UNIVERSITY OF NOVA GORICA
SCHOOL OF ENVIRONMENTAL SCIENCE

REACTIONS OF AVOBENZONE WITH VARIOUS OXIDANTS

MASTER'S THESIS

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IZJAVA

Izjavljam, da je magistrsko delo rezultat lastnega raziskovalnega dela. Rezultati, ki so nastali v okviru skupnega raziskovanja z drugimi raziskovalci, ali so jih prispevali drugi raziskovalci (strokovnjaki), so eksplicitno prikazani oziroma navedeni (citirani) v magistrskem delu.

Kristina Kalister

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Hvala.

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ABBREVIATIONS AND SYMBOLS

DOM	dissolved organic matter
DNA	deoxyribonucleic acid
EC_{50}	median effect concentration
FDA	United States Food and Drug Administration
FID	flame ionization detector
HPLC-DAD	high pressure liquid chromatography coupled with diode array
IARC	INTERNATIONAL AGENCY FOR RESEARCH ON CANCER
LC-MS	liquid chromatography coupled with mass spectrometry
GC-MS	gas chromatography coupled with mass spectrometry
LLE	liquid liquid extraction
MeCN	acetonitrile
MeOH	methanol
MME	micelar - mediated extraction
OH•	hydroxyl radical
ROS	reactive oxygen species
RNA	Ribonucleic acid
SBME	stir bar sorptive extraction
SPE	solid -phase extraction
SPF	The sun protected factor
TCCA	trichloroisocyanuric acid
THF	tetrahydrofurane
UV	ultraviolet
UV-A	ultraviolet A
UV-B	ultraviolet B
UV-C	ultraviolet C
V. fisheri	Vibrio fisheri
WWTP	waste water treatment plants

POVZETEK

V okviru magistrskega dela sem preučevala fotostabilnost UV filtra avobenzona pod dezinfekcijskimi pogoji. Avobenzon spada v skupino UV-filtrov, ki se dodajo izdelkom za osebno nego, kot so kozmetika in senčila za zaščito pred sončnimi žarki. Slednji blokira UVB/UVA del sončnega sevanja z odbojem/sipanjem. Med plavanjem in kopanjem v bazenih/morju se velike količine sončnih krem izperejo v okolje, kjer se lahko razgradijo po različnih poteh razgradnje.

Eksperimente fotorazgradnje smo izvedli v Suntest aparatu, ki simulira naravne pogoje, kot so: intenziteta svetlobe, temperatura in vlaga. Vzorci so bili postavljeni v Suntest komoro, kjer so bili izpostavljeni intenziteti svetlobe; 750, 500 in 250 W/m^2 ter temperaturi 24.4 ℃. Obsevani so bili v različnih časovnih intervalih; od 30 do 240 minut. Razgradnjo avobenzona smo spremljali z uporabo tekočinske kromatografije z diodno detekcijo (HPLC-DAD) pri valovni dolžini 272 in 350 nm. Pripravili smo avobenzon v vodi in klorirani vodi. V obeh primeru smo lahko opazovali nestabilnost oziroma razgradnjo UV filtra s časom. Pri valovni dolžini 272 nm smo odkrili nove klorirane stranske produkte, ki smo jih analizirali z LC–MS tehniko.

Toksičnost vzorcev smo testirali z LUMIStox aparatom s pomočjo morskih luminescencenčnih bakterij *Vibrio fisheri*. Avobenzon deluje kot strupena kemikalija. Najprej je bil opravljen predhoden eksperiment, kjer smo testirali višjo koncentracijo vzorca (125 mg/L) in ga obsevali štiri ure v Suntest aparatu.
Toksičnost vzorca pada v prvi uri obsevanja, ki pa se z daljšim časom obsevanja povečuje do 99.61 % zaviranja luminescence. V primeru majhne količine vzorca (1.25 mg/L) lahko opazimo, da se toksičnost vzorca giblje v razponu od 45.4 – 54.1 %. Po štirih urah izpostavljenost sončnemu obsevanju se njegova vrednost poveča na 57.1 %. V prisotnosti klora se toksičnost vzorca povečuje z daljšim časom obsevanja, do vrednosti 91.1 %.

ABSTRACT

During my master thesis I have examined the photostability of avobenzone in the presence of chlorine. Avobenzone belongs to the group of UV filters, currently added to personal care products such as cosmetics and sunscreens for protection against sunburns. During swimming of bathing they are washed into the environment where they can undergo different degradation pathways and cause impacts on non target organisms.

Photodegradation experiments were performed in Suntest apparatus, which simulates natural conditions (light intensity, temperature and humidity). The samples were exposed in the chamber where the light intensity was set to 750, 500 and 250 W/m^2 and temperature at 24.4 ℃. Samples are irradiated for different time intervals 30, 60, 120 and 240 minutes. The degradation of avobenzone was monitored using liquid chromatography with diode array (HPLC-DAD) at wavelengths 272 and 350 nm. Avobenzone was prepared in water and in chlorinated solutions. In both cases it could be seen that after three hours of irradiation avobenzone was very unstable compound. In chlorinated solution loss of absorption was observed. At wavelength 272 nm new chlorinated by-products were detected, which were analysed with GC-MS.

Toxicity of the samples in the presence of chlorine was tested with LUMIStox using the marine luminescence bacteria *Vibrio fisheri.* Avobenzone acted similar to a toxic chemical. First it was analysed in preliminary experiments where tested higher concentration of sample (125 mg/L), which had been irradiated for four hours in the Suntest apparatus. Here luminescence inhibition was detected, which gradually decomposed over the first one hour. In the fourth hour of irradiation it was observed that the toxicity of the sample increased to 99.61 %. When chlorine was added the toxicity reached a higher percentage, 99.61 %. In the case of small quantities of irradiated sample (1.25 mg/L) luminescence inhibition varied in the range 45.4 to 54.1 %, until after four hours of exposure a value of 57.1 % was reached. In the presence of chlorine, a gradual increase of luminescence inhibition to 91.1 % was observed.

1. INTRODUCTION

Currently, people are exposed to a large number of chemicals, which are accumulating in the environment every day. The greatest problems are caused by organic pollutants, which bio-accumulate in terrestrial and aquatic ecosystems, which are then water-borne and airborne to remote different parts of the world, far from their origin. Among organic chemicals that are released into the environment are also UV-filters. For nearly 75 years they have been used in many sunscreen products (Finkel, 1996) and are present in many products for daily use, such as: skin creams, cosmetics, hair sprays, body lotions, hair dyes, and shampoos (Li et al, 2007). The amount of a specific UV-filter in sunscreens products varies between 0.5 and 10 %, but may reach up to 25 % (Hauri et al., 2003).

