Studies of Sunscreens: Percutaneous Absorption of Benzophenone-3 and Photostability

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2006

Cover: The cover picture shows the Sun of May, replica of an engraving on the first Argentine coin in 1813.

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ISBN-10 91-628-6969-8
ISBN-13 978-91-628-6969-4

To my family

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Abstract

Aim: To learn more about percutaneous absorption of the photoactive compound benzophenone-3 (BZ-3) and to study the excretion pattern of BZ-3 and its metabolite dihydroxy benzophenone (DHB). We also got the opportunity to develop a reverse-phase HPLC method to analyze BZ-3 and DHB. The photostability of seven commercial sunscreens was also studied.

Material and methods: *Paper I:* 11 participants applied a sunscreen, 2 mg/cm^2, containing 4% BZ-3. They collected urine for 48 hours after the application. *Paper II:* 26 participants applied a sunscreen, 2 mg/cm^2, containing 4% BZ-3 morning and night for five days. Half of the participants were exposed to UV radiation (UVR). They collected urine for the five days the sunscreen was applied and an additional five days after the last application. *Paper III:* The assay uses: solid-phase extraction with C8 columns; a Genesis C18 column (4.6 mm x 150 mm); a gradient acetonitrile-water mobile phase; a UV-detector set at 287 nm. *Paper IV:* Seven commercial sunscreens were studied with absorption spectrophotometry. Sunscreen product, 0.5 mg/cm^2, was placed between plates of silica. The area under the curve (AUC) in the spectrum was calculated for the different UV regions. AUC before (AUC_{before}) and after (AUC_{after}) artificial UV exposure and before and after natural UV exposure were calculated. If the AUC Index (AUCI), defined as $AUCI=AUC_{after}/AUC_{before}$, was > 0.80, the sunscreen was considered photostable.

Results: *Paper I:* The average total amount excreted was 11 mg, median 9.8 mg, which is approximately 0.4% of the applied amount BZ-3. *Paper II:* The volunteers excreted 1.2-8.7% BZ-3 of the total applied amount. The mean value found was 3.7%. There was no significant difference between the two groups; $p<0.99$. *Paper III:* The assay was linear r^2 >0.99, with detection limits for BZ-3 and DHB of 0.01 µmol/l and 0.16 µmol/l respectively. Relative standard deviation was less than 10% for BZ-3 and less than 13% for DHB. The excretion pattern varied among the human volunteers, different patterns were discerned among the individuals. *Paper IV:* Three sunscreens were unstable after 90 min of natural UV, in the UVA range the AUCI was between 0.41 and 0.76. In the UVB range, one of these sunscreens was unstable with an AUCI of 0.75 after 90 min. Three sunscreens were photostable after 120 min of natural UV, in the UVA range the AUCI was between 0.85 and 0.99 and in the UVB range between 0.92 and 1.0.

Conclusions: *Paper I:* BZ-3 is absorbed by the skin and excreted in the urine after one topical application of a sunscreen containing 4% BZ-3. There are individual differences in the amount excreted and in the excretion pattern. *Paper II:* Repeated topical applications of a sunscreen containing 4% BZ-3 lead to a higher excretion of BZ-3. There was no statistical difference after exposure to UVR. *Paper III:* The developed reverse-phase HPLC-method was reliable and suitable to handle a large number of samples. BZ-3 and DHB were excreted in a similar pattern. *Paper IV:* Three of the seven investigated sunscreens were photounstable in the UVA region. The combination ethylhexyl methoxycinnamate and butyl methoxydibenzoylmethane was unstable regardless of which other photoactive compound that was included in the sunscreen.

Key words: benzophenone-3, dihydroxy benzophenone, sunscreens, UV radiation, reverse-phase HPLC, photostability

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I H Gustavsson Gonzalez, A Farbrot and O Larkö. Percutaneous absorption of benzophenone-3, a common component of topical sunscreens. *Clinical and Experimental Dermatology 2002; 27, 691-94.*

II H Gonzalez, A Farbrot, O Larkö and A-M Wennberg. Percutaneous absorption of the sunscreen benzophenone-3 after repeated whole body applications - with and without UV irradiation. *British Journal of Dermatology 2006; 154, 337-40.*

III H Gonzalez, C-E Jacobson, A-M Wennberg, O Larkö and A Farbrot. Solid-phase extraction and HPLC: application to study the urinary excretion pattern of benzophenone-3 and its metabolite 2,4-dihydroxybenzophenone in human urine. *Submitted for publication.*

IV H Gonzalez, N Tarras-Wahlberg, B Strömdahl, A Juzeniene, J Moan, O Larkö, A Rosén and A-M Wennberg. Photostability of commercial sunscreens upon sun exposure and irradiation by ultraviolet lamps. *Submitted for publication.*

CONTENTS

ABBREVIATIONS

AK	actinic keratosis
AUC	area under the curve
AUCI	area under the curve index
BCC	basal cell carcinoma
BMDBM	butyl methoxydibenzoylmethane
BSA	body surface area
BZ-3	benzophenone-3
CMM	cutaneous malignant melanoma
DHB	dihydroxybenzophenone
DHMB	dihydroxy methoxybenzophenone
EHMC	ethylhexyl methoxycinnamate
HPLC	high-performance liquid chromatography
IS	internal standard
MED	minimal erythemal dose
NMSC	non-melanoma skin cancer
PFA	the “protection factor UVA”
PPD	persistent pigment darkening
RSD	relative standard deviation
SCC	squamous cell carcinoma
SED	standard erythemal dose
SPF	sun protection factor
THB	trihydroxy benzophenone
TiO_2	titanium dioxide
UPF	ultraviolet protection factor
UVA	ultraviolet A radiation
UVB	ultraviolet B radiation
UVC	ultraviolet C radiation
UVR	ultraviolet radiation
ZnO	zinc oxide

Mad Dogs and Englishmen

by Noel Coward (1899-1999)

In tropical climes there are certain times of day
When all the citizens retire to tear their clothes off and perspire.
It's one of the rules that the greatest fools obey,
Because the sun is much too sultry
And one must avoid its ultry-violet ray.
The natives grieve when the white men leave their huts,
Because they're obviously, definitely nuts!

Mad dogs and Englishmen go out in the midday sun,
The Japanese don´t care to, the Chinese wouldn´t dare to,
Hindus and Argentines sleep firmly from twelve to one
But Englishmen detest-a siesta.
In the Philippines they have lovely screens to protect you from the glare.
In the Malay States, there are hats like plates which the Britishers won't wear.
At twelve noon the natives swoon and no further work is done,
But mad dogs and Englishmen go out in the midday sun.

. . .

INTRODUCTION

And God said, "Let there be light," and there was light - those are among the first words in the Holy Bible. The sun has been worshipped since the early days of mankind and plays an important role in many religions. In ancient Egypt (2700-2270 BC), Ra was the sun god, creator of everything.
The sun is the reason we can live on Earth; it emits visible light, heat and ultraviolet radiation (UVR) which are mandatory for life. UVR is necessary in order to synthesize vitamin D. Vitamin D in turn is essential for our bone health, and deficiency of vitamin D can be related to autoimmune diseases as well to several sorts of cancer.
Visible light plays a crucial role in photosynthesis, the process whereby plants, algae and some bacteria transform carbon dioxide and water to carbohydrate and oxygen. Almost all oxygen in the atmosphere is produced by photosynthesis.

Unfortunately the sun also has negative effects on humans, animals, plants and even inanimate materials such as paint and plastic. The UVR can cause erythema, skin cancer and cataract. Beneficially for us, part of the produced oxygen is transformed to ozone in the stratosphere which protects us from the harmful effects of UVR [1]. Other means of protection is the use of e.g. sunscreens.

This thesis deals with questions about sunscreens. We have studied the percutaneous absorption of BZ-3 and photostability. In the next sections I have presented an overview of some topics which are of relevance when dealing with sunscreens.

THE SKIN

The human skin consists of three layers. The outer part is the *epidermis*, which is usually between 75 and 150 µm in thickness. It consists mainly of keratinocytes, but also of melanocytes, Langerhans cells and Merkel cells. The Langerhans cells are part of the immune system and the Merkel cells are part of the nerve system. The melanocytes contain pigment and are the most important factor for the color of the skin. The outermost part of the epidermis is called the *stratum corneum*. The stratum corneum consists of corneocytes, which are flat, dead keratinocytes with no nucleus The matrix consists of lipids arranged in lamellar sheets.This thin layer provides an effective barrier against water loss, trauma and microorganisms. The second layer, the *dermis*, supports the epidermis. It consists of connective tissue. The third layer, the *subcutis*, consists of loose connective tissue and fat cells [2, 3]. Figure 1 shows a schematic structure of the skin and Figure 2 shows a histological picture of normal skin.

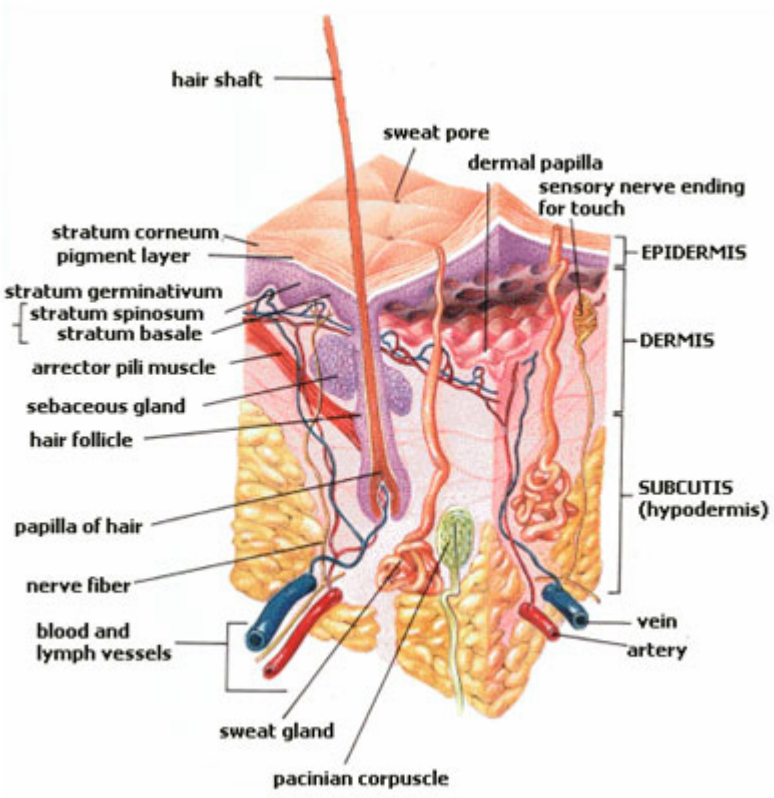


Figure 1 Schematic structure of the skin [4].

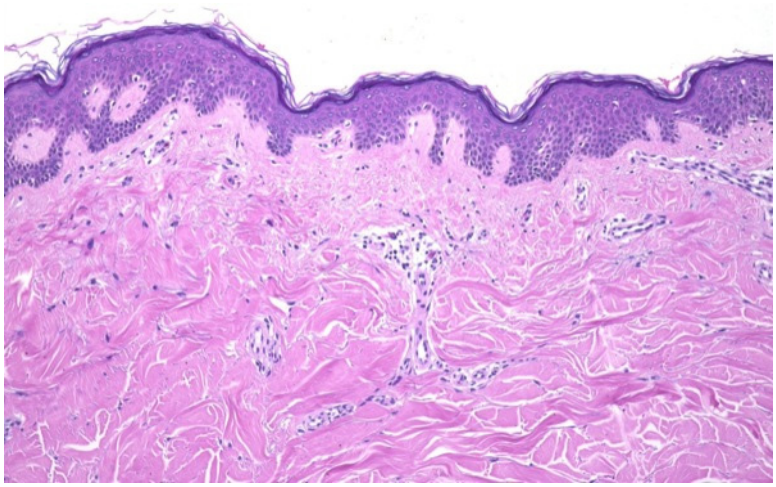


Figure 2 Histological picture of normal skin [5].

In 1975, TB Fitzpatrick developed a classification system for skin (Table 1) [6]. According to its ability to tan, the skin is classified into six different types. This classification can be helpful, but it is important to remember that there are fair-skinned Asians and Indians who may be better classified as skintype II-IV.

Table 1 Fitzpatrick skin types (adapted from MacKie) [7].

Skin type	
I	Fair skinned Caucasians who burn easily and never tan
II	Fair skinned Caucasians who burn easily and tan slowly and with difficulty
III	Medium skinned Caucasians who burn rarely and tan relatively easy
IV	Darker skinned Caucasians who virtually never burn and tan readily, e.g. some individuals with Mediterranean ancestry
V	Asian or Indian skin
VI	Afro-Caribbean or Black skin

ULTRAVIOLET RADIATION

UVR is a type of electromagnetic radiation.
Electromagnetic radiation is a stream of photons, which are massless particles in a wave-like pattern which move at the speed of light [8].
It can be divided into cosmic rays, gamma rays, X-rays, UVR, visible light, micro waves and radio waves. The photons of a radio wave contain less energy than the photons of UVR or gamma rays. The more energy a photon has, the more damage it can cause to cells. The spectrum of electromagnetic wavelengths is shown schematically in Figure 3.

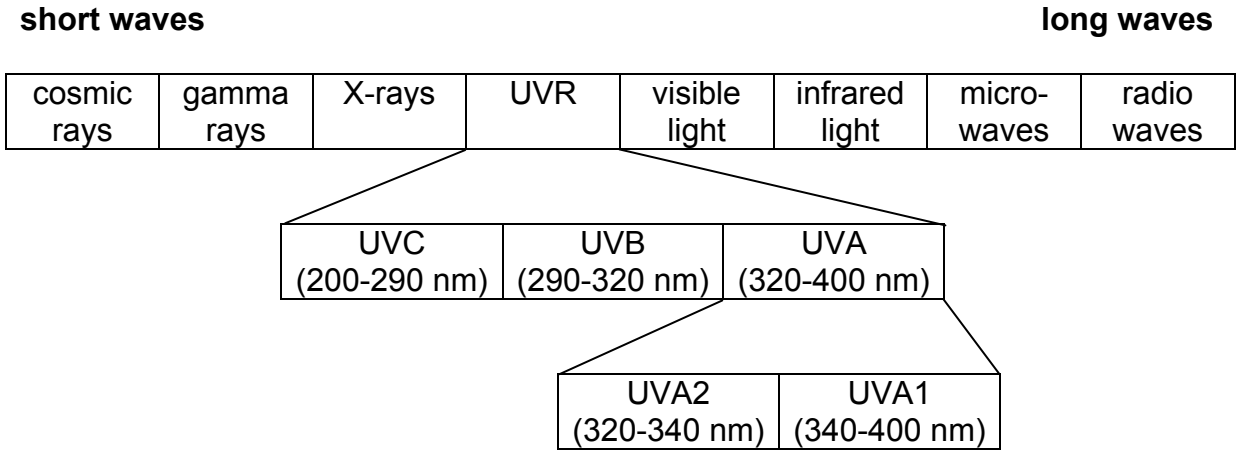


Figure 3 The electromagnetic spectrum.

