

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

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

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*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<sup>2</sup>, containing 4% BZ-3. They collected urine for 48 hours after the application. *Paper II:* 26 participants applied a sunscreen, 2 mg/cm<sup>2</sup>, 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<sup>2</sup>, 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<sub>before</sub>) and after (AUC<sub>after</sub>) 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;  $p < 0.05$ . *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

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## 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.*

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## 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 erythema 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 erythema dose
SPF	sun protection factor
THB	trihydroxy benzophenone
TiO <sub>2</sub>	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 sultry-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.*

...

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MAD DOGS AND ENGLISHMEN © 1930 NC Aventales AG



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## 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.

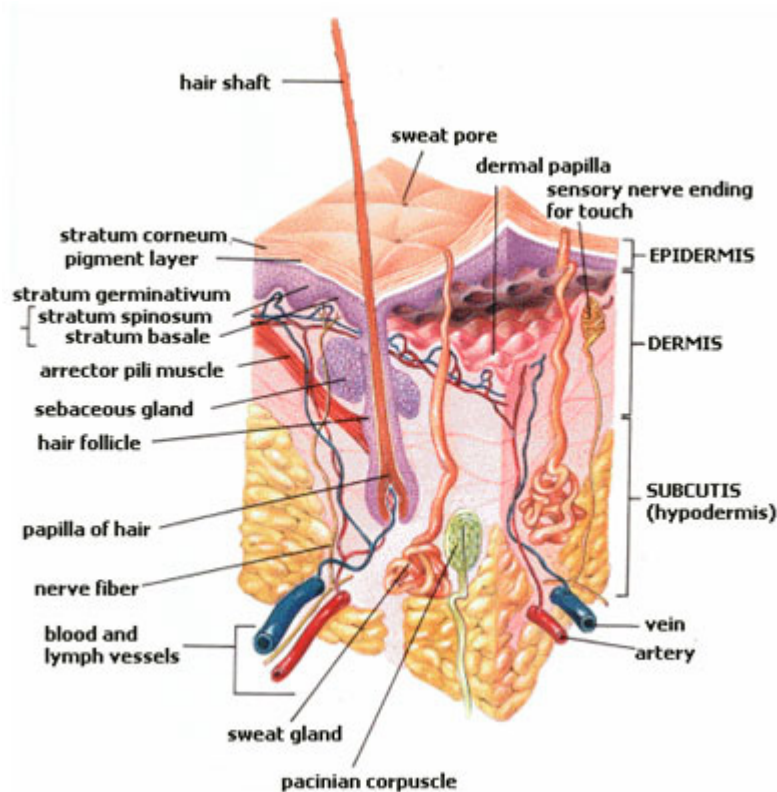
Unfortunately the sun also has negative effects on humans, animals, plants and even inanimate materials such as paint and plastic. The UVR can cause erythema, skin cancer and cataract. Beneficially for us, part of the produced oxygen is transformed to ozone in the stratosphere which protects us from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

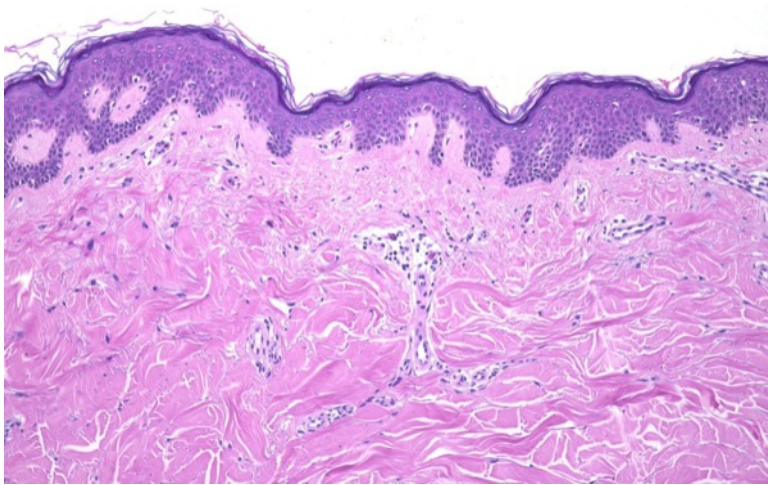
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## THE SKIN

The human skin consists of three layers. The outer part is the *epidermis*, which is usually between 75 and 150  $\mu\text{m}$  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



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 below herself, and the atmosphere will also be thinner.