Exposure to UV radiation can cause sunburn, such as erythema. Prolonged exposure can lead to premature aging of the skin, dermatoses and actinic keratoses as well as inflammatory reaction of the eye. In some cases it may lead to skin cancer (malignant melanomas) and cataracts, therefore the use of sunscreens is recommended (Schauder and Ippen, 1997).

Organic UV filters have the ability to absorb UV radiation and protect human skin from direct effects of sunlight. They act as a shield between the sun and skin and protect from UVA or long-wave rays and UVB or short-wave rays (Dimosthenis et al, 2007). Moreover they have a colour protective ability, which means they could prevent premature fading of hair colour and damage to the hair cuticle. (Schauder and Ippen, 1997)

These properties are obtained by reflection or absorption of solar radiation by UV filters in the sunscreen. Inorganic UV filters, such as titanium dioxide or zinc oxide, are known for their UV protection properties, which include physical protection from the sun. Organic filters are used for absorption of UV radiation.
(http://www.kantonslabor-bs.ch/files/berichte/report0419.pdf)

The public was invited by National and international health authorities to participate in the call for safeguard measures, including sunscreen. Recently it has been estimated by the IARC (International Agency for Research on Cancer) that the use of sunscreens plays an important role in cancer - prevention activity (IARC, 2000). Before a new UV-filter is put on the market it has undertaken rigorous toxicological tests. The molecule has been approved by the SCCNFP (Scientific Committee on Cosmetic Products and Non-food products intended for Consumers) for human use in the case of a safe toxicological profile and a margin of safety of at least 100.
http://www.kantonslabor-bs.ch/files/berichte/report0419.pdf

In recent years, the use of personal care products has constantly grown due to greater public awareness of the harmful effects of solar radiation. Also, larger amounts of UV filters are added into formulations, primarily due to the increased use of higher sunlight protection (Fent et al. 2010). Maximum concentrations of UV filters in the aquatic environment have been measured during the warmest summer days. At this time application of sunscreens is the most frequent, because of increased sunlight irradiation and exposure (Dimosthenis et al., 2007).

Contamination of the aquatic environment with UV-filters may appear through different pathways. Direct input is wash-off from skin during swimming and bathing,

while indirect input is released via wastewater treatment plants, where they may reach detectable and potentially harmful concentrations (Li et al, 2007).
Recently, there is increasing public concern regarding secondary effects of personal care products. Secondary pollutants are created when primary pollutants (UV filters) react with other active substances, present in the environment. The new compound may be more toxic than the starting primary compound. Many studies have shown that UV-filters absorb UV-light and decompose under solar irradiation, due to their unstable properties. This may lead to the formation of certain byproducts with harmful effects. Their decomposition products can cause allergic and toxic reactions to the human skin. I must not neglect the fact that some of these products have lipophilic properties and have the ability to enter through the cell membrane. There are also possible photocatalytic reactions, given the fact that the recommended amount of sunscreens is 2 mg/cm². This is 36 g of sunscreen over the whole body (Baumgartner, 2007).

Based on these studies, it had been found that UV-filters react slowly with chlorine, which is the most commonly, used chemical oxidant for drinking water disinfection**.** Particularly, it is used for hygiene in swimming pools. Numerous transformation products may be formed, due to oxidation/substitution reactions. (Stephen et al, 2012)

1.1 Research goals

The aims of this study were to:

1. Through using the Suntest apparatus carry out the photochemical stability experiments of UV-filter avobenzone in the presence of the chlorinating agent (trichloroisocyanuric acid) at selected lighting conditions (750, 500 and 250 W/m²) and for different periods of irradiation.

2. Monitoring of concentration changes by liquid chromatography HPLC-DAD and UV-Vis spectrophotometer at wavelength 350 and 272 nm.

3. Toxicity determination of irradiated samples using the marine bacteria *V. fisheri* with the apparatus LUMIStox.
4. Determination of chlorinated products by LC–MS

2. THEORETICAL BACKGROUND

2.1 UV – filters general

Ultra Violet (UV) filters are chemically synthesized aromatic compounds most often used as sunscreen products. These compounds are synthesized to block the sunlight in the range of 100 to 380 nm and are designed to protect the skin from direct exposure to UV rays. Ultraviolet filters are substances that absorb UV radiation of wavelengths 200 – 400 nm. Split-range between wavelengths is divided into UV-A (400 – 320 nm), UV-B (320 – 280 nm) and UV-C (280 - 200 nm) (Diffey, 1991; IARC, 1992).

Sunlight on the skin surface is absorbed and may cause a variety of damages, such as burns and erythema. By using sunscreens, the skin burns are avoided, since UV filters absorb UV light instead of the skin and protect against the harmful effects of UV rays (Čufar, 1994).

Under the EU Cosmetics directive, a list of 27 UV–filters has been approved for commercial use. Twenty-five of them are organic and two are inorganic (titanium dioxide and zinc oxide).

2.1.1 Classification

Among organic UV filters differentiated compounds on the basis of their structure or on the basis of which UV light range they absorb.

UV filters are chemically divided into:

1.) BENZOPHENONES
2.) DIBENZOYLMETHANES
3.) CINNAMATES
4.) CAMPHOR DERIVATES
5.) PARA-AMINO BENZOATES
6.) BENZIMODAZOLES (Shaath, 2010; Giokas et al., 2007).

Depending on which part of the UV spectrum they absorb, they are sub-divided into:

a.) UVA
b.) UVB
c.) UVA/UVB

a.) UVA filters

Sunscreens, which contain the UVA filters, provide protection against UVB and UVA radiation, but they are not all equally effective. Beside UVA, UVB filters absorb just some of the UVB radiation. Among UVA filters I included the compound avobenzone, which is extremely unstable with high UV absorption. This substance can undergo photoisomerization to inert, nonphotoprotective compounds (Lowe, 2006).

b.) UVB – filters

Among UV filters are the most effective UVB filters, because they block 90 % of UVB radiation. In this group we have included cinnamates, which are also the most commonly used UVB filters. However, they are known to have very poor sustainability, meaning that they can be washed off very easily, and they are usually found combined with other agents (Perugini, 2002).
Furthermore, the group of salicylates is very soluble in water, stable, nonsensiting and can be used as a solvent for other poorly soluble sunscreen ingredients, for example benzophenones. In hair cosmetics trolamine salicylate is also applied as a photoprotective agent (Kullavanijaya and Lim, 2005).

c.) UVB/UVA filters

Among those filters that absorb UVB and UVA radiation is terephtalylidene dicamphor sulfonic acid. Mostly it is used in the formulations of other compounds that protect skin from the sun, such as avobenzone and other UVB acceptors. From a clinical study it was concluded that the day cream contains a combination of major photostable absorbers UVA and UVB light (octocrylene, avobenzone, and terephtalylidene dicamphor sulfonic acid). The advantage of this product is that it reduces damage to the skin due to UV radiation, and prevents biological changes that are related to the photoaging (Seite et al., 2000).