The German physicist Johann Ritter (1776-1810) is credited with the discovery of UVR in 1801. This was done soon after the discovery of infrared light. Ritter found that there exist invisible rays from the sun that efficiently darken silver chloride, namely UVR [9].
In 1893 the Danish dermatologist, Niels Finsen (1860-1904) introduced phototherapy against lupus vulgaris with good results. In 1903, one year before his death, he was awarded the Nobel Prize in medicine with the motivation "in recognition of his contribution to the treatment of diseases, especially lupus vulgaris, with concentrated light radiation, whereby he has opened a new avenue for medical science" [10, 11].

Normally the UVR is divided into three groups; UVA, UVB and UVC. In 1932 this division was introduced by the International Commission on Illumination (CIE) [12]:

UVA (315-400 nm)
UVB (280-315 nm)
UVC (200-280 nm)

However, it is more common to use a slightly different division:

UVA (320-400 nm)
UVB (290-320 nm)
UVC (200-290 nm)

The division between UVB and UVC is set at 290 nm since it is unlikely that wavelengths below 290 nm reach the Earth. The division between UVB and UVA is perhaps more arbitrary [13].
In the literature one can also see a division of UVA into UVA1 (340-400 nm) and UVA2 (320-340 nm).

The sun is the largest source of UVR. The ozone layer limits the amount of UV that reaches the Earth's surface. UVC is completely filtered by the ozone layer, but we can encounter it from artificial sources such as welding equipment.
About 6% of the UVR that reaches the Earth is UVB and the rest is UVA. The amount of UVR reaching the surface is influenced by the solar zenith angle which varies with time of day and year. The thickness of the ozone layer and the altitude also influences the amount of UVR that reaches the Earth. A person situated at a higher altitude would have more of the atmosphere is below herself, and the atmosphere will also be thinner.

The UV dose that gives a barely noticeable erythema is called the Minimal Erythemal Dose (MED). This dose is in fact individual, and has to be specified in each case but it is nevertheless used to describe doses of about 200-300 J/m^2. The standard erythema dose (SED) is better to use, where 1 SED equals 100 J/m^2 erythema-weighted UVR [14].

UV Index

The UV Index was developed in the 1990s by WHO in collaboration with several other organizations. The UV Index provides information about the UVR level to help us plan outdoor activities in order to prevent overexposure to UVR [15].
The definition is:

$$I_{UV} = k_{er} \int_{250}^{400} E_\lambda S_{er}(\lambda)\, d\lambda$$

where E_λ= solar spectral irradiance in Wm^{-2}nm^{-1}; $d\lambda$= wavelength interval used in the summation; $S_{er}(\lambda)$= erythema reference action spectrum; k_{er}= a constant equal to 40 m^{2}W^{-1}.

The UV Index is normally reported along with the weather forecast in newspapers, on TV and/or on radio. In Sweden the UV Index is measured by the Swedish Meteorological and Hydrological Institute (SMHI) and is normally displayed in the newspapers during summer. On the website of SMHI the current UV Index in Sweden is reported the entire year. Table 2 shows how the UV Index normally is reported.

During summer in Sweden, the UV Index is usually between 4 to7, and during winter below 2.The UV Index varies with the factors mentioned in the previous section about UVR [16].

Table 2 UV Index

Category	UV Index range
Low	0-2
Moderate	3-5
High	6-7
Very High	8-10
Extreme	≥11

Disorders linked to UV radiation

The World Health Organization (WHO) has listed nine diseases with a strong causal relationship to excessive UVR exposure and three diseases due to under-exposure to UVR. The diseases linked to over-exposure are the three most common types of skin cancer, actinic keratosis (AK), sunburn, cortical cataract, pterygium, reactivation of herpes labialis and squamous cell carcinoma (SCC) of the cornea and conjunctiva. The diseases linked to under-exposure are rickets, osteomalacia and osteoporosis. These diseases are all connected with vitamin D, which is produced in the skin after UV-exposure. Vitamin D plays an important role for our bone health. Low levels of vitamin D may be related to autoimmune diseases such as multiple sclerosis and type 1 diabetes. Deficiency of vitamin D may also have a relationship to certain cancers, e.g. prostate and non-Hodgkin lymphoma. The evidence is not yet convincing but in the future we may see a longer list of diseases with a strong causal relationship due to under-exposure to UVR [17].

Skin cancer

It has been known for a long time that UVR can cause skin cancer [18]. UVB can cause DNA damage which leads to the development of skin cancer [19]. One common type of DNA injury is the formation of pyrimidine dimers. This is normally repaired by the enzymes, exonuclease, DNA polymerase and ligase, which excise the damaged DNA and rebuild it to normal DNA. However, this is not 100% effective and sometimes the repair mechanism fails, which can lead to the development of skin cancer. In the rare disorder Xeroderma pigmentosum, the patients lack the enzymes to repair the DNA. They must be extremely careful not to expose themselves to UVR; otherwise they will develop skin cancer at a very young age [20]. Other forms of DNA-damage can also occur such as single strand breaks and DNA crosslinks. UVA can have an indirect DNA-damaging potential through the production of free radicals, which causes oxidative stress.

There are three major types of skin cancer: cutaneous malignant melanoma (CMM), SCC, and basal cell carcinoma (BCC). SCC and BCC are generally called non-melanoma skin cancers (NMSC). NMSC are rarely lethal but can cause severe disfiguration, and they contribute to the economic burden of the health care system.

In Sweden during 2004, 3,420 new cases of SCC were registered, and the estimated number of new cases of BCC was 36,500. Until 2003 there has not been a register in Sweden for BCC. It is the Pathology and Cytology departments which report the BCCs [21]; hence there might be an underestimation since BCCs are commonly treated without a histopathological diagnosis. For CMM, the most serious type of skin cancer,1,950 new cases were registered in Sweden during 2004. The number of deaths in Sweden during 2002 was 380 due to CMM and 63 due to NMSC [22]. There are also precancerous lesions such as AK and SCC in situ (synonym Bowen's disease) which can develop into invasive SCC.
There are several articles that show a causal relationship of UVB with AK and SCC [19, 23, 24]. Which wavelengths are primarily the cause of BCC is still not known [25]. For CMM, there are studies supporting both UVA and UVB as the main cause. It seems that mutations in the tumour suppressor gene *CDKN2A* and in the oncogenes N-*ras* and H-*ras* are the most important cause for developing CMM and that UVR may have a major role in inducing these mutations although the action spectrum is still unknown [26]. Setlow *et al*. showed in a fish model that it was mainly UVA that was responsible for the induction of CMM [27], and other studies support that hypothesis [28, 29], while de Fabo *et al*. showed in a mouse model that only UVB initiated CMM [30].

Probably the truth is somewhere in between. Both UVA and UVB can damage DNA. It is important to remember that there is an interplay between UVR and other factors, e.g. skin type and number of nevi.

SUNSCREENS

General aspects

Protection against the sun has been important as long as there been life on Earth. In the early days people probably used clay or different ointments to put on the skin, and the shade from trees and buildings was presumably also used.

For a long time, pale skin was an ideal. It showed that one did not have to work outdoors in the fields. The women protected themselves with broad-brimmed hats and parasols.

In the 1920s the ideal changed. This is usually ascribed to Coco Chanel (1883-1971), the legendary fashion designer. The story says that she returned from a Palm Beach vacation with a suntan, and all of a sudden it was very fashionable to be tanned. This coincides with the development of the Industrial Revolution when many people worked in the factories, away from the sun. Now a suntan declared that one had time to be outdoors, sailing, travelling etc. [31, 32].
The first commercial sunscreens appeared in the 1920s and 1930s and the most successful was Ambre Solaire containing benzyl salicylate, prepared by Scheuller, who founded the company known as l'Oréal [33]. These sunscreens gave good protection against erythema.

During World War (WW) II, sunscreens were further developed by US government-sponsored programmes. Sunscreens were used to protect the American soldiers fighting in the Pacific [34]. Chemicals like red petrolatum and salicylates were used [35]. Red petrolatum is a product of the process of refining crude oil to gasoline and oil. It was used for veterinary purposes. Its red color is believed to be due to an aromatic hydrocarbon [36]. The sun-protecting properties of red petrolatum were in fact known prior to WW II. Urbach describes in an article that his father used red petrolatum during WW I to protect the hands from sunburn [33].

After WW II, sunscreens became more widespread and also more popular to a lot of consumers. During this period it was more common to go on vacation and spend the holiday at the beach. A golden suntan was equivalent to good health. *Para*-aminobenzoate (PABA) was introduced as a sunscreen, giving its UV-protecting properties in the UVB area, first as a prescription drug, but later as an over-the-counter preparation [37, 38]. Allergy to PABA started to be reported and by the late 1980s it was rare to find PABA in sunscreens. Commercials stated that their products were "PABA-free". PABA-esters are still used and they seem to be less prone to induce contact allergies [39].

In the 1980s benzophenone-3 (BZ-3) was introduced and it soon became very popular. BZ-3 gives good protection also in the UVA range. However, BZ-3 is a common photocontatct allergen [40] and there have been reports about percutaneous absorption and a possible hormone effect [41]. Products with BZ-3 are no longer sold at Swedish pharmacies. Sunscreens are incorporated in many other cosmetic products such as hair spray, face creams and make-up. They are also used to protect e.g. paint from UV degradation.

Sunscreens can be divided into organic chemical absorbers and inorganic chemical absorbers. The protective properties of the sunscreen can be due to absorbing and/ or scattering effects. The organic chemical absorbers have reactive structures that can take up the energy from UVR and then go back to a relaxed state by sending out the energy as heat. They can be classified into different groups, based on their chemical structure: cinnamates, PABA derivates, salicylates, benzophenones, camphor derivates, dibenzoylmethanes, anthranilates and miscellaneous (Figure 4, p.12) [42]. Table 3 shows the ingredients approved for use in Europe. Inorganic chemical absorbers both scatter and absorb UVR. They consist of nanoparticles of titanium dioxide (TiO_2) and zinc oxide (ZnO).

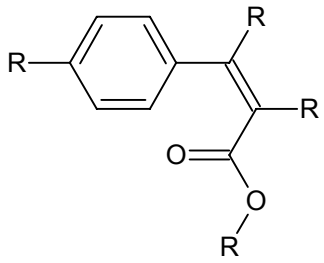
Table 3 Chemical UVR absorbers approved for use in Europe (adapted from IARC Handbook of sunscreens) [43].

INCI name	CAS no	Systematic name*
Organic chemical absorbers		
UVB absorbers		
Cinnamates		
Ethylhexyl methoxycinnamate	5466-77-3	2-Ethylhexyl 4-methoxycinnamate
Isoamyl-*para*-methoxycinnamate	71617-10-2	2-Propenoic acid, 3-(4-methoxyphenyl)-, 3-methylbutyl ester
para-Aminobenzoic acids (PABAs)		
Ethylhexyl dimethyl PABA	21245-02-3	Benzoic acid, 4-(dimethylamino)-, 2-ethylhexyl ester
PABA	150-13-0	Benzoic acid, 4-amino-
PEG-25 PABA	116242-27-4	Poly(oxy-1,2-ethanediyl), alpha,alpha'-(((4-carboxyphenyl)imino)di-2,1-ethanediyl)bis(omega-hydroxy-, ester with alpha-hydro-omega-hydroxypoly(oxy-1,2-ethanediyl) (1:1)
Salicylates		
Ethylhexyl salicylate	118-60-5	Benzoic acid, 2-hydroxy-, 2-ethylhexyl ester
Homosalate	118-56-9	Benzoic acid, 2-hydroxy-, 3,3,5-trimethylcyclohexyl ester
Camphor derivates		
3-Benzylidene camphor	15087-24-8	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-3-(phenylmethylene)-
Benzylidene camphor sulfonic acid	56039-58-8	Benzenesulfonic acid, 4-((4,7,7-trimethyl-3-oxobicyclo(2.2.1)hept-2-ylidene)methyl)-
Camphor benzalkonium methosulfate	52793-97-2	Benzenaminium, N,N,N-trimethyl-4-[(4,7,7-trimethyl-3-oxobicyclo[2.2.1]hept-2-ylidene)methyl]-, methyl sulfate
4-Methylbenzylidene camphor	36861-47-9	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-3-[(4-methylphenyl)methylene]-
Polyacrylamidomethyl benzylidene camphor	113783-61-2	
Miscellaneous		
Diethylhexylbutamido triazone	154702-15-5	Benzoic acid, 4,4'-[[6-[[4-[[(1,1-dimethylethyl)amino]carbonyl]phenyl]amino]-1,3,5-triazine-2,4-diyl]diimino]bis-, bis(2-ethylhexyl) ester
Ethylhexyl triazone	88122-99-0	Benzoic acid, 4,4',4''-(1,3,5-triazine-2,4,6-triyltriimino)tris-, tris(2-ethylhexyl) ester
Octocrylene	6197-30-4	2-Propenoic acid, 2-cyano-3,3-diphenyl-, 2-ethylhexyl ester
Phenylbenzimidazole sulfonic acid	27503-81-7	1H-Benzimidazole-5-sulfonic acid, 2-phenyl-

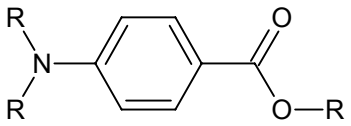
Table 3 cont

UVA absorbers	CAS no	Systematic name*
Benzophenones		
Benzophenone-3	131-57-7	Methanone, (2-hydroxy-4-methoxyphenyl)phenyl-
Benzophenone-4	4065-45-6	Benzenesulfonic acid, 5-benzoyl-4-hydroxy-2-methoxy-
Benzophenone-5	6628-37-1	Benzenesulfonic acid, 5-benzoyl-4-hydroxy-2-methoxy-, monosodium salt
Camphor derivates		
Terephthalylidene dicamphor sulfonic acid	90457-82-2	Bicyclo [2.2.1] heptane-1-methanesulfonic acid, 3,3'-(1,4-phenylenedimethylidene) bis(7,7-dimethyl-2-oxo-**
Dibenzoylmethane		
Butyl methoxydibenzoylmethane	70356-09-1	1,3-Propanedione, 1-[4-(1,1-dimethylethyl)phenyl]-3-(4-methoxyphenyl)-
Miscellaneous		
Bisymidazylate (proposed name)	180898-37-7	1H-Benzimidazole-4,6-disulfonic acid, 2,2'-(1,4-phenylene)bis-, disodium salt
UVA and UVB absorbers		
Miscellaneous		
Anisotriazine (proposed name)	187393-00-6	Phenol, 2,2'-[6-(4-methoxyphenyl)-1,3,5-triazine-2,4-diyl]bis[5-[(2-ethylhexyl)oxy]-
Drometrizole trisiloxane	155633-54-8	
Methylene-bis-benzotriazolyl tetramethylbutylphenol	103597-45-1	Phenol, 2,2'-methylenebis(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-
Inorganic chemical absorbers		
Titanium dioxide	13463-67-7	
Zinc oxide	1314-13-2	

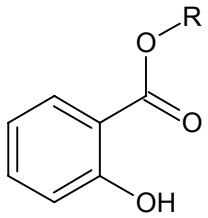
INCI International Nomenclature of Cosmetic Ingredients
CAS Chemical Abstracts Service
* preferred by Swedish Chemicals Inspectorate [44].
** from ChemIDplus [45].