The UV dose that gives a barely noticeable erythema is called the Minimal Erythema 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<sup>2</sup>. The standard erythema dose (SED) is better to use, where 1 SED equals 100 J/m<sup>2</sup> 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  $E_{\lambda}$  = solar spectral irradiance in Wm<sup>-2</sup>nm<sup>-1</sup>;  $d\lambda$  = wavelength interval used in the summation;  $S_{er}(\lambda)$  = erythema reference action spectrum;  $k_{er}$  = a constant equal to 40 m<sup>2</sup>W<sup>-1</sup>.

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.

## UV RADIATION

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During summer in Sweden, the UV Index is usually between 4 to 7, 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 disfigurement, 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. Commercially 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 photocontact 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 derivatives, salicylates, benzophenones, camphor derivatives, 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 ( $\text{TiO}_2$ ) and zinc oxide ( $\text{ZnO}$ ).

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

INCI name	CAS no	Systematic name*
<b>Organic chemical absorbers</b>		
<b>UVB absorbers</b>		
<i>Cinnamates</i>		
Ethylhexyl methoxycinnamate	5466-77-3	2-Ethylhexyl 4-methoxycinnamate
Isoamyl- <i>para</i> -methoxycinnamate	71617-10-2	2-Propenoic acid, 3-(4-methoxyphenyl)-, 3-methylbutyl ester
<i>para</i> -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)
<i>Salicylates</i>		
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
<i>Camphor derivatives</i>		
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	
<i>Miscellaneous</i>		
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**

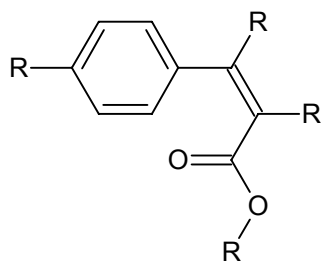
<b>UVA absorbers</b>	<b>CAS no</b>	<b>Systematic name*</b>
<i>Benzophenones</i>		
Benzophenone-3	131-57-7	Methanone, (2-hydroxy-4-methoxyphenyl)phenyl-
Benzophenone-4	4065-45-6	Benzenesulfonic acid, 5-benzoyl-4-hydroxy-2-methoxy-
Benzophenone-5	6628-37-1	Benzenesulfonic acid, 5-benzoyl-4-hydroxy-2-methoxy-, monosodium salt
<i>Camphor derivatives</i>		
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- <sup>**</sup>
<i>Dibenzoylmethane</i>		
Butyl methoxydibenzoylmethane	70356-09-1	1,3-Propanedione, 1-[4-(1,1-dimethylethyl)phenyl]-3-(4-methoxyphenyl)-
<i>Miscellaneous</i>		
Bisimidazylate (proposed name)	180898-37-7	1H-Benzimidazole-4,6-disulfonic acid, 2,2'-(1,4-phenylene)bis-, disodium salt
<b>UVA and UVB absorbers</b>		
<i>Miscellaneous</i>		
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)-
<b>Inorganic chemical absorbers</b>		
Titanium dioxide	13463-67-7	
Zinc oxide	1314-13-2	

INCI International Nomenclature of Cosmetic Ingredients

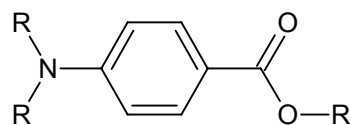
CAS Chemical Abstracts Service

\* preferred by Swedish Chemicals Inspectorate [44].

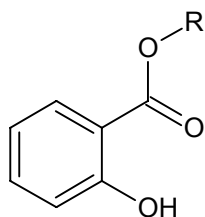
\*\* from ChemIDplus [45].