These compounds provide an efficient electronic delocalization causing the specific maximum absorbance wavelength. They also contain one benzen moiety, conjugated with an electron donor and electron accepting groups in either ortho or para positions. They usually have single or multiple aromatic structures, sometimes conjugated with carbon-carbon double bonds and carbonyl parts (Dimosthenis et al., 2007). They contain molecules, which can scatter, reflect or absorb UV photons. Most of UV filters are highly lipophilic, so they can accumulate both in the human body and in the environment (Li et al., 2007).

Only two inorganic UV filters are allowed for commercial use: titanium dioxide (TiO_2) and zinc oxide (ZnO). Another name for inorganic filters is physical blockers, because they are made of sizeable particles, are micronized and coated. They have the ability to absorb, reflect or scatter solar radiation.
Negative characteristic of inorganic UV filters is that they can cause photochemical changes, which may affect their effectiveness. They have the ability to damage DNA and RNA. To prevent their reactivity and increase their stability, TiO_2 and ZnO particles are coated with dimethicone or silica (Van Reeth, 2006).

The use of organic UV filters constantly increases due to people’s awareness of the harmful effects of the sun's rays. With the increasing use of sunscreens, there is also an increased the risk of exposure to these compounds and their byproducts. (Lambropoulou et al., 2002). Very little is known about the occurrence and fate of UV-filters in the environment. These compounds are lipophilic and show potential for bioaccumulation. The problem is a lack of analytical methods for the determination of some UV-filters, restricting the understanding of the environmental fate of this class of compounds. UV filters may be converted in the environment under the influence of pH, where they may undergo hydrolysis, photodegradation or metabolic degradation (Giokas et al., 2007). The biggest problem occurs when these compounds are transferred through the water system. During the summer months, this percentage increases significantly, due to the large consumption of sunscreens. They are

washed off from the skin into the pools, rivers or sea, so they can be found in water bodies and may enter int wastewater as well (contaminated clothes) (Balmer et al., 2004; Daughton and Ternes, 1999).

How toxic a UV filter is will depend on its toxicity level and on exposure route, and is a function of the level of percutaneous absorption of such topically applied substances, that occur in the human organism (Benech-Kieffer et al., 2003). There are very few reports concerning the potential toxic effects of UV filters on aquatic organisms. Older reports give information on a possible endocrine hormonal disorder caused by absorbed UV filters in the biota. Many UV filters have been found to exhibit oestrogenic, antiestrogenic, androgenic and antiandrogenic activities (Schlumpf et al., 2004).

2.1.2 SPF/Application

The protection against sunrays is ensured by sunscreens by the so called “sun protection factor” (SPF). It could be explained by effectiveness, or how long the skin, covered by sunscreen, takes to burn compared with unprotected skin (Health Education Authority, 1996). Another version of the interpretation of the SPF is: The ratio of the least amount of ultraviolet energy required to produce a minimal erythema on sunscreen protected skin to the amount of energy required to produce the same erythema on unprotected skin (Department of Health and Human Services Food and Drug Administration, 1978).

The most reliable indicator of the effectiveness of sunscreens and UV filters compared with the SPF is the erythema protection factor. This test protocol is easy and non-invasive and determines erythematous response after 24 hours (Hanneman et al., 2006; Lavker, 1995).

At the beginning of the 1990, SPF values for the majority of commercially available sunscreens products was less than ten. Since 2000, the value of SPF has changed, and began to grow. It reached the value of the factor 15 – 30, although there are even products with a factor 50 or higher (Diffey, 2001). Furthermore it is recommended to use a sunscreen with a sun protection factor of 30 or more, which provides a good level of protection.

From the study by Eriksson et al., 2008, the application of UV filters in Denmark could be monitored. These results were compared to the rest of the European Union. The use of UV filters has widely expanded in the personal care products, pet care products and pharmaceuticals products. They are also contained in products for the maintenance of vehicles, pesticides, industrial products, raw materials and in products for cleaning shoes (Eriksson et al., 2008). Using UV-filters is not only to achieve a protection against sunlight, but they are also used as additives in order to achieve stability and durability of many products (Balmer, 2004).

2.1.3 Environmental fate

A large amount of UV filters was found in small rivers, receiving water from waste water treatment plants (WWTP) (Schmid et al., 2006). Remains of sunscreens are found in waste containers that are annually discarded in large quantities. It is well known that domestic plastic residue contains from 10 to 14 % of moisture, which can be a source of residues of UV filters (Riber and Christensen, 2006). The amount of residual sunscreens in containers is mainly dependent on human behaviour and confidentiality of goods. In these products, 10 % of the sunscreen is left as waste. Furthermore, 9 % of solid waste goes on landfills. However, it is cancelling out the fact that UV filters can be emitted from the incitation plants, mainly due to their low evaporation.

Sunscreens can enter in surface water from direct inputs like swimming and bathing pools, or washing clothes, which may contain an UV-filter. It is recommended to use sunscreen every two hours, after swimming or sweating and towelling (Diffey, 2001). This provides maximum protection against the sun (Wright et al., 2001). Furthermore, sunscreens can be applied onto the body, metabolised in the body and excreted via urine and faeces. Only 32 % of the UV filters enter the wastewater treatment plants (WWTP), 27 % are absorbed through the skin and 27 % are washed-off in surface waters. It could be concluded that quantities of UV filter entering the sanitary sewer system are much higher than those, entering the surface water. Less than 1 % of UV filters can be removed by biodegradation and removal in an activated sludge wastewater treatment plant (figure 1).

Another example of released UV filters into the environment are products for cleaning shoes and car care products, which enter into the environment through precipitation. It is possible that these compounds enter to the surface and marine waters, if boat cleaning commodities were applied. In the effluents, four of the six UV filters were detected. Among those mentioned, was also avobenzone (Eriksson, 2008).

