

similar findings for physical filters utilized in a combination with chemical filters was surprising. An explanation may be provided when individual values are inspected. The difference between creams containing only antioxidants and those containing physical filters is measurable and statistically significant. However, mean values are only separated by 0.19 points. The standard deviation for both creams is extremely low, making this an important finding. Yet, when creams containing chemical UV filters were tested, the standard deviation increased from 0.04 to 2.24, disguising minor differences between creams, therefore preventing a noticeable statistically significant difference between them. Despite the protective effect of physical filters, the amount of filter used (2%) may have been too low to show a measurable difference once there was a larger variation in measurement values. This highlights the need to additionally test creams containing a greater concentration of physical filters.

When creams containing antioxidants were investigated, no influence on the spectroscopic efficacy indicator was observed. This was in agreement with the definition of the USPF, as measurements rely solely on optical properties such as reflection or absorption of a substance. Antioxidants are effective by means of various other processes, primarily reducing free radical formation.

6.2.3 USPF measurements in the visible and infrared spectrum of sunlight

In line with the overriding goal to establish a more comprehensive efficacy indicator in order to characterize sunscreen effectiveness in the future, applicability of the USPF method for the visible and infrared ranges of the sunlight is evaluated.

As previously described, the spectroscopic method is an indicator of effectiveness exclusively defined by optical properties of a formulation. Of the total protection achieved for the Vis and IR ranges, only a small portion is attributable to light attenuation, as most formulations lack optical absorbers for these ranges. Therefore, protective efficacy is barely measurable via spectroscopy for this part of the light spectrum.

6.3 The radical formation ratio

During sun irradiation, one important biological response detectable in the skin is free radical formation. It is the first step in a cascade of events, which can eventually lead to premature skin aging [72] and cancer formation [73].

The formation of free radicals can be reliably determined using electron paramagnetic resonance spectroscopy [65] and is a useful tool to determine the effectiveness of sun protection products. During measurements, radical formation is made visible by a loss of signal intensity over time. When no irradiation occurs, a stable signal, indicating no excess free radical formation, is expected. However, a decrease in signal intensity should be apparent during subsequent irradiation of an unprotected skin sample, indicating an increase in free radical formation.

Figure 12 illustrates the radical formation for base formulation without active ingredients and cream 5, containing all active ingredients utilized in this study, before and after irradiation. Nearly stable signal intensity can be observed for both creams before irradiation occurs. Following irradiation initiation, however, it becomes evident that cream 5, containing chemical filters, physical filters and antioxidants shows clearly less signal intensity loss than the base formulation (6% and 39%, respectively). This translates into substantially less radical formation in cream 5 and therefore greater protective efficacy for the cream containing active ingredients, thus validating our method.

6.3.1 Changes in radical formation before and after UV/Vis irradiation

For simplification purposes, a ratio of the signal intensity before and after irradiation illustrating changes in radical formation was formed. The resulting ratios over time are depicted in Figure 13. In line with our expectations, results for changes in radical formation were dividable into two major groups, just as in the case of the results for USPF values: Group A, comprising creams containing no chemical filters (creams 1 and 2) and group B made up of formulations containing chemical UV filters (creams 3, 4 and 5). It was shown that regardless of the presence of antioxidants or physical filters, the radical formation was significantly reduced by the presence of chemical UV filters.

A nearly linear increase in radical formation over time for each formulation could be observed, as indicated by the black dotted trend line in Figure 12. This confirms the

finding of a previous study indicating that radical production in an ex vivo setting remains stable [74].

6.3.2 Influences of active ingredients

Influences attributable to a single active ingredient are discussed and itemized by active ingredient in the following.