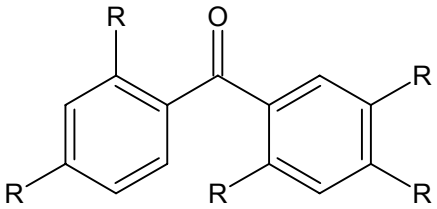
Cinnamate derivates



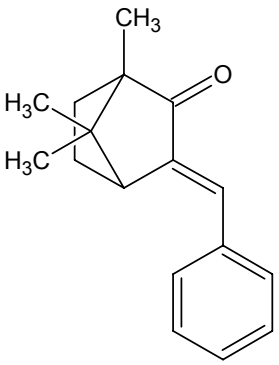
para-Aminobenzoate derivates



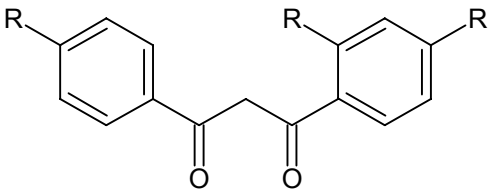
Salicylate derivates



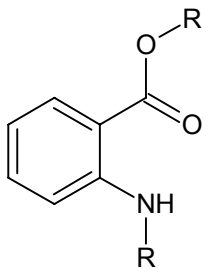
Benzophenone derivates



Camphor derivates



Dibenzoyl methane derivates



Anthranilate derivates

Figure 4 Chemical structure of the seven groups of organic chemical sunscreens.

Benzophenone-3

Benzophenones belong to the aromatic ketone category. They can absorb longer wavelengths, so they give good protection also in the UVA region [42]. They have been used since the 1980s and BZ-3 is the most common compound in the benzophenone group to use for sun protection. It has the molecular weight 228.26 with melting point 66.5°C.

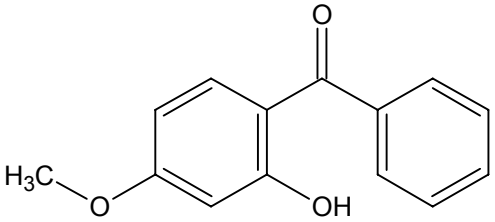


Figure 5 The structure of BZ-3.

Sunscreens and skin cancer

Sunscreens were mainly designed to protect against erythema. There have been studies which indicate that sunscreens increase the risk of getting CMM [46, 47], but other studies report the opposite [48]. Two review articles about sunscreens conclude that there is no relation between sunscreen use and a higher risk of getting CMM [49, 50]. Some studies support the idea that sunscreens are able to protect against skin cancer and actinic keratosis, but there was no evidence that sunscreens were protecting against BCC or CMM [51, 52].

One error which people may make when they use sunscreens is that they stay longer in the sun than if they had not used a sunscreen [53].
Another common error is that the majority of users do not use the recommended amount, 2 mg/cm^2, necessary to obtain the sun protection factor (SPF) value marked on the bottle. Studies show that the average applied amount is much lower, 0.5 to 1.0 mg/cm^2 [54, 55]. Patients with the light-induced skin disease called polymorphic light eruption (a group that is very motivated to obtain good sunprotection) used on average 0.5 mg/cm^2; they also applied the lotion in an uneven manner [56]. Education made the patients use more sunscreen, on average 1.13 mg/cm^2, still too low to obtain the SPF value [57].

Adverse effects of sunscreens

There have been concerns about pulmonary effects of TiO_2 after inhalation. If the TiO_2 particles are too large, the cream will appear white on the skin. For that reason, nanoparticles of TiO_2 are used in sunscreens to make them cosmetically appealing. One study has shown a species difference after inhalation of TiO_2, rats had a marked progression of histopathological lesions while hamsters and mice did not [58]. Boffetta *et al.* studied 15,017 workers in the TiO_2 industry in Europe. They did not find any carcinogenic effect of TiO_2 dust on the human lung [59].

Schlumpf *et al.* reported endocrine activity of several sunscreens. They found estrogenic influence on rats after ingestion of the sunscreen compounds 4-methyl-benzylidene camphor (MBC), ethylhexyl methoxycinnamate (EHMC), and BZ-3. After dermal exposure to rats, MBC gave an increased uterine weight [41, 60]. The rats were exposed to quite large amounts of the substances. One study showed that BZ-3 and its metabolites, dihydroxy methoxybenzophenone (DHMB) and trihydroxy benzophenone (THB), could have estrogenic effects on MCF-human breast cancer cells [61]. However, another study on humans did not show any endocrine effect after dermal exposure to sunscreens [62] and the European Commission concluded in a plenary meeting in 2001 that sunscreens do not have an estrogenic effect which could potentially affect human health [63].

The environment may also be affected by the use of sunscreens. TiO_2 can have ecotoxic effects on algae and daphnids [64]. Octocrylene and MBC have been found in fish from Swiss rivers [65] and those compounds together with BZ-3, EHMC and butyl methoxydibenzoylmethane (BMDBM) have been found in Swiss lakes with a higher concentration during summer. The investigated lakes were used for recreational activities and the sunscreens probably originated from swimmers who had used a sunscreen [66].

Skin problems

Subjective irritation

This is among the most frequent complaints. The symptoms can be stinging, burning and/or itching without any visible skin signs [67].

Contact and photocontact dermatitis

Contact and photocontact dermatitis can be caused by sunscreens. These can be both irritant and allergic reactions. BZ-3, isopropyl dibenzoylmethane and BMDBM were the most common sensitizers in a Swedish study [40]. PABA used to be a common sensitizer, but PABA is practically no longer used and the PABA-esters seem to have less sensitizing properties [39]. There are also reports of contact urticaria, erythema multiforme and anaphylactic shock due to BZ-3 [68-70].

Sensitization from TiO_2 and ZnO is practically non-existent.

Percutaneous absorption

It has long been known that the skin is permeable to different substances, even though we sometimes tend to forget this.
In 1886, over a century ago, there were several cases of cyanosis in newborn children due to aniline toxicity. The diapers were stamped with a 4½ inch oval and the infants who received a newly stamped diaper became cyanotic due to the percutaneous transfer of aniline [71].

There have been lethal outcomes for children when they have been in skin contact with hexachlorophene, an antibacterial substance, but also a known neurotoxin [72, 73].

Several reports of poisoning after topical application of salicylate, some fatal, have also been published. The list of substances that can give systemic effects when applied topically can be much longer [74, 75].

For sunscreens, several studies about percutaneous absorption are available. One of the first studies was conducted in 1970 when 21 organic compounds, among them the sunscreen PABA, were investigated. Carbon^{14} labeled compounds were applied on the forearm and measured in urine. Almost 30% of the applied isotope was present in the urine five days after the application of isotope-labeled PABA [76]. BZ-3 is one of the most bioavailable photoactive compound following dermal application [77]. Between 0.5-9% of applied dose BZ-3 penetrates the skin [78-82]. BZ-3 is extensively conjugated and the main excretion path in rat is urine. This does not mean that BZ-3 has toxicological properties during normal use, but it raises questions about possible toxicological endpoints [77, 83].

Methods for measurement of percutaneous absorption

There are several methods to measure in-vivo absorption. Like all in-vivo methods, they have the advantage of being more true to a real-life situation. Negative aspects are that they are usually more expensive and that people or animals have to be exposed to the compounds. In-vitro methods are more easily controlled but cannot account for processes in the body. Table 4 summarizes the advantages and disadvantages. Some common methods are described in summary.

Table 4 Advantages and disadvantages for in-vivo and in-vitro methods.

	Advantages	**Disadvantages**
In vivo	Biological response	Exposure to humans/animals Expensive
In vitro	Less expensive Less risk for humans/animals Reproducible	No biological endpoint

In-vivo methods for absorption measurements [84]

Radioactivity in blood or excreta

The radioactivity in blood or excreta can be measured after topical application of a labeled compound. It is common to use carbon^{14} or tritium. This method does not take into consideration that the compound can be metabolized.

Stripping method

The compound is applied to the skin. After a certain time (usually 30 min) the stratum corneum is removed by tape application and removal. The tape strippings are assayed with e.g. an HPLC method.

Absolute topical bioavailability

The compound is measured specifically in blood, urine and/or faeces.

Animals can also be used as a model but sometimes animal skin is more permeable than human skin.

In-vitro method for absorption measurements [85]
Diffusion cell
Diffusion cells can be used. They consist of a donor and a receptor chamber with a membrane placed in between. The compounds of interest are dissolved in an appropriate fluid and passed through the chambers. The membrane can consist of human skin, animal skin or an artificial material.

SPF testing

The sun protection factor (SPF) has been used since the 1930s [86]. During the decades some changes have been made, and there were some differences between countries. Since 2002, the European cosmetic toiletry and perfume association (COLIPA), Japan Cosmetic Industry Association (JCIA) and Cosmetic, Toiletry and Fragrance Association of South Africa (CTFA-SA) decided on a joint agreement regarding the international SPF test method [87]. This method is applied worldwide but there are disparities in e.g. protocols, leading to slight differences.
The method in summary is as follows. A test panel of subjects with skin types I, II and III according to Fitzpatrick is included. The back is used as test area, and the area should be between 30 cm^2 and 60 cm^2. A xenon arc lamp with an output in the wavelength region between 290 and 400 nm is used. The applied amount of product should be 2.00 mg/cm^2. The product should be deposited with a syringe and spread with light pressure, using a finger cot. This applies to lotions, liquids, milks, creams and sprays. Exposure to UVR should start 15 to 30 min after the application. The individual MED (MEDi) is calculated, both for unprotected skin (MEDui) and for protected skin (MEDpi). The individual SPF is the ratio of the MEDpi and MEDui.

$$SPFi = \frac{MEDpi}{MEDui}$$

The SPF for the product is calculated as the arithmetic mean of all valid SPFi obtained.

Since the endpoint is erythema, it is mainly the UVB protection that is measured with the SPF method.

UVA testing

In contrast to the SPF method, there is no standard method that is used worldwide for UVA testing or labeling. For UVA there is no clearly defined endpoint as erythema is for UVB.
Several different methods are used, and sometimes a combination of methods. Which method is used differs between continents, countries and companies; no consensus so far exists on the laboratory measurements. The most frequently used methods are explained in summary.

In-vivo methods

The "protection factor UVA" (PFA) [88]

A test panel of subjects, (Fitzpatrick's skin type I to III), receive doses of UVA from a xenon arc lamp with the output between 320 to 400 nm. The minimal response dose (MRD) was measured with protection (MRDp) and without protection (MRDu) with the endpoint minimal erythema or tanning. The MRDp was assessed 16 to 24 h after UVA exposure. The applied amount of sunscreen was 2 mg/cm^2.

$$\text{PFA} = \frac{\text{MRDp}}{\text{MRDu}}$$

Persistent pigment darkening (PPD) [89]

PPD is a photooxidation of melanin or precursors causing a color change of the skin. A test panel of subjects (Fitzpatrick's skin types II to IV) receive doses of UVA from a xenon arc lamp with an output between 320 to 400 nm. The minimal pigmenting dose (MPD) was established. The MPD with protection (MPDp) and without protection (MPDu) were measured. The applied amount of sunscreen was 2 mg/cm^2. The MPDp was assessed 3±1 hour after UVA exposure.

$$\text{UVA protection factor} = \frac{\text{MPDp}}{\text{MPDu}}$$

The UVA protection factor (UVA-PF) is the arithmetic mean of the UVA-PFi values obtained from at least 10 subjects. PFA and PPD are basically the same method but differ slightly. In the PFA method, the endpoint is tanning or erythema and in PPD the endpoint is tanning solely. PPD excludes people with skin type I and PFA excludes people with skin type IV [77, 90, 91].

It also exists methods using immediate pigment darkening and measurements on sensitized skin [92, 93].

In-vitro methods

Critical Wavelength [94]

This is a method developed by Diffey and Robson, using thin-film substrate spectrophotometry. The definition of the critical wavelength (λ_c) is the wavelength at which the integral of the spectral absorbance curve reaches 90% of the integral from 290 to 400 nm.

$$\int_{290}^{\lambda c} A(\lambda)d\lambda = 0.9 \int_{290}^{400} A(\lambda)d\lambda$$

where A=absorption; dλ= wavelength interval used in the summation.