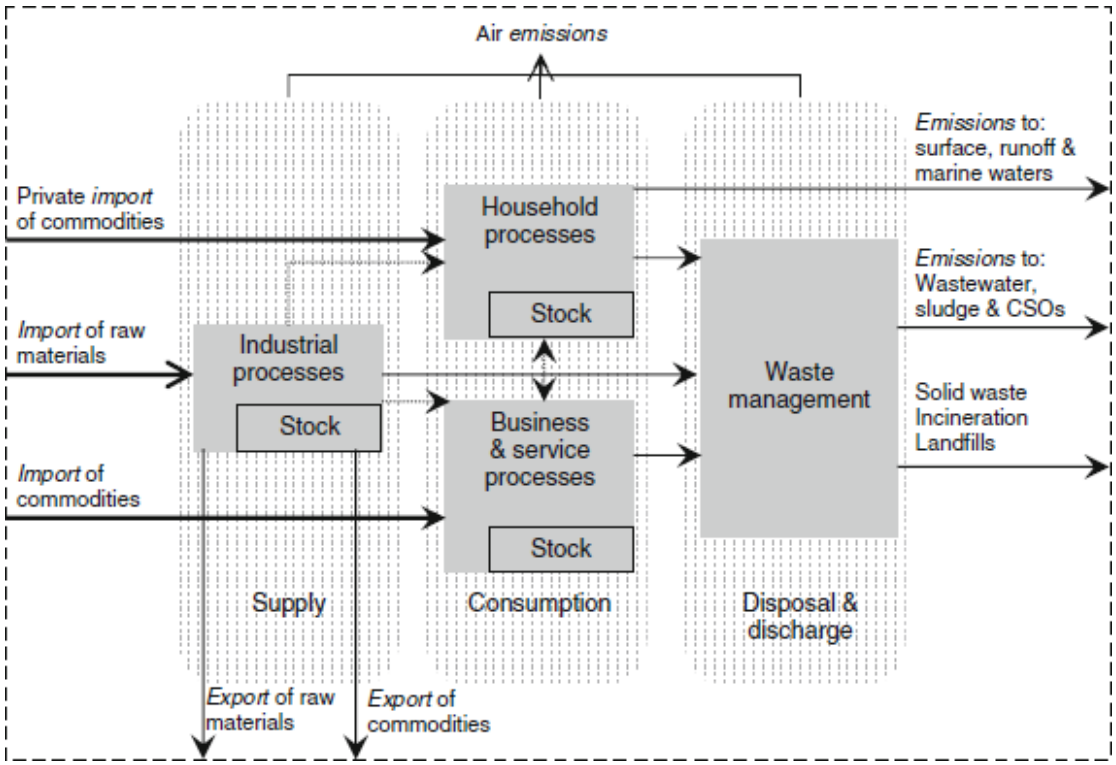


Figure 1: System description of UV-filter flows in Denmark. Businesses and service processes includes shops, day-care centres, beauty and grooming salons, self-tanning salons etc. (Eriksson, 2008)

2.2 Photostability of UV filters

Solar radiation that penetrates the earth's atmosphere is generally divided into ultraviolet light A (UVA) and ultraviolet light B (UVB) radiation. The solar spectrum covers the wavelength of UVB radiation from 290 – 320 nm, while UVA cover longer wavelengths from 320 to 400 nm.
(http://fp.arizona.edu/kkh/nats101gc/PDFs06/medscape.today.uva.pdf)

Groups of UV filters have the ability to reduce transmission of the photons of light, because they usually contain one or more aromatic rings, which are connected with double bonds and a variety of substituents. The light that arrives on the Earth's surface in the form of photons is absorbed by UV filters. With the process of thermal emission of energy, UV filters quickly return to the ground state through a series of vibration transitions. This can lead to the cleavage or degradation of the compound. The process is repeated several times (Kimbrough, 1997).

UV filters are often used in sunscreens to protect people. Most of them are in a dissolved state, and their photochemical stability depends also on the solvent. (Sayre, 1990). Stability of UV filter means prevention of the formation of potentially photooxidant-reactive intermediates on the skin, which may lead to genotoxic effects and photoaging. There are many studies, which indicate the instability of UV filters and many reactions demonstrating the loss of its original form when they are exposed to irradiation. Some organic UV filters may undergo certain reversible and/or irreversible photodegradation reactions. Unstable UV-filter changes the highly energetic UV-radiation into heat by radiationless deactivation. These reactions often

occur in single or multi component solutions as well as in sunscreens (Baumgartner, 2007).

Possible photodegradation reactions of UV-filters when exposed to the irradiation:

- homogenous additions and dimerisation
- radical formation (the radicals can undergo further reactions)
- isomerisation
- heterogeneous additions
- demethylation
- fragmentation (the fragments can undergo further reactions)
- reaction with unsaturated components (shown for cyclohexene, stearic acid methyl ester and EHMC, therefore reactions with common matrix components such as bisabolol, fumaric acid or fragrances are possible)
- reaction with components of the skin (cholesterine reactions could be possible)
- reaction with DNA (dimerisation of thymine in DNA is photosensitized by para-aminobenzoic acid (PABA) (Baumgartner, 2007).

In the case of UV filters that are dissolved in water the photogeneration of reactive oxygen species and free radicals upon irradiation is possible (Blough and Zeep, 1995). In the natural environment, water is full of dissolved organic matter (DOM), bicarbonates, nitrates and chlorides. Photolysis of humic acid, sodium chloride, and nitrate solution can form photosensitizers; oxygen, hydroxyl and/or peroxy radicals. Their presence strongly influenced the degradation of UV filters. On the surface layer of the water system there are two processes: direct photolysis and indirect photolysis.

Degradation of UV filters depends on the presence of disinfectant agents, such as chlorine as well. It is often used in systems for the treatment of drinking water or for the disinfection of water, like swimming pool. Furthermore it may form chlorinated by-products, for example trihalomethanes (Giokas and Vlessidis, 2007).

In some cases unstable filters (for example avobenzone and ethyl-hexyl methoxycinnamate) are additionally supported by the addition of newly developed filters such as Mexoryl SX, Mexoryl XL, Tinosorb M, and Tinosorb S, which improve photostability of unstable filters and better effectiveness is achieved (Chatelain and Gabard, 2001).

2.3 Reactions of UV filters with oxidants

Chlorination of drinking water is currently applied for prevention of infection and chronic diseases. Additionally, the process of chlorination is carried out in swimming pool waters. The negative impact of chlorination processes in addition to bad taste and odor, is also the presence of resistant protozoan cysts and production of toxic carcinogenic products.
However, the addition of chlorine causes many reactions with the presence of substances that are a consequence of human activities. The chlorinating agent are the so-called oxidizing agents and may oxidating various organic compounds, including the organic UV filters. They are present as an oxidation reaction, such as electrophilic substitution.