6.3.2.1 Chemical filters

When creams were analyzed for differences between mean values, several observations were made. For one, as indicated by the changes in radical formation in Figure 13, a statistically significant lower radical formation ratio was observed for creams containing chemical UV filters. The evidenced protective properties are likely due to the absorbance properties of the contained UV filters. Rays are absorbed in the upper layers of the skin, and subsequently, radical formation is prevented. In 2006, Herrling et al investigated the protection against UV-induced radicals in the skin and found that UV filters drastically reduced the total number of free radicals, confirming our findings. However, the authors placed emphasis on the fact that: "the main protection against UV-induced free radicals is provided by UVA filters closely followed by broadband filters. UVB filters contribute only marginally to the radical protection." They reason that UVB filters can only influence the radicals induced by shorter wavelength and generated in the epidermis and consequently do not take part in the reduction of radicals in the dermal layer of the skin [8], suggesting that adequate UVA protection is provided by our tested products. This finding cautions consumers to rely on UV filters containing a high degree of UVB protection and stresses the need to include protection for high radical formation in the UVA ranges of the spectrum. It also implies the need for a comprehensive approach to characterize a sunscreen's protective efficacy, in order to safeguard the consumer by providing a label reflecting the protective properties of a product throughout all spectral ranges.

6.3.2.2 Antioxidants

No significant reduction in radicals attributable to antioxidants was observed when samples were irradiated in the UV and Vis ranges. This is surprising, as we had anticipated to see a protective effect of antioxidants due to their radical scavenging activity in this setting [20]. A possible explanation for the absence of protective properties may be provided by the amount of antioxidants added to the formulation (1%) and the intensity of radiation applied in the ex vivo setting. Previous research indicates a rather high radical protection factor (RPF) for creams 1 and 4 [75]. It should be noted that UV radiation corresponded to a 20-fold minimal erythemal dose to enhance sensitivity of measurements. Thereby, antioxidants may have been obliterated early on. Hence, differences due to antioxidants may just have been too small in scale to show a measurable effect in this setting. A more detailed discussion of RPF values for creams utilized in this study is contained in section 6.4.1.

6.3.2.3 Physical filter

Furthermore, no radical formation reduction was observed when physical filters were added to the formulations, revealing an equally unexpected finding. Physical filters exhibit their protective properties primarily by scattering and reflecting radiation in the upper skin layers. In this manner, penetration of rays into deeper skin stratums and consequently free radical formation is prevented. Previously, Meinke et al found that high scattering properties lead to a significant reduction in radical formation in the near infrared region [76]. A finding not replicable for measurements in the UV range of the present study. Causative factor may be the relatively low amount (2%) of physical filter added. Substantiating this assumption, a prior investigation of this substance by the manufacturer resulted in a substantial decrease in UV-induced radical formation by 86% utilizing a 5% formulation [77]. Additionally, a portion of the finding observed may be attributable to the physiology of the filter itself. Radiation is scattered and reflected within the upper skin layers, protecting the skin from radical formation in the deeper layers, yet excess free radicals on a smaller scale may still be produced within the upper layers.

Further investigations concerning sunscreens with a higher amount of physical filters should be conducted in the future.

6.3.3 The ex vivo setting, advantages and limitations

In the present study, EPR measurements were conducted in an ex vivo setting using porcine ear skin samples. Previous research has shown that fresh porcine ear skin is a suitable alternative to human skin for electron paramagnetic resonance measurements [67]. It is a waste product in the butchering process and is easily and cheaply obtainable. This non-invasive setting made the use of volunteers redundant and spared them from radiation exposure.
However, limitations persist. By employing an ex vivo setting, additional radical formation or protective mechanisms, limiting formation, are not determinable per se. Also, working with porcine rather than human skin samples poses additional limitations.