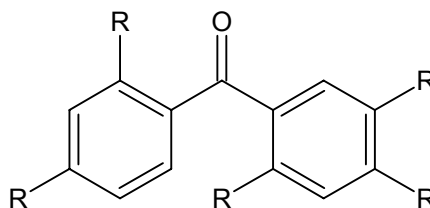
Cinnamate derivatives



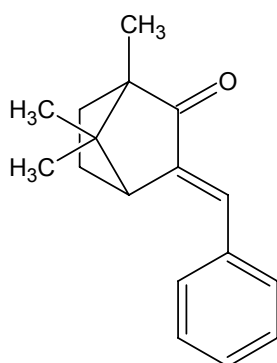
para-Aminobenzoate derivatives



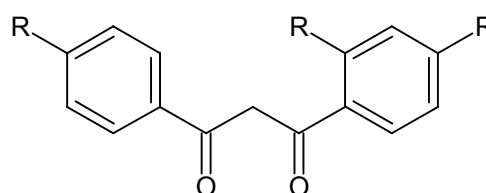
Salicylate derivatives



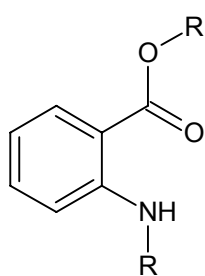
Benzophenone derivatives



Camphor derivatives



Dibenzoyl methane derivatives

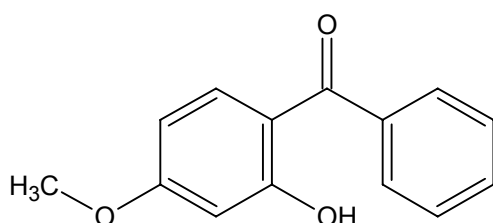


Anthranilate derivatives

**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<sup>2</sup>, 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<sup>2</sup> [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<sup>2</sup>; they also applied the lotion in an uneven manner [56]. Education made the patients use more sunscreen, on average 1.13 mg/cm<sup>2</sup>, still too low to obtain the SPF value [57].

### **Adverse effects of sunscreens**

There have been concerns about pulmonary effects of TiO<sub>2</sub> after inhalation. If the TiO<sub>2</sub> particles are too large, the cream will appear white on the skin. For that reason, nanoparticles of TiO<sub>2</sub> are used in sunscreens to make them cosmetically appealing. One study has shown a species difference after inhalation of TiO<sub>2</sub>, 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<sub>2</sub> industry in Europe. They did not find any carcinogenic effect of TiO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sup>14</sup> 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.

	<b>Advantages</b>	<b>Disadvantages</b>
<b>In vivo</b>	Biological response	Exposure to humans/animals Expensive
<b>In vitro</b>	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<sup>14</sup> 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<sup>2</sup> and 60 cm<sup>2</sup>. 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<sup>2</sup>. 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 (MED<sub>i</sub>) is calculated, both for unprotected skin (MED<sub>ui</sub>) and for protected skin (MED<sub>pi</sub>). The individual SPF is the ratio of the MED<sub>pi</sub> and MED<sub>ui</sub>.

$$\text{SPF}_i = \frac{\text{MED}_{pi}}{\text{MED}_{ui}}$$

The SPF for the product is calculated as the arithmetic mean of all valid SPF<sub>i</sub> 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<sup>2</sup>.

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

### **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<sup>2</sup>. 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 ( $\lambda_c$ ) 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).

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

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<sup>2</sup>). 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]:

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

where  $E_{\lambda}$  = the solar spectral irradiance in  $Wm^{-2}nm^{-1}$ ;  $S_{\lambda}$  = the relative erythral spectral effectiveness ;  $T_{\lambda}$ =spectral transmission coefficient of the textile material;  $\Delta\lambda$ = the bandwidth in nm;  $\lambda$  = the wavelength in nm.