For disinfection of water various types of agents are used such as sodium hypochlorite (NaOCl), ozone (O_3), peracetic acid (CH_3COOOH), gaseous chlorine (Cl_2) and trichloroisocyanuric acid (TCCA).
Trichloroisocyanuric acid or 1,3,5-trichloro-1,3,5-triazine-2,4,6,-(1H,3H,5H)-trione) has been known since 1902. It belongs to the large group of chloroimides. Chlorinated agents are the most widely used disinfectant for drinking water (Deborde and Von Gunten, 2008). It is used in swimming pools and water treatment, as a dishwashing additive in hotel and food services. It protects swimmers from pathogenic organisms and is produced in large quantities; >100.000 t/year.

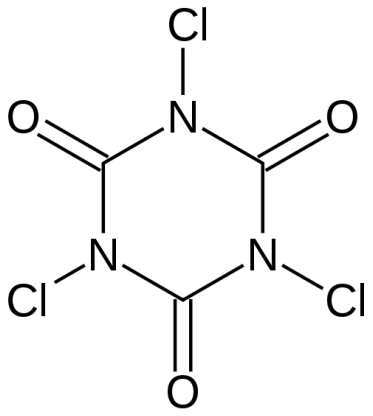


Figure 2: Chemical structure of trichloroisocyanuric acid (TCCA) (source: Products for innovative research, TRC)

Chlorine as an agent consists of chlorine gas, sodium or calcium hypochlorite, chlorinated isocyanurates, bromochlorodimethylhydantoin or chlorine dioxide (Lakind et al., 2010). Depending on the pH, different species of chlorine could be present in aqueous solutions. In drinking water, the chlorine is present in the pH range between 6.5 and 8.5. It can be found in the form of hypochlorous acid (HOCl) and hypochlorite ion (OCl^-), which have different reactivity towards anthropogenic compounds found in drinking water sources (Deborde and Von Gunten, 2008; Duirk et al., 2009).

As mentioned in the previous section, natural waters are full of organic matters. For the aqueous solution of chlorine it is known that chlorine is not a strong enough to oxidant anthropogenic substances that are found in drinking waters. Due to the oxidation/substitution reactions, various transformation products, like chlorinated organic compounds, are formed. (Gallard and Von Gunten, 2002; Dodd et al., 2005; Duirk and Collette, 2006). In swimming pools many disinfection by-products; haloaldeydes, haloketones, trihalomethanes, haloacids, halonitromethanes,

haloamines, haloamides, haloalcohols and halogenated derivates of UV filters have been found (Richardson et al., 2010).

However, the organic matter is not the only substance present in the swimming pools. There are many other substances such as: skin cells, saliva, urine, sweat, hair and personal care products. Thus, the formation of new chlorinated by-products depends on the many factors:

- the amount of disinfectant used,
- disinfectant dose,
- residuals available in the water,
- temperature,
- nitrate and bromide concentrations in water,
- pH,
- DOM (Kanan and Karanfil, 2011).

Swimmers in these pools are exposed to different chlorinated by-products through the skin (absorption), inhalation or ingestion by swallowing water. There are many negative effects on health; incidence of bladder cancer, respiratory diseases and increased risk of asthma (Richardson et al., 2010).

In the literature, little data is available on UV filters in chlorinated waters. Currently, there are only few published studies. In the study Sakkas et al. (2003) they investigated degradation kinetics of the UV filter EHDPABA. They detected five chlorinated products in aqueous solution from swimming pools (2-ethylhexyl dichloro-p-dimethylaminobenzoate, 2-ethylhexyl chloro-p -methylaminobenzoate, 2-ethylhexyl dichloro-p-methylaminobenzoate, 2-ethylhexylp-amino-chlorobenzoate, 2-ethylhexylp-amino-dichlorobenzoate).

The second study was from Negreira et al. (2008). Experiments with three UV-filters (BP3, EHDPABA and ES - ethylhexyl salicylate) were carried out in chlorinated water at neutral pH or at presence of potassium bromide. It was found that the most stable UV filter was ES and the lest BP3. Both filters (BP3 and EHDPABA) show lower stability, when they come into contact with chlorine. Additionally, they studied the stability of UV filters in three pH values (7.2, 8.2, and 6.2). EHDPABA was more stable at pH 8.2, while BP3 was more stable at pH 7.2, 6.2 and less at pH 8.2. Furthermore it was reported the formation of only mono-halogenated compounds in the case of EHDPABA, such as Cl-EHDPABA and Br-EHDPAB and in the case of BP3 also dihalogenated by-product Cl_2BP3 beside ClBP3 was formed.

Nakajima et al. (2009) studied the reaction kinetics of EDHABA and EHMC in swimming pool water. They react slowly with chlorine and form mono- and di-halogenated by-products; Cl-EHDPABA, Cl-EHMPABA, Cl_2-EHMPABA, Cl-EHPABA, Cl_2-EHPABA, Cl_2-EHDPABA. While the EHMC filter formed the following products: Cl-EHMC and CL_2-EHMC.

There is also a study by Zhuang et al. (in press, 2013) who investigated the chlorination of BP3 and BP4 UV filters. They discovered that BP3 with TCCA forms 5-chloro and 3,5-dicloro derivatives. With the chlorinated agent, which was applied in excess, it was determined that another chlorinated by-product 3,5-dicloro-2-hydroxy-4-methoxybenzophenone, was produced. BP4 as well leads to the formation of the two chlorinated products, 5Cl-BP3 and 3,5-diCBP3.

Furthermore, another new UV filter, DHHB, was investigated under disinfection conditions. In chlorinated water it was analysed by LC (HPLC)/MS and HPLC-

MS/MS. Identification by mass spectra of the reaction mixture of DHHB and NaOCl (2.5 eq) revealed formation of new by-products with m/z ratio 404 and 406, indicating the presence of chlorine atom in the molecule. Mass of 404.167 units corresponds to $C_{22}H_{27}NOCl^+$ ion. The another by-products with ion masses of 432.194, 302.055, 274.064 units belongs to $C_{24}H_{31}NO_4Cl^+$, $C_{16}H_{13}NO_3Cl^+$, $C_{15}H_{13}NO_2Cl^+$ ions respectively. Formation of different chloro and dichloro products is explained in details (Grbović et al., 2013).

2.4 Avobenzone

2.4.1 Physical-chemical properies

Avobenzone was, for a long time, the only UVA-blocking compound available commercially, until other UVA sunscreens, such as Uvinul A+ (DHHB, diethylamino hydroxybenzoyl hexylbenzoate) or Tinosorb M and S (BEMT, bis-ethylhexyloxyphenol methoxyphenyltriazine and MBBT, methylene bis-benzotriazolyl tetrametylbutylphenol, respectively) became available on the market (Huong et al., 2008).