The UVA/UVB-ratio [95]

This method is based on the critical wavelength method to calculate the ratio of UVA (320 to 400 nm) and UVB (290-320 nm).

$$\frac{\int_{320}^{400} A(\lambda)d\lambda \Big/ \int_{320}^{400} d\lambda}{\int_{290}^{320} A(\lambda)d\lambda \Big/ \int_{290}^{320} d\lambda}$$

where A=absorption; dλ= wavelength interval used in the summation.

Australian standard/New Zealand standard [96]

There are three alternative methods which can be used depending on the type of sunscreen being tested. In all three a spectrophotometer is used. The transmission is measured in the region 320 nm to 360 nm. If method (a) or (b) is used, maximum 10% of the light may be transmitted and for method (c) maximum 1% of the light may be transmitted, in order to call the product broad-spectrum.

a) solution method
The product is dissolved into a spectroscopic grade solvent and put in a quartz cell. The percentage transmission is calculated.
b) thin film method
This method is used when the product is rather opaque. The product is filled in a quartz cell, constructed to provide an 8 µm thick layer of the sunscreen. The percentage transmission is calculated.
c) plate method
The sunscreen is applied to one surface of a quartz plate in a 20 µm thick layer (which corresponds to 2.0 mg/cm^2. The percentage transmission is calculated.

Labeling in different countries

Japan uses the in-vivo PPD method and the products are marked PA+, PA++ or PA+++.
In Europe no method is adopted officially, so the labeling differs between countries and between brands in the same country.

In the UK it is common to use the Boots star rating system based on the UVA/UVB-ratio (Table 5).

Table 5 The Boots star rating system.

UVA/UVB-ratio	Stars	Category
0.0 to 0.2	no rating	
0.21 to 0.4	one star	minimum
0.41 to 0.6	two stars	moderate
0.61 to 0.8	three stars	good
0.81 to 0.9	four stars	superior
>0.91	five stars	ultra

According to the US Food and Drug Administration (FDA) there is no approved rating system that identifies UVA protection. Scientists are working to create a standardized testing system to measure UVA protection [97].

Protection by clothing

Clothes are widely recommended as UV protection, and they give good protection, but there are some pitfalls. Loosely woven fabrics do not protect as well as tightly woven fabrics and wet material gives poorer protection than dry textiles. [98] There are several reports about the protection factor for clothes [99, 100].
For clothes, the ultraviolet protection factor (UPF) is normally used. There are different test methods for determination of the UPF, such as in-vivo methods similar to the SPF testing, but the most frequently is an in-vitro method using a spectrophotometer. Samples of the fabric are cut out and placed in the spectrophotometer set for 290 to 400 nm.

The definition is [101]:

$$\text{UPF} = \frac{\sum_{290}^{400} E_\lambda S_\lambda \Delta\lambda}{\sum_{290}^{400} E_\lambda S_\lambda T_\lambda \Delta\lambda}$$

where E_λ= the solar spectral irradiance in $Wm^{-2}nm^{-1}$; S_λ = the relative erythemal spectral effectiveness ; T_λ=spectral transmission coefficient of the textile material; Δ_λ= the bandwidth in nm; λ = the wavelength in nm.

There are several brands that manufacture specially designed UV-protecting clothes with UPF 50+.

Sun protection for animals and plants

It is not only humans that are affected by UVR: animals and plants are also concerned. For example, fair-skinned pigs can be sunburnt. Some plants have developed a strategy using flavonoids to become more pigmented, they also may have enzymes which can perform DNA repair. Cyanobacteria are one of the first existing life forms on earth; they contain UV-absorbing pigments, mycosporinelike amino acids [102].

The hippopotamus excretes a fluid that contains a pigment which at first is red and then turns brown. The function of this is not fully understood, but the fluid has both antibiotic and UV-absorbing properties [103].

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) was first developed around 1900 by the Russian botanist Michail Tsvet (1872-1919). He used it to separate different plant colors, such as chlorophyll. In the 1940s the method was rediscovered and further developed by the British chemists Archer JP Martin (1910-2002) and Richard Synge (1914-1994) who received the Nobel Prize for their achievements in 1952 [1, 104]. Since the 1970s, HPLC has been used to separate different chemical compounds. A mobile phase is forced with high pressure through a stationary phase, a column. The sample is injected and the solution goes through the stationary phase. The different components go through the column at different speeds and are then separated.
Normally a UV detector with variable wavelength is used. The detector registers each component as a peak in a graph called a chromatogram.

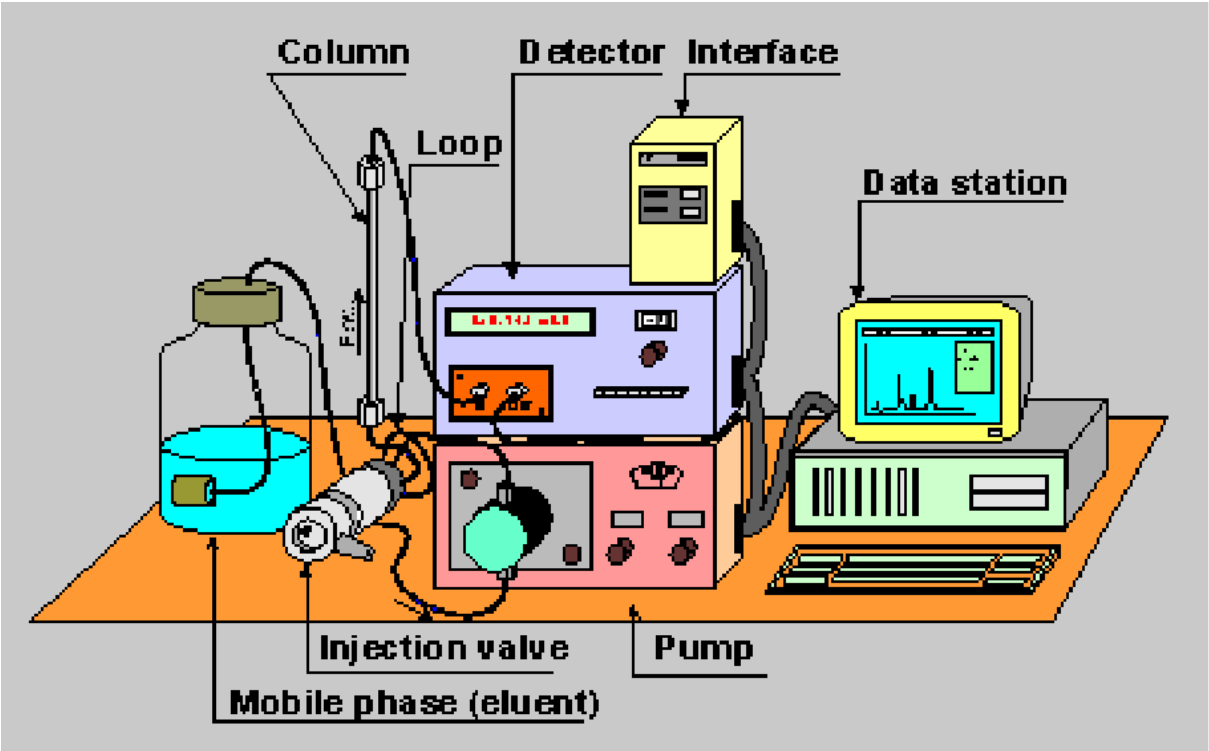


Figure 6 Schematic picture of HPLC [105].

Usually a reverse-phase HPLC is used; the stationary phase is nonpolar (hydrophobic), and the mobile phase is a polar liquid, such as mixtures of water and methanol or water and acetonitrile. In our set-up we used a reverse-phase HPLC. Detectors with infrared light, fluorescence or mass spectrometry can also be used. There have been several studies about sunscreens and HPLC [106-109]. However, few of them dealt with human urine and none of them suited our purpose completely. In Table 6 an overview of studies about BZ-3 in biological materials is presented. Only studies with detailed information about the analytical set-up have been included. Some studies dealt with the issue but did not provide sufficient information to reproduce their experimental set-up.

Table 6 An overview of studies about BZ-3 (and its metabolites) and HPLC, applied on biological samples. Dermal exposure unless otherwise mentioned. None of the methods used an internal standard.

reference	compound	minimum detectable limit	biological sample	mobile phase	column	hydrolysis	wavelength	comment
Abdel-Nabi *et al.* (1992) [110]	BZ-3	2.0 ng/ml	urine blood tissue***	methanol:acetic acid (60:40)	Hypersil ODS C18	β-glucuronidase HCl	305	rat, oral exposure
Jiang *et al.* (1996) [111]	BZ-3*	0.05 µg/ml	spiked plasma	methanol-water (88:12)	Novapak C18 RCM		315	human
Potard *et al.* (1999) [107]	BZ-3*	20 µg/ml	skin tissue	methanol:water (69:31)	Novapak C18		291	human
Sarvieya *et al.* (2004) [81]	BZ-3* DHB** THB**	0.8 ng (0.08µg/ml)	spiked samples (urine,skin tissue,plasma) urine, skin tissue, plasma	methanol-water gradient 75:25-92:8	Symmetry C18	β-glucuronidase	289	human
Kasichayanula *et al.* (2005) [112]	BZ-3 DHB THB DHMB	BZ-3 0.5 ng DHB 0.7 ng THB 0.5 ng DHMB 0.6 ng	plasma urine skin tissue	methanol:water gradient(50:50-90:10)	Milford C18		289	piglet

* Other compounds were also investigated.
** Only trace amounts were found.
*** Liver, kidney and testes.

SPECTROPHOTOMETER

A spectrophotometer is used to measure the absorption of electromagnetic radiation at different wavelengths. It consists of two instruments, a spectrometer that produces light of a selected wavelength and a photometer to measure the intensity of light.
It is mainly used to measure the absorbance of a substance at different wavelengths but also to determine the concentration of a compound by using the Lambert-Beer law [9].

Figure 7 A spectrophotometer [113].

AIMS OF THE STUDY

Paper I

To investigate the excretion of BZ-3 in urine after one whole-body application of a sunscreen containing 4% BZ-3.

Paper II

To examine the excretion of BZ-3 in urine after repeated whole-body applications of a sunscreen containing 4% BZ-3, and to investigate whether UVR had any impact on the excretion.

Paper III

To develop and validate a reverse-phase HPLC method to determine the amounts of BZ-3 and DHB in human urine. The assay was applied to study the urinary excretion pattern of BZ-3 and DHB after repeated whole-body applications of a commercial sunscreen.

Paper IV

To examine the photostability of seven commercial sunscreens before and after exposure to artificial UVR and before and after exposure to UVR from the sun.

MATERIAL AND METHODS

Paper I
The study was performed on 11 volunteers (mean age 26 years, range 22-37 years, 4 women, 7 men). They provided a reference urine sample prior to the application of the sunscreen.
They applied a commercially available sun-protecting lotion containing 4% BZ-3 over the whole body except the scalp and genital area. Urine samples were collected during a 48-hour period after the application. They used the recommended amount 2 mg/cm^2 and the body surface area (BSA) was estimated to be 2 m^2. BZ-3 in urine was analyzed with a reverse-phase HPLC method.

Paper II
The study was performed on 25 healthy participants (mean age 27, range 22-42 years, 16 women, 9 men). They provided a reference urine sample prior to the first application of the sunscreen. They applied 2 mg/cm^2 of a commercially available sun-protecting lotion containing 4% BZ-3, morning and night for five days. The sunscreen was distributed in plastic containers. The urine was measured during those five days and for a further five days after the last application. The individual BSA was calculated [114]; hence each participant applied a different amount of sunscreen. They were randomized into two groups: **A** and **B** (Table 7). One participant in Group A was excluded due to lack of compliance.

Table 7 Demographics of volunteers in group A and B.

Group	Women	Men	Mean age (years)	Range (years)
A (n=11)	6	5	26	22-37
B (n=14)	9	5	28	22-42

Group A did not receive any UVR. **Group B** received UVR during the five days the sunscreen was applied, according to skin type: total amount UVA between 400 and 707 J/cm^2, and total amount UVB between 0.46 and 2.0 J/cm^2. For UVA irradiation a Dermalight Ultra A1, equipped with six light tubes Dr Hönle 200 W (Martinsreid, Germany), was used. For UVB irradiation an Esshå Corona IV, equipped with 28 light tubes, Philips UVB TL 40 W/12 (Eindhoven, the Netherlands), was used.
The BZ-3 in urine was analyzed with the reverse-phase HPLC method described in Paper III.

Paper III
Urine samples were analyzed regarding both conjugated/non-conjugated BZ-3 and conjugated/non-conjugated DHB since both BZ-3 and DHB are extensively conjugated in the body. In Figure 8 the structure of DHB is shown. Solid-phase extraction (SPE) with C8 columns was followed by reverse-phase HPLC. For separation a HIChrom C18 column was used with an acetonitrile-water mobile phase and the detector was set at 287 nm. An internal standard was used to provide a more correct determination of the amounts.

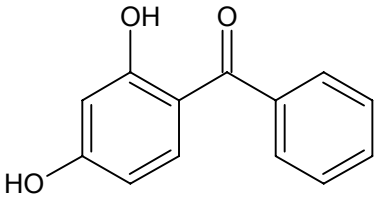


Figure 8 The structure of DHB.

Paper IV

Seven commercial sunscreens were studied with absorption spectroscopy. In Table 8 an overview of the photoactive compounds is presented. Sunscreen product, 0.5 mg/cm^2, was placed between plates of silica. The area under the curve (AUC) in the spectrum was calculated for UVA (320-400 nm), UVA1 (340-400 nm), UVA2 (320-340 nm) and UVB (290-320 nm) before (AUC_{before}) and after (AUC_{after}) artificial UVA (980 kJ/m^2) and artificial UVB (12 kJ/m^2). If the AUC Index (AUCI) defined as AUC_{after}/AUC_{before}, was higher than 0.80, the sunscreen was considered photostable. For UVA irradiation, a UVASUN 2000 (MUTZHAS, Germany) was used. The output was mainly between 340-400 nm. For UVB irradiation an Esshå Corona Mini (Sweden), equipped with 2 light tubes, Philips TL 12 20 W, was used.
For natural UV the samples were placed horizontally outdoors when the weather was sunny. This was done in July in Gothenburg. Spectra were measured after 30 min, 90 min and 120 min of natural UV exposure.

The spectra were recorded by a Cary 4 spectophotometer (Varian, USA) and we received the natural UV doses from SMHI.

Table 8 The photoactive compounds in the investigated sunscreens, CAS no and SPF of the product.