Recently, Arndt et al compared radical protection in an in vivo versus ex vivo setting for the Vis and IR ranges. They found that neither absolute values nor kinetics were comparable for ex and in vivo radical formation [74]. Absolute values were shown to be higher in vivo rather than ex vivo utilizing the same irradiation intensity. This prompted the use of a higher irradiation intensity in the ex vivo setting of our study, in order to enhance sensitivity of measurements. However, this in turn may lead to the previously proposed reduction in antioxidant effectiveness, as irradiation is so high that antioxidants may be depleted in the UV region before results are measureable. An additional finding by Arndt et al was that kinetics differed for in and ex vivo measurements. While the ex vivo radical formation was stable over time, a decrease was noted for radical formation in vivo [74]. They propose that the body may increase endogenous antioxidative protection mechanisms leading to the decrease observed. A similar mechanism is not observable in the ex vivo setting, limiting its applicability for this purpose.
Furthermore, given the study set-up, differences between human and porcine skin should not be neglected. Haag et al found that human skin was different from bovine udder and porcine ear skin concerning levels of carotenoids, catalase activity as well as radical formation following UV irradiation [67]. Free radical formation in human skin was found to be lower than in porcine ear skin evaluated under similar conditions. Nevertheless, differences in radical formation for both were comparable, justifying the use of porcine ear skin for this purpose.

While the ex vivo setting is still the standard employed for most EPR studies [16, 65], in vivo investigations are increasing [74, 78] and will provide additional opportunities in the future.

6.3.4 Radical formation in the infrared spectrum of sunlight

In the present study, experiments were conducted utilizing UV and Vis irradiation. However, recent research suggests that excess free radical formation is detectable even in the near infrared (NIR) range of the sunlight [16].

In light of these findings, Meinke et al conducted research comparing different commercial sunscreens for protective efficacy in the IR range and found that while a significant radical reduction in the UV and Vis ranges could be achieved by sunscreens containing chemical UV filters, radical formation in the IR range was demonstrated to follow a different pattern. Here, production was shown to be primarily reduced due to scattering properties of physical filters and antioxidant additives [76]. These findings were affirmed when creams exclusively containing either antioxidants or physical filters were investigated. A clear protective effect in the NIR range was found for the formulation containing antioxidant additive. The same was true for the cream with physical filters, exhibiting a high scattering coefficient [75].

These results not only indicate the need to include filters and additives capable of utilizing optical, as well as biochemical properties to provide protection against the entire range of the sunlight spectrum, but also reinforce EPR as a suitable method for the detection of free radicals in all ranges.

6.4 The sun protection factor

The SPF has been the main label for protective efficacy of sunscreens for the past decades. Its calculation is based on erythema formation of irradiated skin. It gives consumers a quantitative measure of how much longer they can stay in the sun when using sun protection without having to fear the consequences of sunlight, expressed by the development of a sunburn.
In this study, four out of five different, specially developed sunscreen formulations were investigated for their SPF values.

6.4.1 Individual SPF values and influences of active ingredients

Table 8 summarizes all determined values. Cream 2, containing 2% physical filters exhibits a mean SPF value of 3.7. While this corresponds to a protective value of almost four times the amount of protection compared to no sunscreen product, when put in perspective, this corresponds to a labeling category of less than 'low protection' according to the European Commission System for SPF Labeling (Table 2). Therefore, in this context, formulation 2 would be labeled as having no protective efficacy, although some protective value was evident during testing. However, employing higher amounts of physical filter has been shown to increase protective efficacy in previous research [79]. Also, the manufacturer states that the recommended amount of this specific physical filter is 2 - 15%, thus putting our product with 2% filter at the low end of protection [80]. They further add that for every percent of active ingredient, an increase of 2 SPF units is expected. This is consistent with our findings.
When the same system is applied for the other three creams, cream 3 containing chemical UV filters (SPF value of 11.3) would be categorized as a sunscreen with low protection and creams 4 and 5 would be considered to have a medium level of protective efficacy (SPF values of 16.0 and 15.2, respectively). This is on a par with our expectations, as the 2% physical filter was expected to be less powerful in terms of protection when compared to creams containing 10% chemical UV filter. Creams 4 and 5 comprising, in addition to the chemical filters, active ingredients of physical filter as well as antioxidants were shown to exhibit the highest SPF values, also in line with our expectations.