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

### **Sun protection for animals and plants**

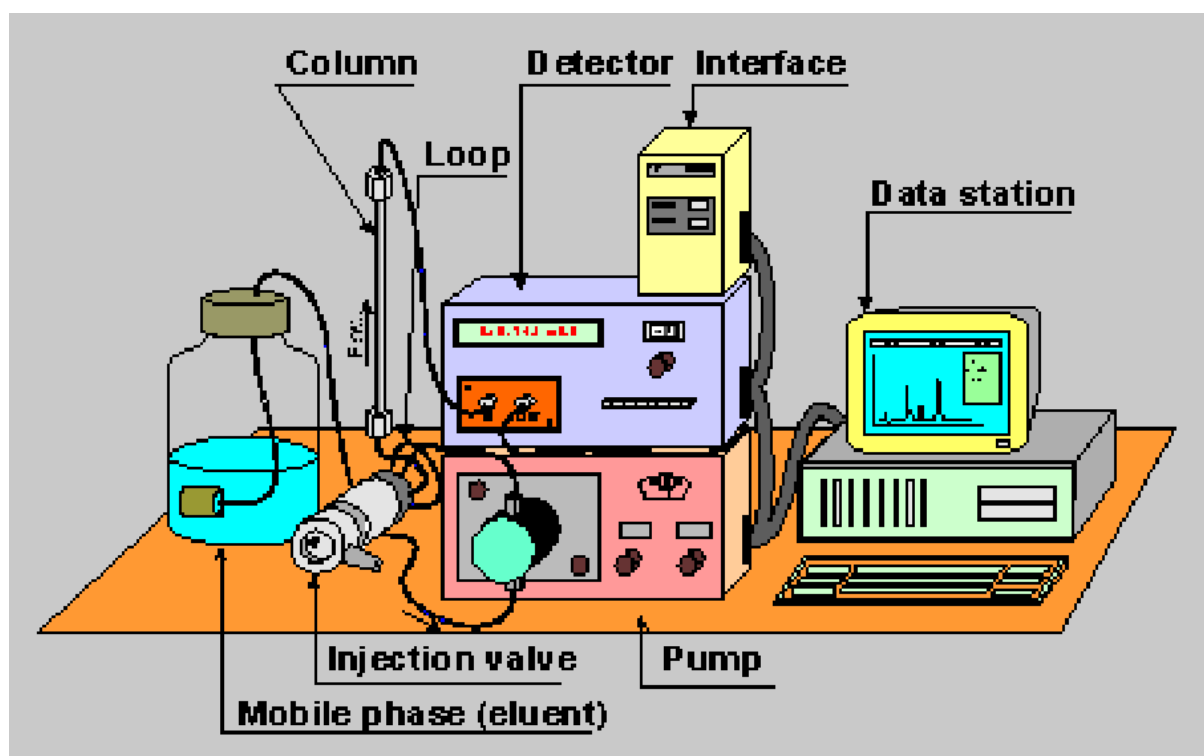
It is not only humans that are affected by UVR: animals and plants are also concerned. For example, fair-skinned pigs can be sunburnt. Some plants have developed a strategy using flavonoids to become more pigmented, they also may have enzymes which can perform DNA repair. Cyanobacteria are one of the 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 <i>et al.</i> (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 <i>et al.</i> (1996) [111]	BZ-3*	0.05 µg/ml	spiked plasma	methanol-water (88:12)	Novapak C18 RCM		315	human
Potard <i>et al.</i> (1999) [107]	BZ-3*	20 µg/ml	skin tissue	methanol:water (69:31)	Novapak C18		291	human
Sarvieya <i>et al.</i> (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 <i>et al.</i> (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.

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## 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].

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## **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.