Avobenzone or butyl methoxydibenzoylmethane is a commonly used UVA absorber (320 – 400 nm) with phenyl ketone group. It was approved by the United States Food and Drug Administration (FDA) in 1998. Avobenzone is known to be one of only three active sunscreen ingredients available that protect skin from the entire UVA spectrum (Jing et al., 2008). It could absorb both rays, UVA (which cause long term skin damage) and UVB (causes sunburn).

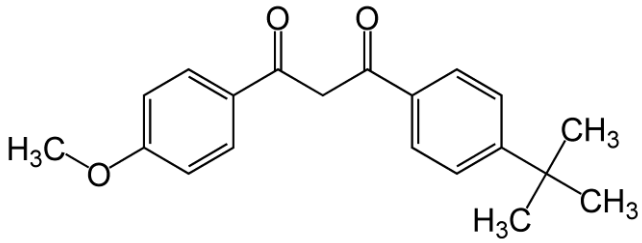


Figure 3: Chemical structure of avobenzone

Table 1: Physical - chemical properties of avobenzone

Name	avobenzone
Other names	butylmethoxydibenzoylmethane;4-tert-butyl-4'-methoxydibenzoylmethane
IUPAC name	1-(4-Methoxyphenyl)-3-(4-*tert*-butylphenyl)propane-1,3-dione
Molecular formula	$C_{20}H_{22}O_3$
Molecular mass	310.39 $gmol^{-1}$
Appearance	colourless crystal
Melting point	80 – 85°C
Specific gravity	1.037 – 1.041
Solubility in water	Insoluble
Stability	Stable under normal conditions.

2.4.2 Application

Avobenzone has been authorized for use in cosmetics by the European Commission for Health and Consumers with the Cosmetics Directive 93/47/EEC. Permitted amounts of avobenzone in sunscreen products reach the amount of 5 % (table 2). But the true value of permitted UV-filters is less than the permissible. In this way, better safety can be provided due to several known undesirable dermatological side-effects caused by the use of sunscreen (Funk et al., 1996, Schauder et al., 1997; Berne et al., 1997; Cook et al., 2002).

Table 2: Avobenzone allowed in cosmetic products (Source: European Commission Health and Consumers:
http://ec.europa.eu/consumers/cosmetics/cosing/index.cfm?fuseaction=search.results&annex_v2=VI&search

Chemical name / INN / XAN	Name of Common Ingredients Glossary	CAS Number	EC Number	Maximum concentrati-on in ready for use preparatio n
1-(4-tert-Butylphenyl)-3-(4-methoxyphenyl) propane-1,3-dione / Avobenzone	BUTYL METHOXYDIBENZOYL METHANE	70356-09-1	274-581-6	5 %

From the study of Poiger et al. (2004) it can be concluded that avobenzone has been found to be the most frequently used UV filter in Denmark (Poiger et al., 2004). Furthermore it is used in 40 % of the sunscreens. From the table 3 it could be seen that avobenzone is used in sun-screening pharmaceutical skin care products and in many highest selling perfumes, eau-de-toilettes for men and women (SPT, 2007).

Table 3: Presence of avobezone in sunscreens (in %) in different countries (Eriksson, 2008)

Presence of avobenzone in sunscreens and other cosmetics in Denmark and other countries (in %)		
Country	Presence in personal care products	avobenzone
DK	Lip stick	
	Perfume	29
	Shampoo and hair care	
	Sunscreens	41
	Sunscreens (DTU)	40
DE	Anti – aging	
	Perfume	
	Shampoo	
	Sunscreens	
CH	Sunscreens	81
US	Anti – aging	1
	Perfume	5
	Skincare incl. sunscreens	29

2.4.3 Photostability of avobenzone

Avobenzone in sunscreen products is not photoprotective, because of its photoinstability. Many studies have shown that exposure of avobenzone to natural or artificial sunlight may lead to photodegradation reactions that can change its physical properties. It is known that avobenzone forms photo-products (by-products) that absorb mainly in the UVC region, depending on the solvent (Georges et al., 2008).

It exists in the enol form, which absorbs in the UVA region or in the keto form. Under irradiation the enol form is photoisomerised to the keto form, where a large loss of absorption was observed.

The keto form appears only in one geometric form and absorb in UVC region, between 260 – 280 nm. While the enol form has many geometric configurations. The enol form also exists in two isomeric form; cis enols, and are stabilised by the hydrogen bond. It may exist in solid or aqueous phase. Such forms may be the result of rotation around a single bond (between carbon C8 and C9 in first enol form, C7 and C8 in second enol form) or isomerisation at the double bond in first enol form (between C7 and C8 and in second enol form (between C8 and C9) (Cantrell et al., 2001).

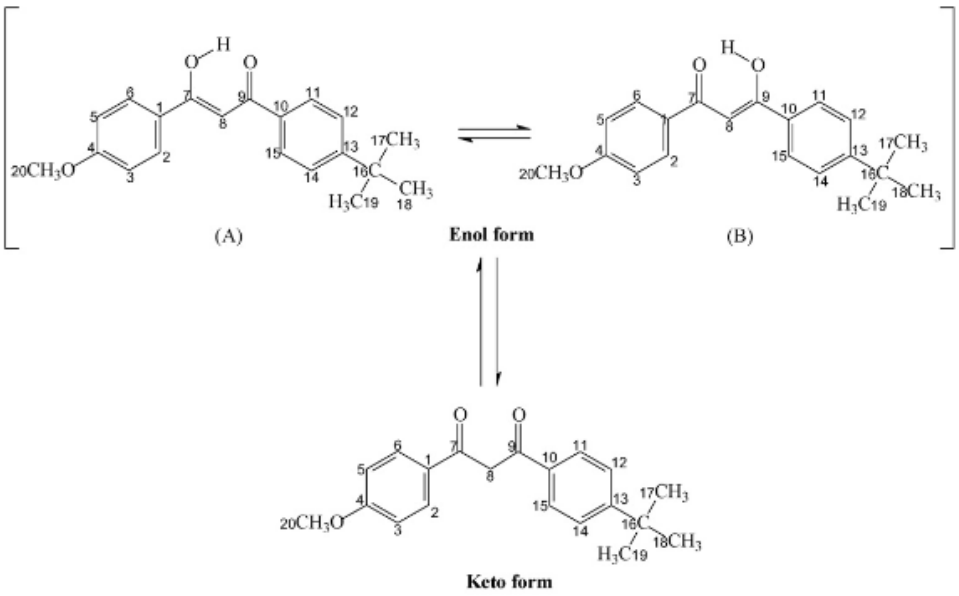


Figure 4: The keto-enol tautomerism of avobenzone (Cantrell et al., 2001)

A study performed by Schwack and Rudolph (1995) reported numerous photoproducts; dibenzoyl ethanes, dibenzoylmethanes, and substituted benzoic acids. The formation of these photoproducts involves primary α-bond cleavages of carbonyl groups of the 1,3-diketo form, followed by either hydrogen abstraction, oxidation and/or radical recombination.