Photoactive compound	**CAS no**	**Mainly protection against**		**Active ingredients in the seven investigated sunscreen products**						
		UVA	**UVB**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
EHMC	5466-77-3		x	x	x	x				
MBC	36861-47-9		x			x	x	x		
EHT	88122-99-0		x							
OC	6197-30-4		x						x	
BMDBM	70356-09-1	x		x	x	x	x	x	x	
BZ-3	131-57-7	x			x	x				
TLDCSA	90457-82-2	x							x	
TiO_2	13463-67-7		x			x	x		x	x
ZnO	1314-13-2	x								x
SPF				4	14	10	10	6	10	15

CAS Chemical Abstracts Service
EHMC ethylhexyl methoxycinnamate **MBC** 4-methylbenzylidene camphor
EHT ethylhexyl triazone **OC** octocrylene **BMDBM** butyl methoxydibenzoylmethane
BZ-3 benzophenone-3 **TLDCSA** terephthalylidene dicamphor sulfonic acid
TiO_2 titanium dioxide **ZnO** zinc oxide
SPF Sun Protection Factor

Statistical methods

Paper II

Differences were compared with Student's t-test. A p-value of less than 0.05 was considered to indicate statistical significance.
Correlations were calculated using Pearson's correlation coefficient.

Paper III

The minimum detectable limit was defined as three times the baseline noise level.
The within-day and between-days precision was calculated as relative SD (RSD).
RSD=(SD/mean) x 100.

Ethics

Papers I and **II**

The regional ethical review board in Gothenburg approved the studies.
The volunteers participated after informed consent was obtained from them.

RESULTS

Paper I

A single whole-body application of a sunscreen containing 4% BZ-3 resulted in excretion of BZ-3 in the urine. The average total amount excreted was 11 mg, median 9.8 mg, which is approximately 0.4% of the applied amount BZ-3.

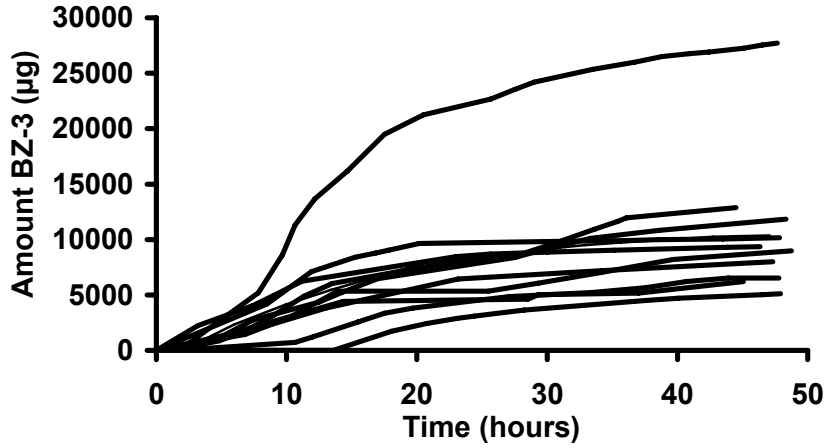


Figure 9 Accumulated amount of BZ-3 (µg) recovered in urine during a 48-hour period after topical application to 11 human volunteers. Each line represents one volunteer.

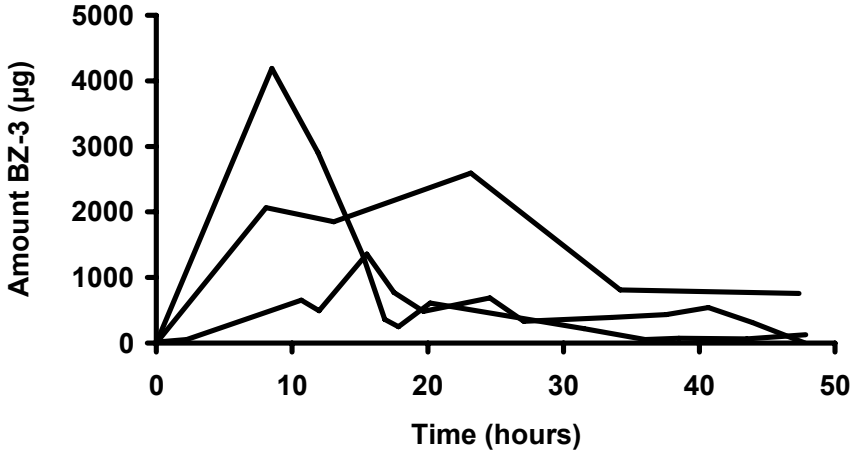


Figure 10 Three different excretion profiles to illustrate the variation in excretion pattern between the volunteers. Each line represents one volunteer.

Paper II

Repeated whole-body applications of a sunscreen containing 4% BZ-3 resulted in a higher excretion of BZ-3 in urine. The mean amount was 3.7% (range 1.2-8.7%) of the total amount applied BZ-3. There was no significant difference between groups A and B ($p < 0.99$). Figure 11 shows the individual urinary excretion of BZ-3 (%).

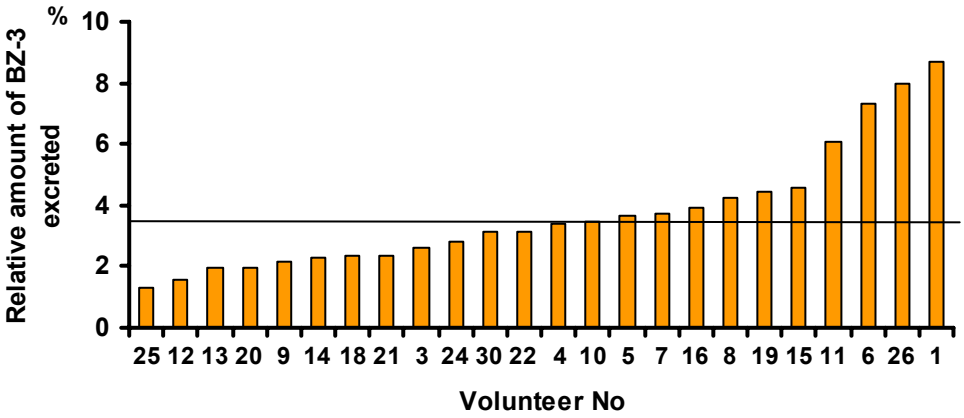


Figure 11 Urinary excretion of BZ-3 after 10 days. Range 1.2%-8.7% of the total amount applied. The mean value of 3.7% is shown as a horizontal line.

Paper III

The detection limits for BZ-3 and DHB were 0.01 µmol/l (0.1 ng) and 0.16 µmol/l (2 ng) respectively. RSD was less than 10% for BZ-3 and less than 13% for DHB. The assay was linear r^2 >0.99.

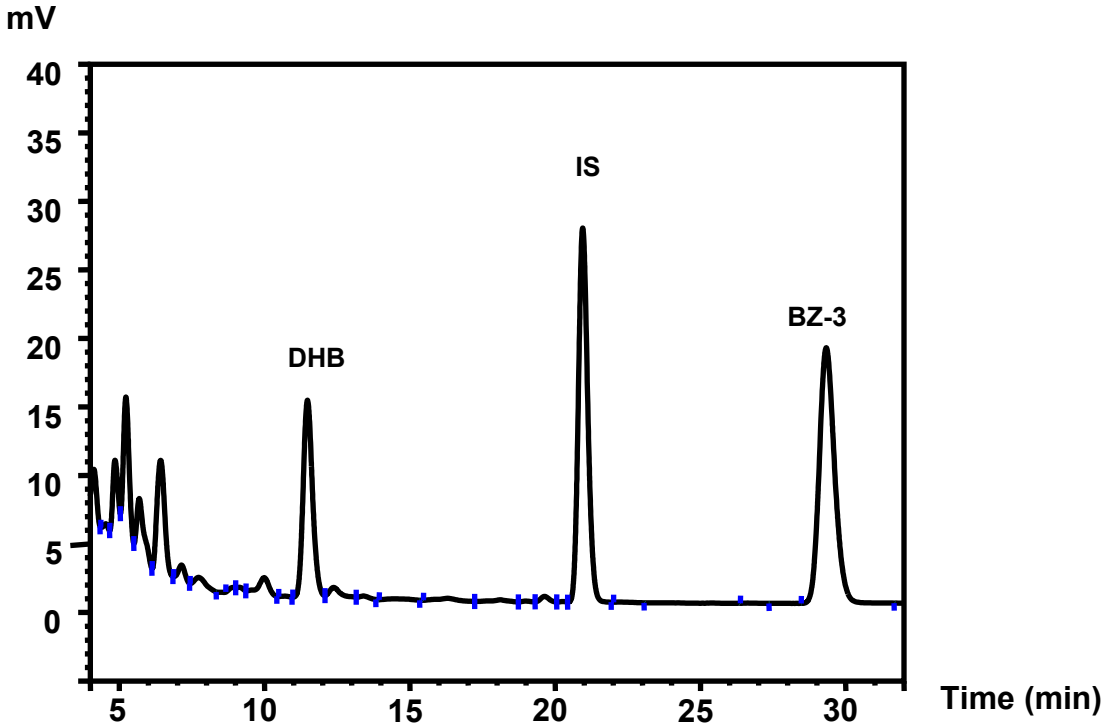


Figure 12 Example of a chromatogram from person 8. Internal standard (IS).

BZ-3 and DHB were extensively conjugated and only a smaller part was excreted in the non-conjugated form, mean value 5.9% and 8.8% respectively (Figure 13).

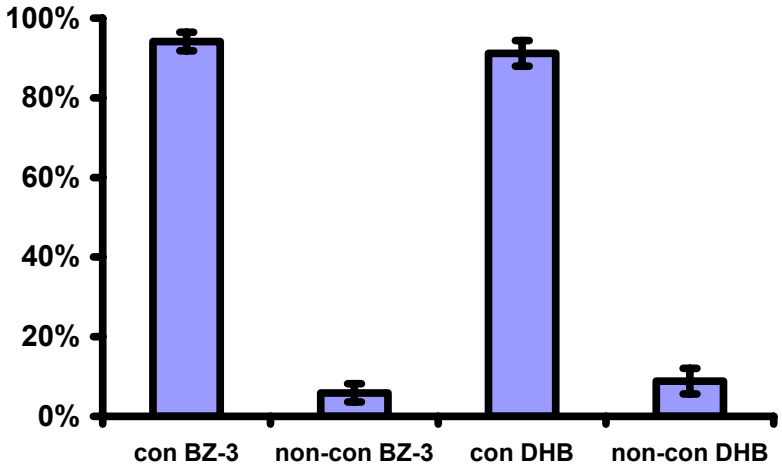


Figure 13 The relations between conjugated/non-conjugated BZ-3 and conjugated/non-conjugated DHB.

The excretion pattern varied among the human volunteers; we discerned different patterns among the individuals (Figure 14).

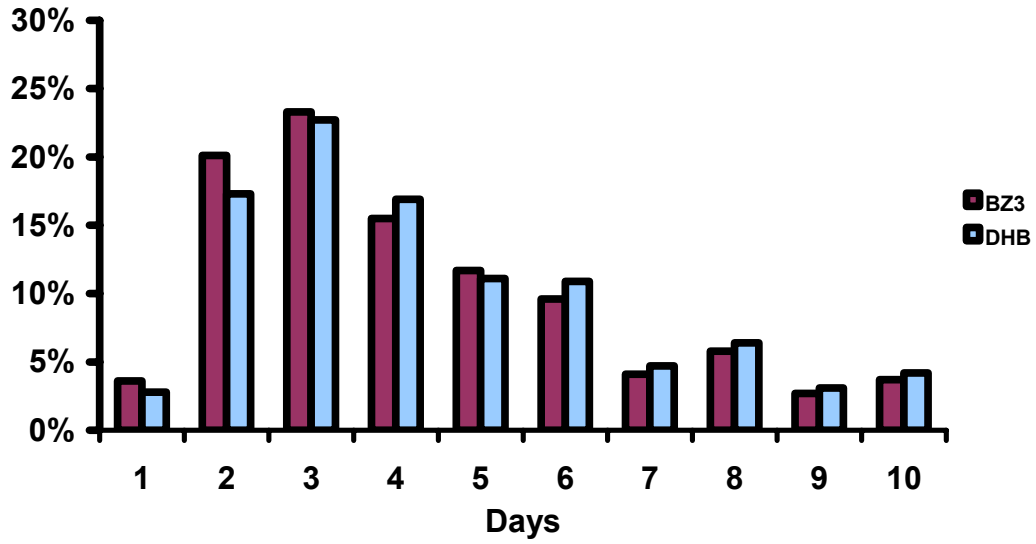


Figure 14 Example of excretion pattern of conjugated BZ-3 and DHB.

Paper IV

Three sunscreens were photounstable after exposure to natural UV, in the UVA range the AUCI was between 0.36 and 0.76. In the UVB range one of these sunscreens showed an AUCI of 0.63. Three sunscreens were photostable after natural UV irradiation, with AUCI >0.80. Five of the sunscreens were photostable in the UVB region after artificial UV exposure. The combination of EHMC and BMDBM was always unstable regardless of which other photoactive compound was included. Table 9 shows an overview of the AUCI of the investigated sunscreens, and Figure 15 shows UV absorbance spectra for Sunscreen 1.