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## 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<sup>2</sup> and the body surface area (BSA) was estimated to be 2 m<sup>2</sup>. 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<sup>2</sup> 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

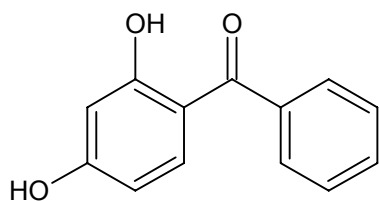
**Group A** did not receive any UVR. **Group B** received UVR during the five days the sunscreen was applied, according to skin type: total amount UVA between 400 and 707 J/cm<sup>2</sup>, and total amount UVB between 0.46 and 2.0 J/cm<sup>2</sup>. For UVA irradiation a Dermalight Ultra A1, equipped 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 HICrom 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<sup>2</sup>, 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<sub>before</sub>) and after (AUC<sub>after</sub>) artificial UVA (980 kJ/m<sup>2</sup>) and artificial UVB (12 kJ/m<sup>2</sup>). If the AUC Index (AUCI) defined as AUC<sub>after</sub>/AUC<sub>before</sub>, 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 spectrophotometer (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
<b>EHMC</b>	5466-77-3		x	x	x	x				
<b>MBC</b>	36861-47-9		x			x	x	x		
<b>EHT</b>	88122-99-0		x							
<b>OC</b>	6197-30-4		x						x	
<b>BMDBM</b>	70356-09-1	x		x	x	x	x	x	x	
<b>BZ-3</b>	131-57-7	x			x	x				
<b>TLDCSA</b>	90457-82-2	x							x	
<b>TiO<sub>2</sub></b>	13463-67-7		x			x	x		x	x
<b>ZnO</b>	1314-13-2	x								x
<b>SPF</b>				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<sub>2</sub>** 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).

$RSD = (SD/mean) \times 100$ .

#### **Ethics**

##### **Papers I and II**

The regional ethical review board in Gothenburg approved the studies.

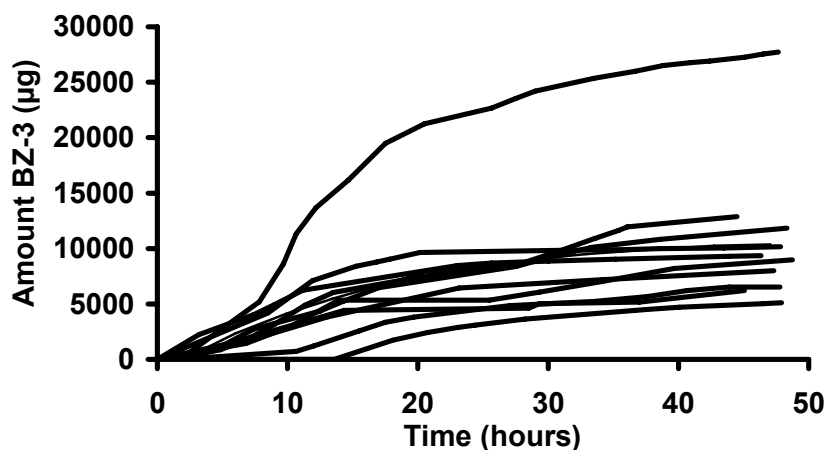
The volunteers participated after informed consent was obtained from them.

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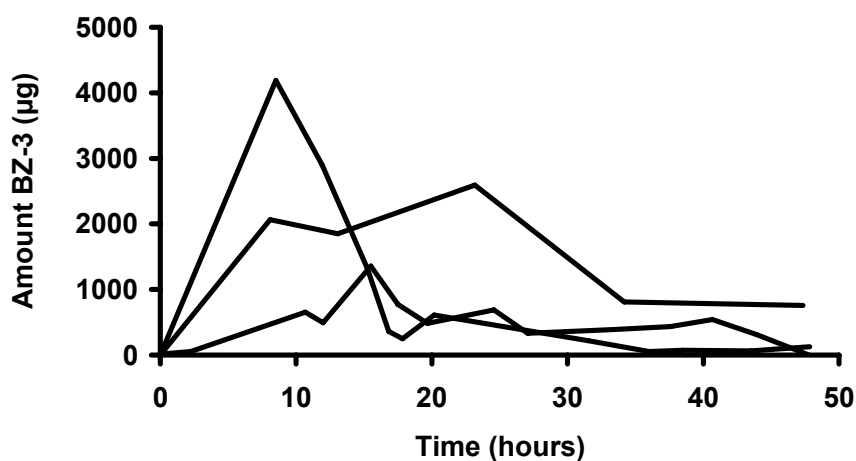
## RESULTS