Statistical analyses of the mean values of the formulations yielded significant differences between cream 2, containing physical filters, and creams 3, 4, and 5 containing the common denominator of chemical UV filters (Figure 15). Also, a significant difference between cream 3 on the one hand and creams 4 and 5 on the other was determined. A clear effect of the added antioxidants in creams 4 and 5 was shown. However, no effect of the additionally added physical filter in cream 5 could be discerned.

In previous studies, a synergistic effect of chemical filters in combination with physical filters has been described [53]. This effect could not be reproduced in this setting. Explanation may be provided by the radical protection factor (RPF) additionally determined for the formulations. In a prior study using the same formulations as utilized in this study, Meinke et al determined an RPF value of $(444 \pm 22)\ 10^{14}$ radicals/mg for cream 1, containing only antioxidants as active ingredient. An RPF of $(459 \pm 28)\ 10^{14}$ radicals/mg for cream 4 (chemical filters and antioxidants) and an RPF value of $(29 \pm 1)\ 10^{14}$ radicals/mg was determined for cream 5 containing chemical- as well as physical filters and antioxidants. While the value was similar for the formulation containing antioxidants (cream 1), as well as antioxidants in addition to chemical filters (cream 4), the low RPF value for cream 5 suggests that the antioxidant capacity may change when combined with physical filters.
Hence, the SPF value determined for cream 5 (mean SPF: 15.2) may constitute a combination of protective effects of physical filters (cream 2 – mean SPF: 3.7) and chemical filters (cream 3 – mean SPF: 11.3) rather than antioxidants, as indicated by the respective RPF value.

In this study a clear protective effect of antioxidants in the in vivo setting was observed. Similar results using topically applied antioxidants have been previously described by several authors [81] [82]. Additionally, Stahl et al have shown that a diet rich in antioxidants may also protect against UV-induced erythema [23].
It should be noted that due to the financial burden involved in the SPF testing, mean values of only four out of five formulations were determined in this study. This allows no conclusions to be drawn concerning the protective qualities of antioxidants independent of the presence of chemical filters or for that matter, allows no assumptions of protective

properties for antioxidants in relation to physical filters when used as single active ingredient in this study.

To evaluate findings concerning influences of active ingredients in context, it is imperative to apprehend that a vast array of processes is implicated in erythema formation. While the discussion of each process would exceed the scope of this study, it can be deduced that also a number of processes have the potential to lead to a significant reduction in erythema formation, implying the risk involved using the MED as a singular efficacy indicator [83].

6.4.2 Limitations of the sun protection factor

Substantial limitations of the SPF are caused by the narrow consideration of only part of the sunlight spectrum. As previously stated, the erythema formation is the single biological response evaluated when the SPF is determined- a response primarily elicited by UVB radiation. It is accepted today that UVA radiation contributes to a large extend to immunosuppression [41] and skin ageing [48] and may even contribute to skin cancer formation [84]. Also, as research progresses, 50% of harmful radical formation in the skin was found to be produced by radiation of the visible and infrared spectrum of the sunlight [16].

Though some degree of protection is ensured by the revised Colipa guidelines, urging to include at least one-third of the total protection in the UVB range in UVA filters [59]; for the consumer the amount of protection provided in these ranges is not easily deducible from the SPF label.

6.4.2.1 Individual variation

The single biological response analyzed for the SPF determination also implies that a high individual variation between measurements may be encountered. This is in part due to variation between laboratories when analyzing erythema formation [62] but can be largely attributed to individual skin type, skin structure, age and diet.