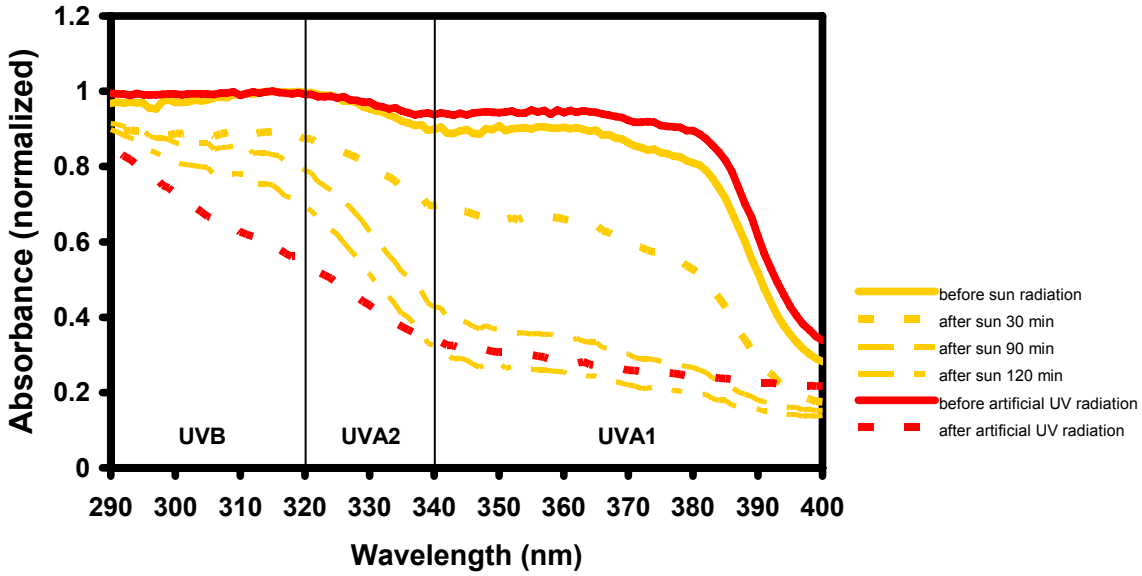


Figure 15 UV absorbance spectra of UVA **photounstable** Sunscreen1 (AUCI <0.80). Before and after natural UV exposure, and before and after artificial UV exposure.

Table 9 An overview of the AUCI of the investigated sunscreens.

Sunscreen	After natural UV exposure												After artificial UV exposure			
	UVA			UVA1			UVA2			UVB			UVA	UVA1	UVA2	UVB
	30 min	90 min	120 min	30 min	90 min	120 min	30 min	90 min	120 min	30min	90 min	120 min				
1	**0.72**	**0.46**	**0.36**	**0.69**	**0.38**	**0.29**	0.83	**0.65**	**0.54**	0.91	0.87	0.81	**0.36**	**0.32**	**0.45**	**0.69**
2	0.84	**0.76**	**0.75**	0.83	**0.69**	**0.67**	0.86	0.92	0.97	0.86	0.92	0.97	**0.63**	**0.53**	0.88	0.89
3	**0.67**	**0.41**	**0.41**	**0.59**	**0.30**	**0.34**	0.81	**0.58**	**0.52**	0.92	**0.75**	**0.63**	**0.40**	**0.31**	**0.58**	**0.73**
4	0.92	0.86	0.87	0.91	0.85	0.83	0.94	0.91	0.91	0.95	0.93	0.95	**0.72**	**0.69**	0.81	0.83
5	0.96	0.89	0.85	0.94	0.87	0.83	0.99	0.95	0.93	0.99	0.99	0.98	0.90	0.88	0.97	0.97
6	0.98	0.94	0.94	0.97	0.93	0.93	0.98	0.96	0.97	0.99	0.99	1.00	0.85	0.82	0.92	1.00
7			0.99*			1.00*			0.96*			0.92*	0.99	0.99	1.00	0.99

The AUCI is defined as AUC_{after}/AUC_{before}. The bold numbers show when AUCI is <0.80.

* Sunscreen 7 was exposed to natural UV during 240 min.

DISCUSSION

Methodological considerations

Papers I and **II**

There was no control that the volunteers applied all of the sunscreen they received. Follow-up questions were asked when the urine samples were delivered, to learn whether there had been any difficulties. The volunteers had to measure the volume of urine every time they urinated. Two persons in Paper II forgot to do this on one or two occasions. Since that would lead to an underestimation of excreted BZ-3, they were also included. One person in Paper II was excluded due to obstacles in following the instructions.

In Paper II, half of the volunteers were exposed to UV. For practical reasons this was done at lunchtime but the applications were done morning and night. The general recommendation is to apply the sunscreen 15 to 20 min prior to UV exposure. The delay between application and UV exposure may have influenced the results.

Paper III

For the standard solutions, methanol/ultrafiltrated water was used instead of urine. We tried to do the standard solutions in urine but the BZ-3 precipitated in the high concentrations. THB had unstable properties and it was therefore not analyzed. A previous study has shown that THB was was found only in trace amounts in human urine [81].

Paper IV

The absorbance was too high for proper measurements when the recommended amount of 2 mg/cm^2 was applied, causing distortion in the absorption spectrum. For this reason a thinner layer was applied.
The number of silica plates was limited, so we were not able to expose all the sunscreens to natural sunlight at the same time. Hence, the sunscreens were exposed to different amounts of natural UV.

The spectrophotometer did not have an integrated sphere, but since we are interested in changes in the absorbance, the absence of an integrating sphere is not a major problem.

General discussion

Many of us encounter sunscreens every day. They are not only incorporated in products specifically designed to protect against UVR, but also contained inproducts such as day-creams, lipsticks and foundations.
Percutaneous absorption of different sunscreen compounds is important to investigate in order to aquire more knowledge about how much of a compound is absorbed. It is also essential to remember that the metabolites of a product can have properties different from the parent compound. When a new drug is tested on the market this is taken into consideration, but when it comes to sunscreens this is not the case.

Several studies have reported that BZ-3 is absorbed by the skin [76, 78, 79, 81]. Our data (Paper I) show that, after one whole-body application of a sunscreen containing 4% BZ-3, BZ-3 can be found in human urine up to 48 hours after application. The previous studies about BZ-3 and metabolism have been performed on rats, mice or cell cultures [61, 115, 116]. Since we did not know for how long BZ-3 was going to be excreted in the urine, we chose a 48-hour period which had been used in a previous study [78]. Four of the volunteers did not reach their reference value at the end of the study; they still excreted BZ-3 48 hours after the application.

In our next study (Paper II) we investigated the absorption of BZ-3 after repeated whole-body applications of a sunscreen containing 4% BZ-3. This time, the urine collection was performed during the five days the sunscreen was applied with an additional five days after the last application. The time of investigation was more than twice as long as in Paper I. Now we saw that none of the participants reached their reference value at the end of the study. We did not see any statistically significant influence by UV exposure. This may be due to factors such as time of irradiation, mentioned previously. BZ-3 is quite photostable as indicated in Paper IV, this can also be part of the explanation why there was no difference between the groups.

Most of the BZ-3 excreted in the urine was in the conjugated form; this is consistent with previous studies showing that BZ-3 undergoes extensive conjugation in the body [78, 115]. Strassburg *et al.* have studied the development aspects of human hepatic drug glucuronidation in young children and adults. Their findings showed that the hepatic glucuronidation activity in children under the age of 24 months was lower than for adults for several drugs. Even though BZ-3 was not included in their study, BZ-3 undergoes extensive conjugation and these findings may be relevant for BZ-3 as well. The development of hepatic glucuronidation enzymes is significant for the prevention of adverse drug effects [117]. Okereke *et al.* found BZ-3 in liver, spleen, heart and testes in rats after oral administration of BZ-3 [115]. There were large individual differences in excretion of BZ-3 among the volunteers, and this may be due to differences in enzyme activity. There is also enzyme activity in the skin, and both esterases and conjugation enzymes are present in the skin [118].

During the time of the second study we encountered many technical obstacles when we were analyzing BZ-3. Several studies describe methods about HPLC and BZ-3 but none of them fitted our purposes exactly. Sometimes the method did not work as described in the articles, or the method was not described in enough detail to enable us to reproduce it; for these reasons we developed our own method (Paper III). Many of the previously described studies are developed for product evaluation and not for in-vivo analysis. In some methods the biological samples were spiked with BZ-3; hence no biological influence such as metabolism etc. needed to be taken into consideration [111].
Our study had detection limits of 0.01 µmol/l and 0.16 µmol/l for BZ-3 and DHB respectively. These are at the same (or lower) levels as in other methods (Table 6), and the detection limits were sufficient to analyze the samples on day 10. We have described the method in detail to facilitate its reproduction by other researchers. The method was applied to measure BZ-3 and DHB and to study the excretion pattern. DHB was also extensively conjugated and excreted in the urine.

BZ-3 and DHB were always excreted in a similar pattern but the excretion pattern differed among the individuals. This may be due to different enzyme activity.

There are few studies about metabolism of BZ-3 in humans. Hopefully, our work has made a small contribution to the research field. In rat, BZ-3 is metabolized to DHB, DHMB and THB. Urine seems to be the major metabolic pathway and DHB and THB the major metabolites in urine. DHMB was found solely in trace amounts. Only a smaller part of BZ-3 was excreted in faeces, and in faeces DHMB was the major metabolite [115, 119]. Okereke *et al*. also found differences between rat and mouse after oral administration of BZ-3. Tissue studies showed the highest amount of BZ-3 in the liver for both rat and mouse, but in mice the elimination was split between urine and faeces with THB as the major metabolite. This may be due to enzyme differences between the species [116].
Toxicity studies have not shown any mutagenicity in *Salmonella typhimurium* strains for BZ-3 or DHB [120]. LD50 for rat was 7,400 mg/kg after oral administration of BZ-3 [45]. For THB and DHMB no records of toxicity were available at the HSDB Hazardous Substances Data Bank [121].

Shall we forbid products with BZ-3? I do not think so; after all, sunscreens are supposed to be used as a complement to other forms of sun protection, such as clothes and shade. BZ-3 is a relatively photostable compound and it gives good protection in the UVA region, so it has its place in the sunscreen market. Hayden *et al*. showed no keratinocyte toxicity after incubation of human keratinocytes and BZ-3 in a normal dose [122]. However, it is prudent not to use it for children under the age of 24 months, for the reasons mentioned earlier.

Photostability has been an issue for sunscreens for many years. It is well-known that some photoactive compounds and combinations of photoactive compounds are photounstable. There are mainly four reactions that can occur: photoaddition/substitution, cycloaddition, isomerization and photofragmentation. After the excited molecule has absorbed the UV, it can return to its ground state or produce isomers or new photoproducts. Evidently the former is preferred [123]. Schwack and Rudolph showed in 1995 that BMDBM is photounstable and about a dozen photoproducts were identified [124]. Bonda *et al*. showed 1999 that the combination of EHMC and BMDBM is photounstable, which other studies also show [125, 126].

BMDBM is a very popular compound since it is a UVA absorber. It was included in six of our seven investigated sunscreens (Table 8, p.25). A previous study has shown that photoactive ingredients in petrolatum are photounstable [127]. In commercially available products stabilizing agents may be added to the vehicle, and some combinations of photoactive compounds may be more stable than others.
In our study (Paper IV) we could clearly see that this was not always the case. The combination of EHMC and BMDBM was used in three products. FDA does not recommend this combination [128] and it has been known for several years that this combination is photounstable, regardless of which other photoactive compounds are included in addition. Irradiation of EHMC and BMDBM leads to photocycloaddition creating cinnamate dimers and cyclobutylketone photoadducts which fragmented into new compounds [129].

Green *et al*. showed that regular use of a sunscreen, containing EHMC and BMDBM, gave protection against AK and SCC, but they did not find any protective effect on BCC or CMM. This may be due to the fact that the combination is photounstable and if the study had been done with a photostable product, the result might have differed [51].

There is no standard method to measure photostability, and several methods are currently in use, both in-vivo and in-vitro methods [130-133]. Most of them use a spectrophotometer, but there are also studies with HPLC [134] or combinations with HPLC and spectrophotometry [135]. Few studies have been done using natural sunlight [133]. The photostability of a sunscreen is rarely presented on the final product.

Our proposed method, the AUCI, can be a helpful tool in measuring the photostability. We chose to set the limit for photostability at AUCI >0.80 but this can of course be modified depending on the set-up.

There are already many excellent commercially available sunscreens, and much research is being done to enhance photostability [136-139]. Many patent applications about enhancement of photostability have been published. TiO_2 may also work as a stabilizer for photounstable drugs such as ketoprofen [140].

In the future I hope it will be easier for the consumer to choose a sunscreen by also adding photostability testing on the final product. As mentioned before, sunscreens are a complement to clothes and shade in order to decrease our exposure to UVR.

CONCLUSIONS

BZ-3 is absorbed by the skin and excreted in the urine after one topical application of a sunscreen. There are individual differences in the amount excreted and in the excretion pattern. (Paper I)

Repeated topical applications of a sunscreen containing BZ-3 lead to a higher excretion of BZ-3. There was no statistical difference after exposure to UVR
As in Paper I there are large individual differences. (Paper II)

The developed HPLC method was reliable and suitable for handling a large number of samples. Three different excretion patterns were discerned among the volunteers. BZ-3 and DHB were excreted in a similar pattern in each volunteer. (Paper III)

Three of the seven investigated sunscreens were photounstable in the UVA region. The combination of EHMC and BMDBM was unstable regardless of which other photoactive compounds were included. Our proposed method, the AUCI, can be a helpful tool in measuring the photostability. (Paper IV)

FUTURE PROSPECTS

Few studies have been done about the metabolism of BZ-3 in humans. Both skin metabolism and genotyping would be interesting fields to further investigate.

Several non-invasive methods e.g. multi photon microscopy, exist to study the skin structure, and some of these methods can also be used to investigate different compounds through the skin.

Some photoactive compounds, e.g. BMDBM form photoproducts after irradiation. There are few studies about the impact of these products on humans. Many interesting studies are waiting to be done in this area. Can the photoproducts cause photoallergic reactions or even be able to damage the DNA?

What will be the new generation of sunscreens? Cyanobacteria might help us to invent new products.

Organ transplant recipients are a group especially vulnerable to skin cancer, because of their immunosuppressive therapy. In the future I hope we will develop programmes to protect them better, and effective sunscreens may be a part of such a programme.

ACKNOWLEDGEMENTS

There are many people who have made this thesis possible. I would like to express my sincere gratitude to all of you. In particular I thank the following:

My supervisor *Ann-Marie Wennberg*, for your willingness to share your knowledge, excellent research skills and support, both in my research and in my clinical practice. In addition, your great sense of humour enlightens the work atmosphere.
Olle Larkö, my assisting supervisor for leading me into the field of research. Even though you are a very busy person, you have always had time when I needed it. It has been good to know that your door always is open in those moments.
My co-worker and supervisor at the HPLC lab *Anne Farbrot*, for helping me tremendously when my chemistry knowledge has been insufficient, for making HPLC interesting, and in the end even fun. I would never have thought that when I started. Thank you also for being more than just a great co-worker, but also a good friend.

Ing-Marie Bergbrant for the opportunity to do research.