### 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.

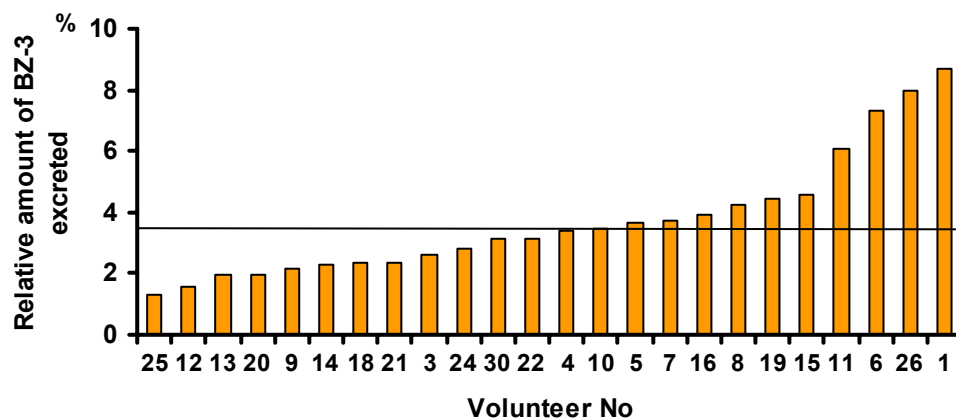


**Figure 10** Three different excretion profiles to illustrate the variation in excretion pattern between the volunteers. Each line represents one volunteer.

## RESULTS

### Paper II

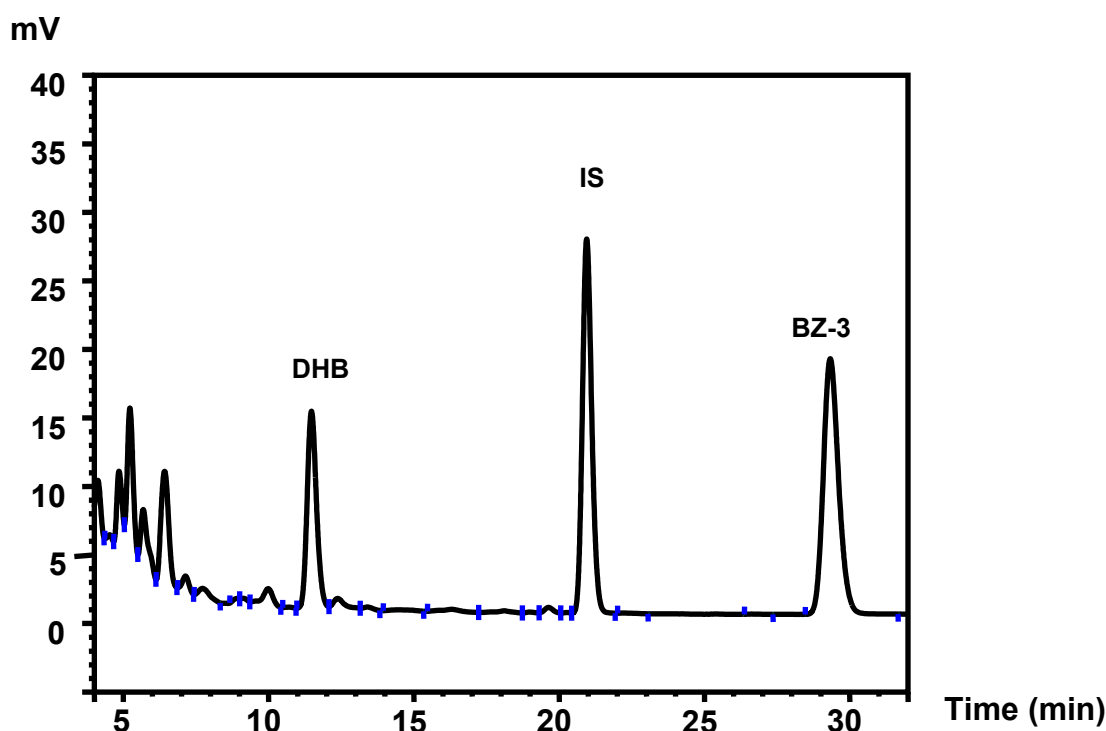
Repeated whole-body applications of a sunscreen containing 4% BZ-3 resulted in a higher excretion of BZ-3 in urine. The mean amount was 3.7% (range 1.2-8.7%) of the total amount applied BZ-3. There was no significant difference between groups A and B ( $p < 0.99$ ). Figure 11 shows the individual urinary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