In our study, when SPF values are considered individually for each formulation (Table 7), the vast differences between volunteers become apparent. Creams 4 and 5 provide the highest variations, demonstrating individual values ranging from 11.4 to 22.6 for cream 4 and 10.7 through 21.1 for cream 5. This corresponds to almost twice the

amount of protection provided by the same cream to two different volunteers. According to Brown the higher the mean SPF, the higher the variability between individual SPF values [62]. Similar results were shown in this study. While the standard deviation for cream 2, exhibiting the lowest mean SPF value, was only 0.8, the deviation increased up to 3.5 at higher SPF values. In a direct comparison with USPF and RF measurements for creams 4 and 5, demonstrating the highest standard deviation (Figure 19), the disparity between the different methods becomes apparent.

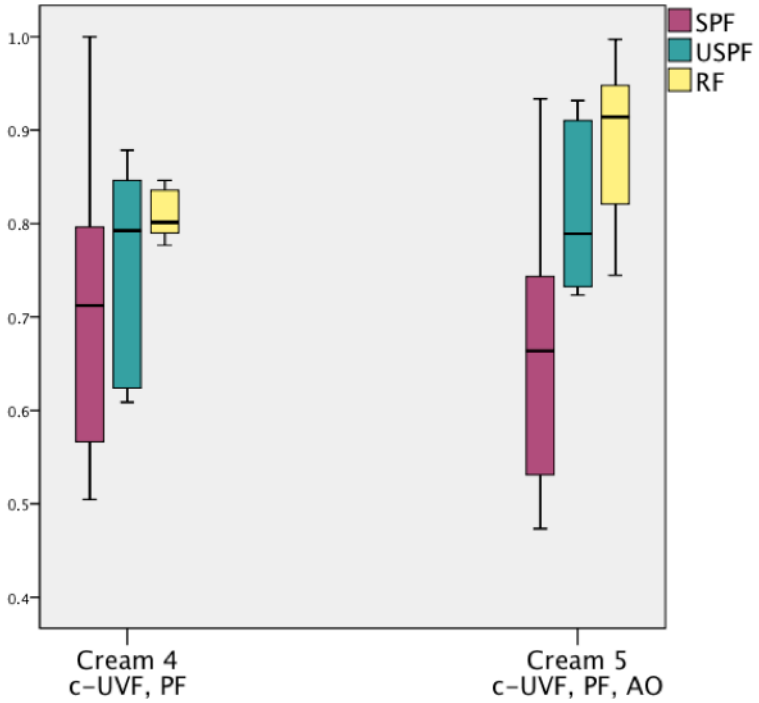

Figure 19: Relative changes in SPF, USPF and RF measurement values demonstrated by the example of creams 4 and 5. The highest individual value of each measurement method was set to 1. c-UVF = chemical UV filter, PF = physical filter, AO = Antioxidants.

In combination with the discrepancies of SPF measurement means between laboratories of up to 40% [62], values may lose validity when regarded in their totality.

6.4.2.2 Invasiveness of the method

Finally, the invasiveness of the method should be considered. In order to achieve reliable measurements for a product, for every cream tested there should be at least 10-20 volunteers. Each volunteer is subject to radiation that produces erythema, reflecting

part of the individual skin damage. When the SPF method is revised it should be a priority to limit volunteer exposure and reduce harmful effects of the sunlight.

6.5 Correlations

A specific aim of this study was to evaluate the different methods for correlations. The graphic display of the results for each formulation pair is illustrated in section 5.4.

6.5.1 Correlations between the universal sun protection factor and radical formation ratio

As demonstrated in Figure 16, a statistically highly significant correlation (Pearson correlation -0.998) between USPF and RF mean values was found.
This is a rather unexpected result due to the different properties of sunscreen evaluated by the two methods. As discussed previously antioxidants were expected to increase the radical scavenging activity of a product, which was not demonstrable in this setting due to the high radiation intensity employed. No effect on the optical properties represented by the USPF value was anticipated. However, when no significant effect of antioxidants was noted during EPR measurements, a clear correlation between the two methods could be demonstrated.