I would like to thank my co-authors *Carl-Eric Jacobson*, *Asta Juzeniene*, *Johan Moan* and *Arne Rosén* for good collaboration. Thank you *Nils Tarras-Wahlberg*, for patiently explaining things over and over again when I needed it.

Lena Berntsson, *Ellinor Mattsson* and *Agneta Söderberg* who have performed the laboratory work with such excellence.

There have been many times when the HPLC system did not work as we wanted. I wish to thank *Christian Hüls*, *Göran Oresten* and *Gunnar Stenhagen* for providing invaluable guidance during those times. *Mats Ohlson* has not only contributed with advice about HPLC, but has also provided the chemical structures in this thesis.

The whole staff at the HPLC lab, Sahlgrenska University Hospital, for welcoming me warmly into your circle. We have shared many good moments during coffee breaks.

The staff at the Department of Dermatology at Sahlgrenska University Hospital, especially *Barbro Pettersson* who has been very helpful with the UV lamps.

The volunteers who patiently endured the trials.

All members in our Research group for interesting Monday meetings and fruitful discussions.

Inger Forsell for excellent administrative and secretarial help. Thank you for keeping track of all details.

Morgan Carlsson, *Marica Ericson* and *Martin Gillstedt* for skilful help with photos and technical issues.

All wonderful colleagues and friends at the Department of Dermatology and Venereology for making a pleasant atmosphere at work.

I am most grateful to all my dear *friends* and *relatives*, thank you for being there through good as well as bad times. Especially I would like to mention *Charlotte Björklund* and *Jenny Axelsson* for the many good laughs and enjoyable times we have shared.

Martin Bergendahl for helping me with my physics in senior high school, without your help I would never been able to attend medical school.

Our pets,Twinkie and Cupcake, also deserves to be mentioned. To see your little faces when I come home at night makes me very happy.

Finally I would like to thank my wonderful, supporting family.
Mina kära föräldrar *Ulla* och *Arne Gustavsson* som alltid stöttar och tror på mig, även när mina mål inte är solklara. Tack för att ni ALLTID ställer upp vad det än gäller, barnvakt, skjutsning, tvätt, städning……listan kan göras lång. Bättre föräldrar kan man inte önska sig.
My husband *Michael Gonzalez* for your extreme patience, even in times when I have not deserved it. Thank you for your support, love and care. I am grateful to have you in my life.
Philip, my adorable, intense and supporting son who makes me smile every day, and who reminds me who the real boss is.

The project has been financially supported by the Welander Foundation, the Swedish Research Council, local university funds (ALF, LUA) and the Bergendahl Foundation, for which I am very grateful.

REFERENCES

1. **http://www.NE.se**; 2006.
2. Junqueira LC, Carneiro J, Kelley RO (eds.): **Basic histology**, seventh edn: Appleton & Lange; 1992.
3. **http://www.aad.org/professionals/Residents/MedStudCoreCurr/DCStratumCorneum.htm**
4. **http://en.wikipedia.org/wiki/Image:Skin.jpg**.
5. **http://missinglink.ucsf.edu/lm/DermatologyGlossary/normal_skin.html**; 2006.
6. Fitzpatrick TB: **The validity and practicality of sun-reactive skin types I through VI**. *Arch Dermatol* 1988, **124**(6):869-871.
7. MacKie RM: **Effects on ultraviolet radiation on human health**. *Radiat Prot Dosimetry* 2000, **91**(1):15-18.
8. **http://imagine.gsfc.nasa.gov/docs/science/know_l1/emspectrum.html**
9. **Encyclopædia Britannica Online**
10. Lyngbye J: **Niels Finsen och hans team på Finseninstituttet**. Copenhagen: Nordisk Forlag A/S; 2003.
11. **http://nobelprize.org/nobel_prizes/medicine/laureates/1903/**
12. CIE: **International Lighting Vocabulary. 17.4**; 1987.
13. Diffey BL: **Sources and measurement of ultraviolet radiation**. *Methods* 2002, **28**(1):4-13.
14. CIE: **Standard: Erythema reference action spectrum and standard erythema dose. CIE S007/E-1998**.
15. WHO: **Global solar UV Index, WHO/SDE/OEH/02.2** ; 2002.
16. **http://www.smhi.se**; 2006.
17. Lucas R, McMichael T, Smith W, Armstrong B: **Solar Ultraviolet Radiation, Global burden of disease from solar ultraviolet radiation** *Environmental Burden of Disease Series.* Edited by Prüss-Üstün A, Zeeb H, C. M, Repacholi M. Geneva: WHO; 2006.
18. Findlay GM: **Ultra-violet light and skin cancer**. *The Lancet* 1928, **2**:1070-1073.
19. Ziegler A, Jonason AS, Leffel DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, Brash DE: **Sunburn and p53 in the onset of skin cancer**. *Nature* 1994, **372**(6508):730-731.
20. Burns T, Breathnach S, Cox N, Griffiths C (eds.): **Rook´s Textbook of Dermatolgy**: Blackwell Science; 2004.
21. **Basalcellscancer**. The national board of health and welfare; 2006.
22. **Cancer Incidence in Sweden 2004.** The national board of health and welfare; 2006.
23. Brash DE, Ziegler A, Jonason AS, Simon JA, Kunala S, Leffel DJ: **Sunlight and sunburn in human skin cancer: p53, apoptosis, and tumor promotion**. *J Invest Dermatol Symp Proc* 1996, **1**(2):136-142.
24. Wang L-E, Xiong P, Strom SS, Goldberg LH, Lee JE, Ross MI, Mansfield PF, Gershenwald JE, Prieto VG, Cormier JN *et al*: **In vitro sensitivity to ultraviolet B light and skin cancer risk: A case-control Analysis**. *J Natl Cancer Inst* 2005, **97**(24):1822-1831.
25. Lacour JP: **Carcinogenesis of basal cell carcinomas: genetcis and molecular mechanisms**. *Br J Dermatol* 2002, **146**(Suppl 61):17-19.

26. Ortonne JP: **Photobiology and genetics of malignant melanom**. *Br J Dermatol* 2002, **146**(Suppl 61):11-16.
27. Setlow RB, Grist E, Thompson K, D. WA: **Wavelengths effective in induction of malignant melanoma**. *Proc Natl Acad Sci USA* 1993, **90**(14):6666-6670.
28. Agar NS, Halliday GM, Barnetson R, Ananthaswamy HN, Wheeler M, Jones AM: **The basal layer in human squamous tumors harbors more UVA than UVB fingerprint mutations: A role for UVA in human skin carcinogenesis**. *PNAS* 2004, **101**(14):4954-4959.
29. Moan J, Dahlbäck A, Setlow RB: **Epidemiological support for an hypothesis for melanoma induction indicating a role for UVA radiation**. *Photochem Photobiol* 1999, **70**(2):243-247.
30. De Fabo EC, Noonan FB, Fears T, Merlino G: **Ultraviolet B but not ultraviolet A initiates melanoma**. *Cancer Res* 2004, **64**(18):6372-6376.
31. Holubar K, Schmidt C: **Historical, anthropological, and biological aspects of sun and the skin**. *Clin Dermatol* 1998, **16**:19-22.
32. Wolf R, Wolf D, Morganti P, Ruocco V: **Sunscreens**. *Clin Dermatol* 2001, **19**(4):452-459.
33. Urbach F: **The historical aspects of sunscreens**. *J Photochem Photobiol B* 2001, **64**(2-3):99-104.
34. Phillips SJ: **The role of federal programs in the transfer of biomedical technology**. *Artif Organs* 2004, **28**(5):432-436.
35. Luckiesh M, Taylor AH, Cole HN, Sollman T: **Protective skin coatings for the prevention of sunburn**. *JAMA* 1946, **130**(1).
36. MacEachern WN, Jillson OF: **A Practical Sunscreen-"Red Vet Pet"**. *Arch Dermatol* 1964, **89**:147-150.
37. Rothman SH, B.A.: **The sunburn-protecting effect of para-aminobenzoic acid**. *J Invest Dermatol* 1947, **9**:317-313.
38. Mackie BS, Mackie LE: **The PABA story**. *Australas J Dermatol* 1999, **40**(1):51-53.
39. Dromgoole SH, Maibach HI: **Sunscreening agent intolerance: contact and photocontact sensitization and contact urticaria**. *J Am Acad Dermatol* 1990, **22**(6 Pt 1):1068-1078.
40. Berne B, Ros AM: **7 years experience of photopatch testing with sunscreen allergens in Sweden**. *Contact Dermatitis* 1998, **38**(2):61-64.
41. Schlumpf M, Cotton B, Conscience M, Haller V, Steinmann B, Lichtensteiger W: **In vitro and in vivo estrogenicity of UV sunscreens**. *Environ Health Perspectives* 2001, **109**:239-244.
42. Lowe NJ, Shaath NA, Pathak MA (eds.): **Sunscreens, development, evaluation, and regulatory aspects**. New York: Marcel Dekker; 1997.
43. Vainio H, Bianchini F (eds.): **Sunscreens**. Lyon: International Agency for Research on Cancer; 2001.
44. **http://www.kemi.se**; 2006.
45. **http://chem.sis.nlm.nih.gov/chemidplus**; 2006.
46. Westerdahl J, Olsson H, Masback A, Ingvar C, Jonsson N: **Is the use of sunscreens a risk factor for malignant melanoma?** *Melanoma Res* 1995, **5**(1):59-65.
47. Westerdahl J, Ingvar C, Masback A, Olsson H: **Sunscreen use and malignant melanoma**. *Int J Cancer* 2000, **87**(1):145-150.

48. Naldi L, Gallus S, Imberti GL, Cainelli T, Negri E, La Vecchia C: **Sunscreens and cutaneous malignant melanoma: an Italian case-control study**. *Int J Cancer* 2000, **86**(6):879-882.
49. Huncharek M, Kupelnick B: **Use of topical sunscreens and the risk of malignant melanoma: a meta-analysis of 9067 patients from 11 case-control studies**. *Am J Public Health* 2002, **92**(7):1173-1177.
50. Dennis LK, Beane Freeman LE, VanBeek MJ: **Sunscreen use and the risk for melanoma: a quantitative review**. *Ann Intern Med* 2003, **139**(12):966-978.
51. Green A, Williams G, Neale R, Hart V, Leslie D, Parsons P, Marks GC, Gaffney P, Battistutta D, Frost C *et al*: **Daily sunscreen application and betacarotene supplementation in prevention of basal-cell and squamous-cell carcinomas of the skin: a randomised controlled trial**. *Lancet* 1999, **354**(9180):723-729.
52. Darlington S, Williams G, Neale R, Frost C, Green A: **A randomized controlled trial to assess sunscreen application and beta carotene supplementation in the prevention of solar keratoses**. *Arch Dermatol* 2003, **139**(4):451-455.
53. Gasparro FP, Mitchnick M, Nash JF: **A review of sunscreen safety and efficacy**. *Photochem Photobiol* 1998, **68**:243-256.
54. Stenberg C, Larko O: **Sunscreen application and its importance for the sun protection factor**. *Arch Dermatol* 1985, **121**(11):1400-1402.
55. Bech-Thomsen N, Wulf HC: **Sunbathers' application of sunscreen is probably inadequate to obtain the sun protection factor assigned to the preparation**. *Photodermatol Photoimmunol Photomed* 1992, **9**(6):242-244.
56. Azurdia RM, Pagliaro JA, Diffey BL, Rhodes LE: **Sunscreen application by photosensitive patients is inadequate for protection**. *Br J Dermatol* 1999, **140**(2):255-258.
57. Azurdia RM, Pagliaro JA, Rhodes LE: **Sunscreen application technique in photosensitive patients: a quantitative assessment of the effect of education**. *Photodermatol Photoimmunol Photomed* 2000, **16**(2):53-56.
58. Hext PM, Tomenson JA, Thompson P: **Titanium dioxide: inhalation toxicology and epidemiology**. *Ann Occup Hyg* 2005, **49**(6):461-472.
59. Boffetta P, Soutar A, Cherrie JW, Granath F, Andersen A, Anttila A, Blettner A, Gaborieau V, Klug SJ, Langard S *et al*: **Mortality among workers employed in the titanium dioxide production industry in Europe**. *Cancer Causes Control* 2004, **15**(7):697-706.
60. Schlumpf M, Jarry H, Wuttke W, Ma R, Lichtensteiger W: **Estrogenic activity and estrogen receptor β binding of the UV filter 3-benzylidene camphor Comparison with 4-methylbenzylidene camphor**. *Toxicology* 2004, **199**:109-120.
61. Nakagawa Y, Suzuki T: **Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells**. *Chem Biol Interact* 2002, **139**:115-128.