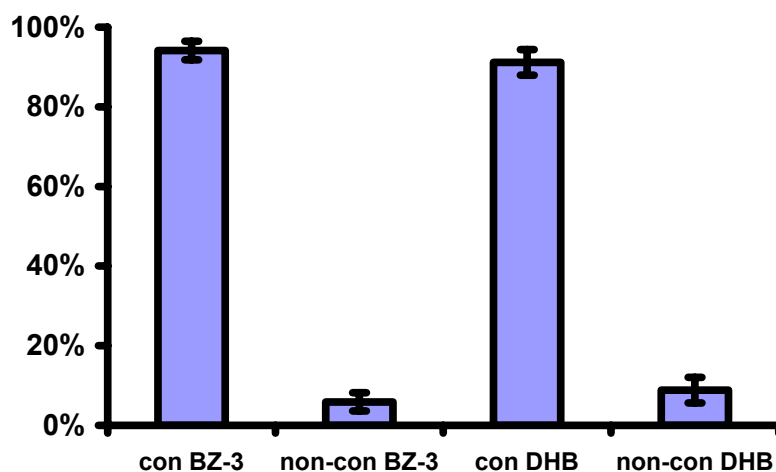
### Paper III

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



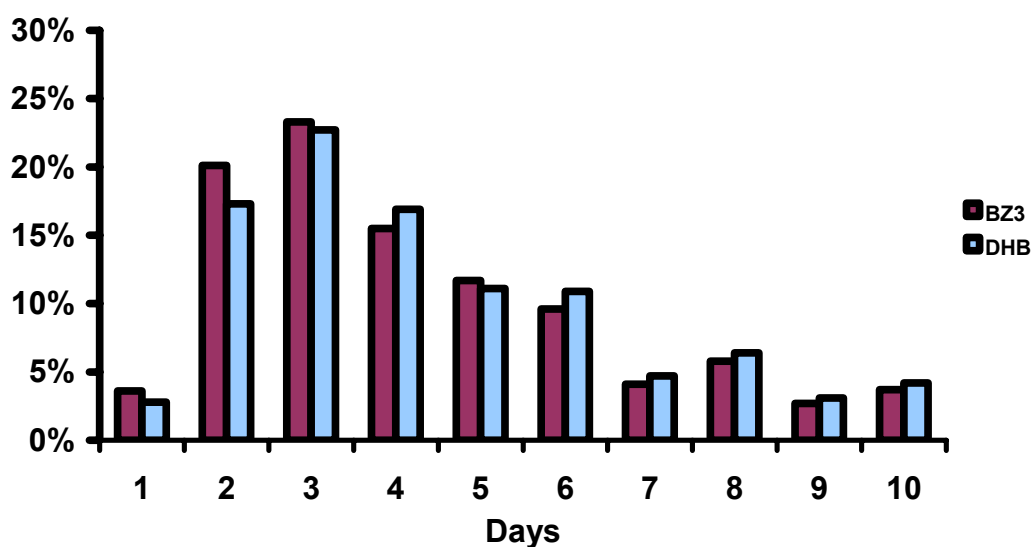
**Figure 12** Example of a chromatogram from person 8. Internal standard (IS).

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.