2.4.4 Avobenzone transformation products - overview

Photostability of avobenzone in cyclohexane was investigated in the study by Schwack and Rudolph (1995). Because of recombination or oxidation reaction many photoproducts such as benzaldehydes, benzoic acids, phenylglyoxals, acetophenones, benzils, dibenzoylmethane and a dibenzoylethane were detected. All of these products are the result of α-cleavage of the keto form of avobenzone (Mturi and Martincigh, 2008).

Some of these radicals may be the result of the formation of dibenzoyl ethane and 1,4-bis (4-methoxyphenyl)butane-1,4-dione). In the study, the ester which was detected, was set of photoproducts of avobenzone and cyclohexane (Schwack and Rudolph, 1995). Therefore GC–MS analysis showed that avobenzone photodegraded from the keto form (Mturi and Martincigh, 2008).

Photolysis of avobenzone was studied by Roscer et. al. (2005) and Schwack and Rudolph (1995). In the first paper the authors identified derivatives, such as t-butyl and methoxy-benzene derivatives. On the other hand, Schwack and Rudolph (1995) noted two products of photolysis: unsymmetrical 1,2 - and 1,4-diketones, which initially loose protection against UVA rays. However, the experimental conditions for both studies varied and were not comparable.

Huong et.al (2008) studied the photoreactivity of the UV filter avobenzone under various experimental conditions. Avobenzone shows as a stable compound in dioxane, acetonitrile, ethyl acetate, THF, ethanol and isopropanol. Furthermore, they

determined that, if avobenzone was located in a non-polar medium, and this compound is also irradiated, it does not form degradation products.

Using LC-MS, degradation products in the sample were detected. From figure 5, it could be seen that there are seven products, with molecular mass 284, 152, 178, 298, 378, 326, 296 and 310. There is just one, that has a molecular mass greaterr than avobenzone, 326. This larger value of molecular mass is due to the addition of oxygen atoms in the molecule.

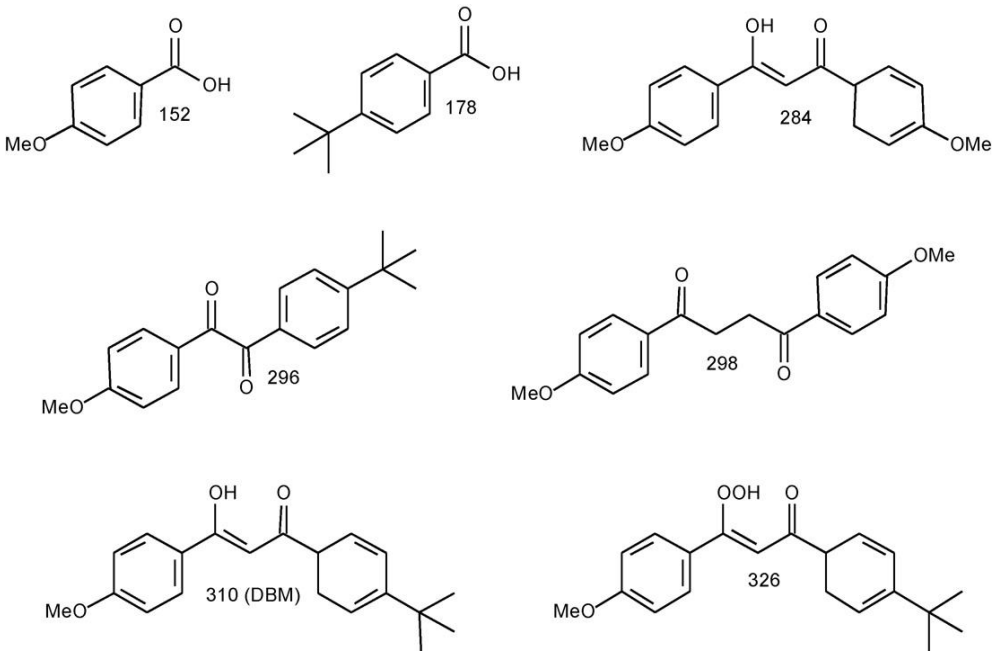


Figure 5: Photodegradation products of avobenzone in water (Huong et al., 2008)

2.4.5 Avobenzone and toxicity

Most sunscreens currently contain chemical UV filters, which provide protection against the sun. However, this increases the possibility of population exposure to this chemical substances and thus raises concerns regarding the use of these products.

Organic UV filters cause negative effects to the human body, due to the direct contact of these compounds and their byproducts. UV filters may penetrate the human skin and were found in human urine. In swimming pools in the presence of solar light they may form byproducts with potentially more harmful effects than the parent compounds. There are a significant number of studies reporting on allergic and photoallergic reactions to UV filters on the human skin. It was reported that allergic reaction to the active ingredient in creams, photoallergic contact dermatitis and allergic contact dermatitis in humans are very rare, although 20 % of people observed allergic reactions in the case when they used protective cream with sun protection factor 15 and over a period of seven months (Foley et al., 1993).
Avobenzone was shown a causal agent in these allergic and photoallergic reactions. Furthermore it could lead to the formation of new molecules with unknown toxicological properties (Schauder and Ippen, 1997; Lodén et al., 2011).

Very little is known about the fate of UV-filters in the environment. To ensure appropriate UV protection, three to eight different UV filters are usually added into sunscreen. Therefore, the risk of accumulation of some UV filters in fish and humans increased, because of their lipophilic nature, quite similar to the nature of polychlorinated biphenyls and dichloro-diphenyl-trichloroethane. An example is a study by Gonzalez et al., (2002), in which 0.5 % of the overall amount of benzophenone-3 in the human urine of volunteers who have been exposed to UV filter for 48 hours was detected. Toxicological effects of UV-filters on aquatics organisms are very heterogeneous. There is some concern over possible endocrine-disrupting and long-terms effects on living organisms. Some studies confirm the presence of UV filters in fish from recreational lakes. (Dimosthenis et al., 2007).

From the study of Kunz and Fent, (2006), it was found out that the mixture of 19 UV filters caused abnormal hormonal activity, among which the most expressed ones were: antiestrogenic and antiandrogenic activities of phenyl and benzyl salicylate, benzophenone-1 and benzophenone-2, and of 4-hydroxybenzophenone (Kunz and Fent, 2006)
The European Union Cosmetics Advisory Committee confirms that sunscreens, presently on the EU market, do not exhibit any estrogenic affects. This statement was denied by the study of Heneweer et al., (2005), where they proved that certain UV filters (OMC, octyl dimethyl PABA, and 4-methyl-benzilidene canphor) activated estrogen receptors.