62. Janjua NR, Mogensen B, Andersson AM, Petersen JH, Henriksen M, Skakkebaek NE, Wulf HC: **Systemic absorption of the sunscreens benzophenone-3, octyl-methoxycinnamate, and 3-(4-methyl-benzylidene) camphor after whole-body topical application and reproductive hormone levels in humans**. *J Invest Dermatol* 2004, **123**(1):57-61.
63. SCCNFP: **Opinion on the evaluation of potentially estrogenic effects of UV-filters adopted by the SCCNFP during the 17th plenary meetin**. In: *Scientific Committee for Cosmetic Products, and non-food products intended for consumers: 12 June 2001*; 2001.
64. Hund-Rinke K, Simon M: **Ecotoxic effect on photocatalytic active nanoparticles (TiO_2) on algae and daphnids**. *Environ Sci Pollut Res Int* 2006, **13**(4):225-232.
65. Buser H-R, Balmer ME, Schmid P, Kohler M: **Occurence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various Swiss rivers with inputs from wastewater treatment plants**. *Environ Sci technol* 2006, **40**:1427-1431.
66. Poiger T, Buser H-R, Balmer ME, Bergqvist P-A, Müller MD: **Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes**. *Chemosphere* 2004, **55**:951-963.
67. Pustisek N, Lipozencic J, Ljubojevic S: **A review of sunscreens and their adverse reactions**. *Acta Dermatovenerol Croat* 2005, **13**(1):28-35.
68. Zhang XM, Nakagawa M, Kawai K: **Erythema-multiforme-like eruption following photoallergic contact dermatitis from oxybenzone**. *Contact Dermatitis* 1998, **38**(1):43-44.
69. Yesudian PD, King CM: **Severe contact urticaria and anaphylaxis from benzophenone-3 (2-hydroxy 4-methoxy benzophenone)**. *Contact Dermatitis* 2002, **46**(1):55-56.
70. Bourrain JL, Amblard P, Beani JC: **Contact urticaria photoinduced by benzophenones**. *Contact Dermatitis* 2003, **48**(1):45-46.
71. Rayner W: **Cyanosis in newly born children caused by aniline marking ink**. *Br Med J* 1886, **I**:294.
72. Powell H, Swarner O, Gluck L, Lampert P: **Hexachlorophene myelinopathy in premature infants**. *J Pediatr* 1973, **82**(6):976-981.
73. Martin-Bouyer G, Lebreton R, Toga M, Stolley PD, Lockhart J: **Outbreak of accidental hexachlorophene poisoning in France**. *Lancet* 1982, **1**(8263):91-95.
74. Lindsey CP: **Two cases of fatal salicylate poisoning after topical application of an antifungal solution**. *Med J Aust* 1968, **1**(9):353-354.
75. Aspinall JB, Goel KM: **Salicylate poisoning in dermatological therapy**. *Br Med J* 1978, **2**(6148):1373.
76. Feldmann RJ, Maibach HI: **Absorption of some organic compounds through the skin in man**. *J Invest Dermatol* 1970, **54**(5):399-404.
77. Nash JF: **Human safety and efficacy of ultraviolet filters and sunscreen products**. *Dermatol Clin* 2006, **24**(1):35-51.
78. Hayden CG, Roberts MS, Benson HA: **Systemic absorption of sunscreen after topical application**. *Lancet* 1997, **350**(9081):863-864.
79. Jiang R, Roberts MS, Collins DM, Benson HA: **Absorption of sunscreens across human skin: an evaluation of commercial products for children and adults**. *Br J Clin Pharmacol* 1999, **48**(4):635-637.

80. Gustavsson Gonzalez H, Farbrot A, Larkö O: **Percutaneous absorption of benzophenone-3, a common component of topical sunscreens**. *Clin Exp Dermatol* 2002, **27**:691-694.
81. Sarveiya V, Risk S, Benson HA: **Liquid chromatographic assay for common sunscreen agents: application to in vivo assessment of skin penetration and systemic absorption in human volunteers**. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004, **803**(2):225-231.
82. Gonzalez H, Farbrot A, Larko O, Wennberg AM: **Percutaneous absorption of the sunscreen benzophenone-3 after repeated whole-body applications, with and without ultraviolet irradiation**. *Br J Dermatol* 2006, **154**(2):337-340.
83. Benson HA: **Assessment and clinical implications of absorption of sunscreens across skin**. *Am J Clin Dermatol* 2000, **1**(4):217-224.
84. Wester RC, Maibach HI: **In vivo methods for percutaneous absorption measurement** *Topical absorption of Dermatological products.* Edited by Bronaugh RL, Maibach HI. New York: Marcel Dekker; 2002: 145-156.
85. Bronaugh RL, Hood HL, Kraeling MEK, Yourick JJ: **Determination of percutaneous absorption by in vitro techniques** *Topical absorption of dermatological products.* Edited by Bronaugh RL, Maibach HI. New York: Marcel Dekker; 2002: 157-161.
86. Blum HF, Eicher M, Terus WS: **Evaluation of protective measures against sunburn**. *Am J Physiol* 1945, **146**(1):118-125.
87. CTFA-SA, COLIPA, JCIA: **International sun protection factor (SPF) test method**. 2003.
88. Cole C, VanFossen R: **Measurement of sunscreen UVA protection: an unsensitized human model**. *J Am Acad Dermatol* 1992, **26**(2 Pt 1):178-184.
89. Moyal D, Chardon A, Kollias N: **Determination of UVA protection factors using the persistent pigment darkening (PPD) as the end point. (Part 1). Calibration of the method**. *Photodermatol Photoimmunol Photomed* 2000, **16**(6):245-249.
90. Cole C: **Sunscreen protection in the ultraviolet A region: how to measure the effectiveness**. *Photodermatol Photoimmunol Photomed* 2001, **17**(1):2-10.
91. Nash JF, Tanner PR, Matts PJ: **Ultraviolet A radiation: testing and labeling for sunscreen products**. *Dermatol Clin* 2006, **24**(1):63-74.
92. Gange RW, Soparkar A, Matzinger E, Dromgoole SH, Sefton J, DeGryse R: **Efficacy of a sunscreen containing butyl methoxydibenzoylmethane against ultraviolet A radiation in photosensitized subjects**. *J Am Acad Dermatol* 1986, **15**(3):494-499.
93. Kaidbey KH, Barnes A: **Determination of UVA protection factors by means of immediate pigment darkening in normal skin**. *J Am Acad Dermatol* 1991, **25**(2 Pt 1):262-266.
94. Diffey BL, Tanner PR, Matts PJ, Nash JF: **In vitro assessment of the broad-spectrum ultraviolet protection of sunscreen products**. *J Am Acad Dermatol* 2000, **43**(6):1024-1035.
95. Diffey BL: **A method for broad spectrum classifications of sunscreens** *: Int J Cosmet Sci 1994,* **16**:47-52.96.
96. AS/NZS: **Australian/New Zealand Standard**. 1998.
97. **http://www.aad.org/aad/Newsroom/factsunscreen.htm**; 2006.
98. Morison WL: **Photoprotection by clothing**. *Dermatol Ther* 2003, **16**(1):16-22.

99. Berne B, Fischer T: **Protective effects of various types of clothes against UV radiation**. *Acta Derm Venereol* 1980, **60**(5):459-460.
100. Robson J, Diffey BL: **Textiles and sun protection**. *Photodermatol Photoimmunol Photomed* 1990, **7**(1):32-34.
101. Gies HP, Roy CR, McLennan A, Diffey BL, Pailthorpe M, Driscoll C, Whillock M, McKinlay AF, Grainger K, Clark I *et al*: **UV protection by clothing: an intercomparison of measurements and methods**. *Health Phys* 1997, **73**(3):456-464.
102. Björn LO, Widell S, Wang T: **Evolution of UV-B regulation and protection in plants**. *Adv Space Res* 2002, **30**(6):1557-1562.
103. Saikawa Y, Hashimoto K, Nakata M, Yoshihara M, Nagai K, Ida M, Komiya T: **Pigment chemistry: the red sweat of the hippopotamus**. *Nature* 2004, **429**(6990):363.
104. **http://nobelprize.org/nobel_prizes/chemistry/laureates/1952/**
105. Kazakevich Y, McNair H: **http://hplc.chem.shu.edu/NEW/HPLC_Book/index.html**; 1996.
106. Vanquerp V, Rodriguez C, Coiffard C, Coiffard LJ, De Roeck-Holtzhauer Y: **High-performance liquid chromatographic method for the comparison of the photostability of five sunscreen agents**. *J Chromatogr A* 1999, **832**(1-2):273-277.
107. Potard G, Laugel C, Baillet A, Schaefer H, Marty J-P: **Quantitative HPLC analysis of sunscreens and caffeine during in vitro percutaneous penetration studies**. *Int J Pharm* 1999, **189**(2):249-260.
108. Shih Y, Cheng F-C: **Determination of sunscreen agents in cosmetic products using microwave-assisted extraction and liquid chromatography**. *J Chromatogr A* 2000, **876**:243-246.
109. Chisvert A, Pascual-Marti MC, Salvador A: **Determination of UV filters worldwide authorized in sunscreens by high-performance liquid chromatography. Use of cyclodextrins as mobile phase modifier**. *J Chromatogr A* 2001, **921**(2):207-215.
110. Abdel-Nabi IM, Kadry AM, Davis RA, Abdel-Rahman MS: **Development and validation of a high-performance liquid chromatographic method for the determination of benzophenone-3 in rats**. *J Appl toxicol* 1992, **12**(4):255-259.
111. Jiang R, Hayden CG, Prankerd RJ, Roberts MS, Benson HA: **High-performance liquid chromatographic assay for common sunscreening agents in cosmetic products, bovine serum albumin solution and human plasma**. *J Chromatogr B Biomed Appl* 1996(682):137-145.
112. Kasichayanula S, House JD, Wang T, Gu X: **Simultaneous analysis of insect repelland DEET, sunscreen oxybenzone and five relevant metabolites by reversed-phase HPLC with UV detection: application to an in vivo study in a piglet model.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2005, **822**(1-2):271-277.
113. **http://en.wikipedia.org/wiki/Image:Spektrofotometri.jpg**.
114. DuBois D, DuBois EF: **A formula to estimate the approximate surface area if height and wight be known**. *Arch Int Med* 1916, **17**:863-871.
115. Okereke CS, Kadry AM, Abdel-Rahman MS, Davis RA, Friedman MA: **Metabolism of Benzophenone-3 in rats**. *Drug Metab Dispos* 1993, **21**(5).

116. Okereke CS, Abdel-Rahman MS: **Species differences in the disposition of benzophenone-3 after oral administration in rat and mouse**. *Toxic Substances Journal* 1994, **13**(4):239-251.
117. Strassburg CP, Strassburg A, Kneip S, Barut A, Tukey RH, Rodeck B, Manns MP: **Development aspects of human hepatic drug glucuronidation in young children and adults**. *Gut* 2002, **50**:259265.
118. Boehnlein J, Sakr A, Lichtin JL, Bronaugh RL: **Characterization of esterase and alcohol dehydrogenase activity in the skin: metablism of retinyl palmitate to retinol (vitamin A) during percutaneous absorption**. *Pharm Res* 1994, **11**:1155-1159.
119. Okereke CS, Abdel-Rahman MS, Friedman MA: **Dispostition of benzophenone-3 after dermal administration in male rats**. *Toxicol Lett* 1994, **73**:113-122.
120. Bonin AM, Arlauskas AP, Angus DS, Baker RS, Galagher CH, Greenoak G, Brown MM, Meher-Homji KM, Reeve V: **UV-absorbing and other substances: genotoxicity of 2-ethylhexyl P-methoxycinnamate**. *Muat Res* 1982, **105**(5):303-308.
121. **http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB**; 2006.
122. Hayden CG, Cross SE, Anderson C, Saunders NA, Roberts MS: **Sunscreen penetration of human skin and related keratinocytes toxicity after topical application**. *Skin Pharmacol Physiol* 2005, **18**:170-174.
123. Bonda C, Marinelli P: **The photochemistry of sunscreen photostability**. In: *European UV sunfilters international conference: 3-4 Nov 1999; Paris*: Step Publishing; 1999: 46-51.
124. Schwack W, Rudolph T: **Photochemistry of dibenzoyl methane UVA filters Part 1**. *Photochem Photobiol B* 1995, **28**:229-234.
125. Johncock W: **Sunscreen interactions in formulations**. *Allured´s Cosmetic and Toiletries® magazine* 1999, **114**(9):75-82.
126. Sayre RM, Dowdy JC, Gerwig AJ, Shields WJ, Lloyd RV: **Unexspected photolysis of the sunscreen octinoxate in the presence of the sunscreen avobenzone**. *Photochem Photobiol* 2005, **81**:452-456.
127. Tarras-Wahlberg N, Stenhagen G, Larkö O, Rosén A, Wennberg A-M, Wennerström O: **Change of ultraviolet absorbance of sunscreens by exposure to solar-simulated radiation**. *J Invest Dermatol* 1999, **117**(2):256-262.
128. **http://www.cfsan.fda.gov/~lrd/fr990521.html**; Sunscreen drug products for over-the-counter human use, Final Monograph, Federal register 64 27666, US Food and Drug Administration.
129. Dondi D, Albini A, Serpone N: **Interactions between different solar UVB/UVA filters contained in commercial suncreams and consequent loss of UV protection**. *Photochem Photobiol Sci* 2006, **5**:835-843.
130. Diffey BL, Stokes RP, Forestier S, Mazilier C, Rougier A: **Suncare product stability: a key parameter for a more realistic in vitro efficacy evaluation**. *Eur J Dermatol* 1997, **7**:226-228.
131. Sayre RM, Dowdy JC: **Photostability testing of avobenzone**. *Allured´s Cosmetic and Toiletries® magazine* 1999, **114**(5):85-91.
132. Maier H, Schauberger G, Brunnhofer K, Hönigsman H: **Chancge of ultraviolet absorbance of sunscreens by exposure to solar-simulated radiation**. *J Invest Dermatol* 2001, **117**(2):256-262.

133. Moyal D, Refrégier J-L, Chardon A: **In vivo measurement of photostability of sunscreen products using diffuse reflectance spectroscopy**. *Photodermatol Photoimmunol Photomed* 2002, **18**:14-22.
134. Cambon M, Issachar N, Castelli D, Robert C: **An in vivo method to assess the photostability of UV filters in a sunscreen**. *J Cosmet Sci* 2001, **52**:1-11.
135. Gaspar LR, Campos M: **Evaluation of the photostability of different UV filter combinations in a sunscreen**. *Int J Pharm* 2006, **307**:123-128.
136. Chatelain E, Gabard B: **Photostabilization of butyl methoxydibenzoylmethane (avobenzone) and ethylhexyl methoxycinnamate by bis-ethylhexyloxyphenol methoxyphenyl triazine (Tinosorb S), a new UV broadband filter**. *Photochem Photobiol* 2001, **74**(3):401-406.
137. Perugini P, Simeoni S, Scalia S, Genta I, Modena T, Conti B, Pavanetto F: **Effect of nanoparticle encapsulation on the photostability of the sunscreen agent, 2-ethylhexyl-*p*-methoxycinnamate**. *Int J Pharm* 2002, **246**:37-45.
138. Chaudhuri RK, Lascu Z, Puccetti G, Desphande AA, Paknikar SK: **Design of a photostabilizer having built-in antioxidant functionality and its utility in obtaining broad-spectrum sunscreen formulations**. *Photochem Photobiol* 2006, **82**:823-828.
139. Iannuccelli V, Sala N, Tursilli R, Coppi G, Scalia S: **Influence of liposphere preparation on butyl-methoxydibenzoylmethane photostability**. *Eur J Pharm biopharm* 2006, **63**(2):140-145.
140. Lodén M, Åkerström U, Berne B: **Novel method for studying photolability of topical formulations: A case study of titanium dioxide stabilization of ketoprofen.** *J Pharm Sci* 2005, **94**(4):781-787.