6.5.2 Correlations between the universal sun protection factor and sun protection factor

A different picture emerged when the SPF was added to the equation. Antioxidants were shown to have significant protective properties in regard to erythema formation (Figure 15).
When SPF and USPF values are compared side by side it is essential to understand that several parameters can influence measurement values [69]. While USPF values are solely based on light attenuation, the SPF is affected by many variables such as UVB radiation intensity, the UVB/UVA extinction ratio of the filters utilized, as well as by the addition of antioxidative and anti-inflammatory substances, ultimately limiting erythema formation.
In this study, formulations differing in regard to filters and antioxidants added were

used. Therefore, no significant correlation between SPF and USPF values was predicted. However, a trend for a correlation was observed (Figure 17).

To adequately classify results it would be necessary to consider formulations containing similar filters and additives for a valid correlation between USPF and SPF values. Previous results comparing USPF and SPF values of commercial sunscreen formulations containing similar UV filter substances and antioxidative additives were graphically illustrated (Figure 20) [6]. Creams 4 and 5 (red triangles, Figure 20) containing chemical UV filters and antioxidants and chemical UV filters, physical filters and antioxidants, respectively, were added to the illustration. As depicted in Figure 20, both show proximity to the linear trend line of the previously investigated commercial sunscreens. On the other hand, when cream 3 (green circle, Figure 20), containing only chemical filters, was added to the illustration no immediate relationship with the trend line was observed, illustrating the importance of matched filter substances and additives for USPF and SPF correlation.

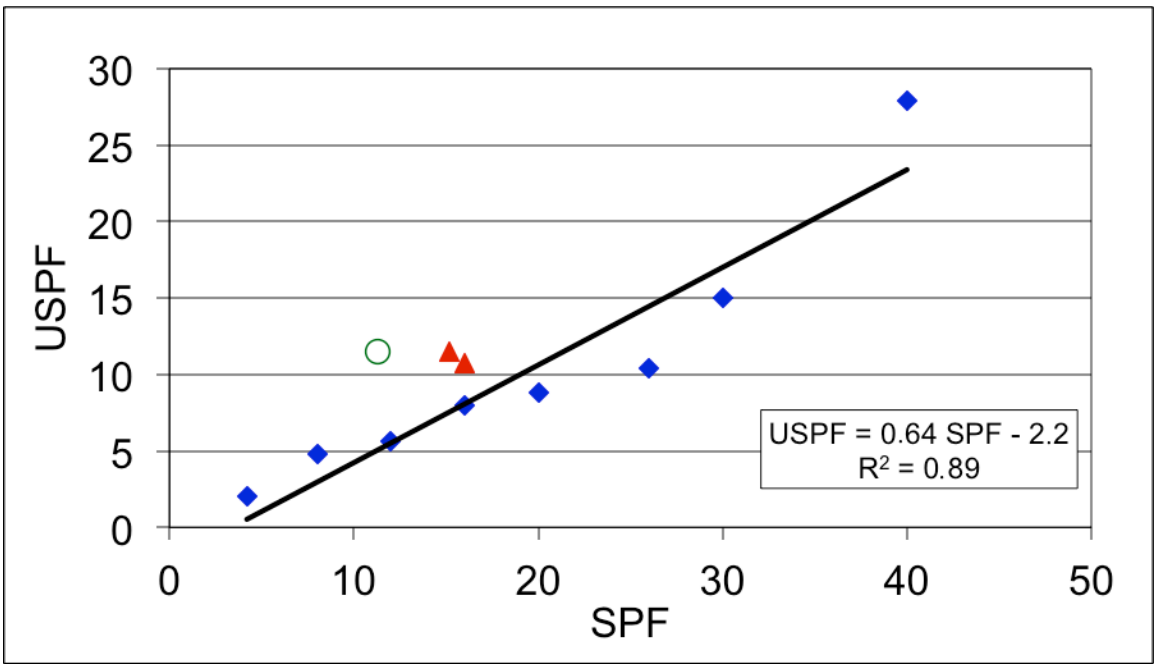

Figure 20: Visual correlation between mean USPF and SPF values for creams 3, 4 and 5 and a group of commercial sunscreens containing similar UV filters and antioxidants as used in the present study. *n* = 6 or more. Red triangles represent cream 4 (chemical filters, antioxidants) and 5 (chemical filters, physical filters, antioxidants), blue squares - commercial sunscreens containing a comparable UVB/UVA proportion and antioxidants, green circle - cream 3 (chemical filters).