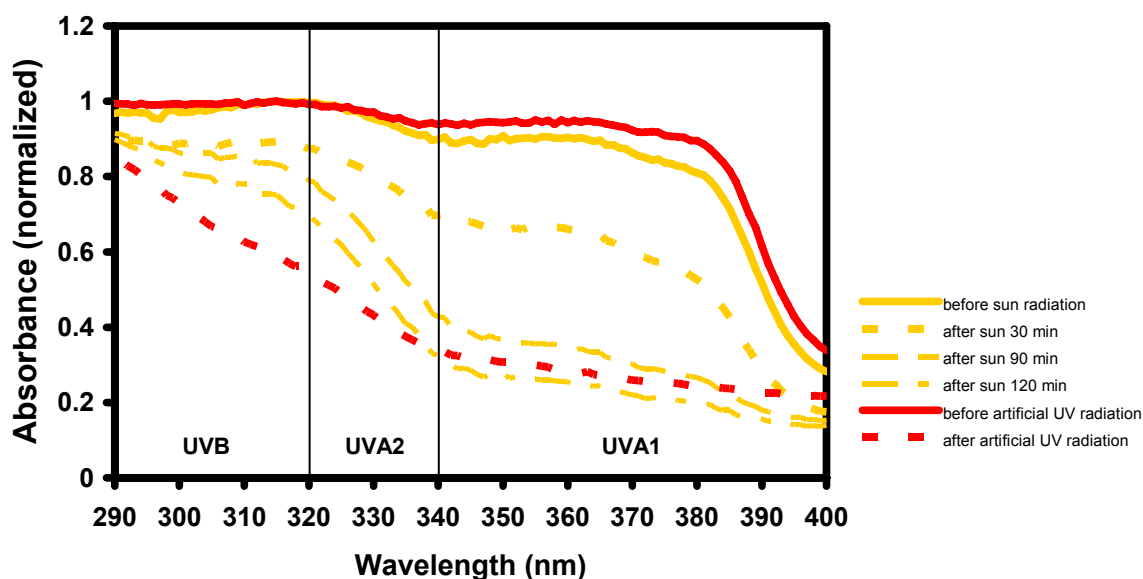
The excretion pattern varied among the human volunteers; we discerned different patterns among the individuals (Figure 14).



**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				
<b>1</b>	<b>0.72</b>	<b>0.46</b>	<b>0.36</b>	<b>0.69</b>	<b>0.38</b>	<b>0.29</b>	0.83	<b>0.65</b>	<b>0.54</b>	0.91	0.87	0.81	<b>0.36</b>	<b>0.32</b>	<b>0.45</b>	<b>0.69</b>
<b>2</b>	0.84	<b>0.76</b>	<b>0.75</b>	0.83	<b>0.69</b>	<b>0.67</b>	0.86	0.92	0.97	0.86	0.92	0.97	<b>0.63</b>	<b>0.53</b>	0.88	0.89
<b>3</b>	<b>0.67</b>	<b>0.41</b>	<b>0.41</b>	<b>0.59</b>	<b>0.30</b>	<b>0.34</b>	0.81	<b>0.58</b>	<b>0.52</b>	0.92	<b>0.75</b>	<b>0.63</b>	<b>0.40</b>	<b>0.31</b>	<b>0.58</b>	<b>0.73</b>
<b>4</b>	0.92	0.86	0.87	0.91	0.85	0.83	0.94	0.91	0.91	0.95	0.93	0.95	<b>0.72</b>	<b>0.69</b>	0.81	0.83
<b>5</b>	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
<b>6</b>	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
<b>7</b>			0.99*			1.00*			0.96*			0.92*	0.99	0.99	1.00	0.99

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

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

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## 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 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<sup>2</sup> 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 in products such as day-creams, lipsticks and foundations.

Percutaneous absorption of different sunscreen compounds is important to investigate in order to acquire 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  $\mu\text{mol/l}$  and 0.16  $\mu\text{mol/l}$  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.

## DISCUSSION

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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<sub>2</sub> 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.

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## 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)

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## **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.

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