6.5.3 Correlations between the radical formation ratio and sun protection factor

While a trend for correlation between SPF and RF values was observed, the expected clear correlation could not be confirmed.
This may in part be due to the lack of effect of antioxidants during EPR measurements previously discussed. However, the small quantity of only four different formulations tested may also hinder a significant finding.

A total of five different formulations were used in this study. When SPF values were determined for four formulations, mean values ranged from 3.7 to 16. For correlation purposes, besides an increase in the quantity of formulations tested, SPF values extending over the entire range from no (SPF 1) to very high protection (SPF 50+) would be valuable.
Unfortunately, this was not feasible in this setting due to cost, time restrictions and the scope of the study.

7 Conclusion

The primary goal of the study was to compare two alternative sunscreen efficacy indicators to the sun protection factor and to evaluate their value in a more comprehensive sunscreen efficacy evaluation.

In line with this aim, a series of sunscreen formulations containing different active ingredients was investigated. Their protective effectiveness was determined by three different methods resulting in USPF, RF and SPF as efficacy indicators. The results allow conclusions to be drawn regarding influences of the utilized sunscreen components on the investigated protective efficacy indicators and provide an insight into correlations between them. Concluding, in consideration of the current state of research, information regarding applicability and boundaries of these indicators in the future can be deduced.

Three active ingredients, i.e., chemical filters, physical filters and antioxidants, were investigated in the present study. As expected, chemical filters were shown to influence efficacy indicator values of all three methods. Physical filters, when used as a single active ingredient, exerted no measurable effect on RF values, however they exhibited qualities measurable by the USPF and SPF methods. Nevertheless, these effects were not replicable when physical filters were added in combination with chemical filters, most likely due to the low amount (2%) of physical filters utilized in the formulations examined in this study. Furthermore, previous studies suggest that a combination of physical filters and antioxidants may lead to a decrease in antioxidant capacity [75]. As the only formulation in this study containing both chemical and physical filters also includes antioxidants, this may lead to a camouflage effect hindering a discrimination concerning the protective efficacy of either solely antioxidants or physical filters in this combination.

In agreement with the erythema formation as the basis for SPF determination, antioxidants lead to significantly higher SPF values. In line with the exclusively spectroscopical reference of the USPF, the USPF value was not influenced by addition

of antioxidants to the formulations. Although also anticipated, no effect on RF measurements was observed. This may be attributable to the high radiation intensity used in the ex vivo setting, therefore obliterating antioxidants early on.

A highly significant correlation between USPF and RF was observed, indicating that though the mechanism by which the protective efficacy is determined differs, both efficacy indicators show comparable results for each formulation. Available data suggests only a trend for correlations of USPF-SPF and RF-SPF. However, when USPF values were plotted in results for sunscreen formulations containing filters with similar amounts of UVB/UVA extinction and antioxidants, adequate correlation was seen.

Based on the results achieved for the investigated efficacy indicators, the following fundamental statements can be derived. Prospectively, the USPF, measuring efficacy of sunscreens based on their optical properties, is well suited to objectively evaluate the efficacy of sunscreen products in the UV range. It is independent of biological responses and represents light attenuation over both the UVB and the UVA ranges. Therefore, this label may lead to safer sun exposure and at the same time prompt manufacturers to move away from the one-third required UVA filter and increase filters according to their protective value. Unlike the sun protection factor determination, the tape stripping method utilized in this study employs no harmful radiation and is therefore non-invasive by nature. However, despite its great usefulness for the UV range, the USPF is limited in its applicability for the visible and infrared ranges of the sunlight, given that adequate protection in this range is currently not achievable by a spectroscopically measurable intensity reduction.

Conversely, radical formation is the primary biological response evoked by radiation for the Vis and IR ranges. Excess formation can be efficiently detected using electron paramagnetic spin resonance technology. Hence, radical formation ratios represent an underlying biological response over the entire UV, Vis and IR ranges. The disadvantage of this method, however, lies in its invasive nature. In this study EPR experiments were conducted in an ex-vivo setting leading to the use of higher irradiation intensities, thereby possibly lessening the potency of antioxidants added and consequently diminishing the significance of the findings. An in vivo setting would be the preferred

method to achieve the most reliable results, but would in turn pose the danger of irradiation.

Overall, both methods provide valuable information concerning the protective efficacy of sunscreen products throughout different wavelength ranges. Hence, it may be proposed that a combination of both can provide the consumer with the most comprehensive and reliable possibility of determining a sunscreen's protective capacity while utilizing the least invasive setting. By using the USPF for characterization of the entire UV range, biological responses can be neglected and the protective properties of sunscreens can be evaluated purely by attenuation abilities of the added filters. Using the RF in an in vivo setting exclusively for the visible and infrared ranges would lead to a significant reduction in overall radiation, limiting exposure to merely therapeutic doses, while effectively accounting for the primary biological response of the Vis and IR ranges.

Lastly, the SPF and its meaning has been an accepted and well-known indicator throughout the history of sun protection. The idea of a simple label informing the consumer of how many times longer one can stay in the sun without exposing oneself to the increased health risks of sun exposure has stuck in the minds of many. Yet, the relevance of this well-established factor is too small when evaluated in the context of the entire sunlight spectrum. Leading to the conclusion that the concept of this factor should be preserved, however, the method of determining light protection factors should be reviewed and subsequently replaced by more comprehensive approaches to determine the efficacy of sun protection.

This study was conducted as a baseline investigation and may be considered as proof of concept for the feasibility of a new sun protection factor. The results of this study point in the right direction but highlight the need for further research on a larger scale to find favor with the industry and eventually lead to permanent acceptance in European sunscreen evaluation standards.

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Eidesstattliche Versicherung

„Ich, Felicia Maria Syring versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The Future of Sunscreen Efficacy Evaluation – a Comparative Study of Universal Sun Protection Factor, Radical Formation Ratio and Sun Protection Factor Assessing the Protective Value of Sunscreen Formulations Containing Chemical-, and/or Physical Filters as well as Antioxidant Additives“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Anteilserklärung an etwaigen erfolgten Publikationen

Felicia Syring hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Meinke MC, Syring F, Schanzer S, Haag SF, Graf R, Loch M, Gersonde I, Groth N, Pflücker F, Lademann J, Radical Protection by Differently Composed Creams in the UV/VIS and IR Spectral Ranges, Photochemistry and Photobiology, 2013

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Publikation 2: Syring F, Schanzer S, Weigmann HJ, Meinke MC, Lademann J, The future of sunscreen efficacy labeling, Posterkongress: „Wissenschaftliches Arbeiten im Reformstudiengang Medizin“, 5. Juli 2013

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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publikationsliste

1. Meinke MC, Syring F, Schanzer S, Haag SF, Graf R, Loch M, Gersonde I, Groth N, Pflücker F, Lademann J., Radical Protection by Differently Composed Creams in the UV/VIS and IR Spectral Ranges, Photochemistry and Photobiology, 2013

2. Syring F, Schanzer S, Weigmann HJ, Meinke MC, Lademann J, The future of sunscreen efficacy labeling, Posterkongress: „Wissenschaftliches Arbeiten im Reformstudiengang Medizin“, 5. Juli 2013

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