Under the influence of sunlight and the presence of sunscreens reactive oxygen species (ROS), such as hydroxyl radicals (OH•) may be formed as well. In one of the studies on animals it was reported that these radicals can cause nucleated epidermis, depending on the type of used UV filter and the persistence of the skin. This study confirmed the development of cancer, instead of being preventative (Wang et al., 2001).

Recently, there is an increased demand for water-resistant sunscreens. More lipophilic substances enhance their dermal absorption. Microfine metallic oxides that are used in sunscreens and are capable of absorption through the skin could cause adverse effects on human health. However in one of the studies it was found that

ZnO or TiO_2 particles are not capable of penetration of the porcine stratum corneum (Gamer et al., 2006; Borm et al., 2006; Schulz et al., 2002).

Furthermore, toxicity testing of DHHB and chlorinated products (3-chloro and 5-chloro DHHB) were performed with liquid- dried bacterial. EC_{50} for 3-chloro DHHB was 0.83 mg/L and for 5-chloro DHHB, 0.85 mg/L. After 30 minutes of exposure in the Suntest apparatus 20 % of luminescence inhibition was detected. (Grbović et al., in submission).

2.4.5.1 Toxicity testing with luminescence bacteria ***V. fisheri***

The most commonly used toxicological tests are tests with bacteria. They are characterized by the easiness and speed of the assays. Bacteria have an integral part of the cellular system and most chemicals exert their affects by interfering with common cellular process (i.e. energetics, macromolecular synthesis, etc.) (Cronin and Schultz, 1996).

Bacterial toxicity tests (Block et al., 1989) are divided into five groups:

- bioluminescence assays
- respiration assays
- substrate consumption assays
- energy assays
- population growth assays;

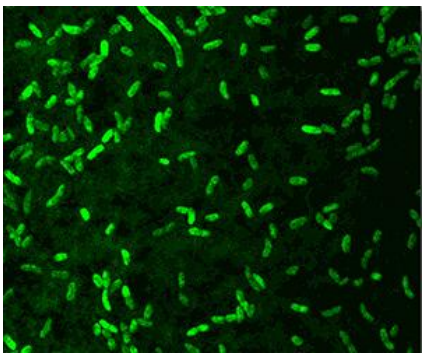


Figure 6: V. fisheri bacteria (E. Nelson and L. Sycuro, source: http://microbewiki.kenyon.edu/index.php/File:Vibrio_fischeri_1145457864.jpg)

One of the possible methods for determining the toxicity and evaluating the bioactivity of photodegradation products in sunscreens is the test with the luminescent bacterium *V. fischeri.* This is a gram negative bacterium with bioluminescence properties. It is used in order to check the toxicity of the bacterium based on its bioluminescence measurements. The method is used for testing the toxicity of water, sediment and soil. Bacterial bioluminescence is directly related to cellular respiration, so any change in cell activity with altered level of breathing, affects the amount of light emitted. The more toxic the sample, the less light bacteria emits in the test suspension. So the level of inhibition is proportional to toxicity.

2.5 Determination of UV filters

For the process of determination of UV filters, the first step usually applied is extraction/preconcentration step followed by chromatographic and mass spectrometry analysis. There are some analytical techniques for samples preparation, including liquid-liquid extraction (LLE), solid-phase extraction (SPE), stir bar sorptive extraction (SBME) liquid-liquid membrane assisted extraction (Mall), single drop microextraction (SDME) and micelar-mediated extraction (MME) (Giokas et al., 2007 and Peck, 2006).

In 1972 the first article about detection of UV-filters was published, where gas chromatography played an important role (GC) (Paulus et al, 1972). Following this technique cinnamates and salicylates were identified (Paulus et al., 1972).

However, the GC is not used often, because for the identification of compounds with GC, volatilization and termostability are necessary. Furthermore, for the UV-filter it should not be forgotten that they have a relatively high boiling point. For that reason GC analysis can be used for identification (Masse et. al., 1982, Paulus et al., 1972) of only some ionizable UV filters, such as PAB or sulfonic acids (PBS BZ4, TDS). They are characterized by low volatility. Their volatility and sensitivity may be increased through derivatization (Cumpelik 1982, Ro et al., 1994). On-line identification of the UV filters present in a cosmetic formulation (Masse et al., 2001, Ikeda et al., 1990, Ro et al., 1994) could be achieved by combination of GC with mass spectroscopy detection (GC-MS). Moreover, the flame ionization detection (FID) was also used.

For quantitative determination of UV filters it was preferred to use liquid chromatography (LC), in order to cope with low volatile substances. In liquid chromatography, some solvents for reversed-phase separations were used, such as water, acetonitrile (MeCN), methanol (MeOH) and tetrahydrofurane (THF) or combinations of them. Isocratic or gradient elution modes can be used.

Another technique used is UV/Vis spectrometry with single wavelength or with diode-array detection. For UV filters it is known to have significant absorbance in the UV range. The whole UV spectrum for each peak could be seen through diode-array detector (DAD), and this has been used for identification purposes (Rastogi et al., 1998)

In the study Lambropoulou et al. (2002), GC was used with FID detection and GC-MS for the determination of BZ3 (Benzophenone-3) and EDP (2-ethylhexyl 4-(N,N-dimethylamino)benzoate) at ng/ml level in water samples, such as swimming pool water, shower waste and seawater. Solid-phase microextraction (SPME) was used for the extraction of selected compounds.

Furthermore, GC-MS was also used for the determination of EDP by-products coming from chlorinated waters (Sakkas et al., 2003). The same authors studied the formation of EDP by-products in chlorinated waters (Sakkas et al., 2003).

3. EXPERIMENTAL WORK

3.1 Reagents and solutions

- Avobenzone, product of USA, Sigma-Aldrich,
- Double deionised water (<18 MΩ cm) was prepared by NANOpure use of the water system (Barnstead, USA)
- Acetonitrile, Sigma Aldrich, for HPLC > 99.9 %,
- Trichloroisocyanuric acid (97 % purinity), from Aldrich
- Methanol
- Sodium chloride from Carlo Erba Reagenti.

3.2 Sample preparation

3.2.1 Sample preparation for SUNTEST

a.) Higher concentration solution for preliminary experiments

Stock solution of avobenzone was prepared by dissolving of 12.5 mg in 50 mL of acetonitrile. For further experiments this solution was diluted by deionised water (50 mL). For Suntest experiments I prepared 500 mL of solution with the final concentration of 125 mg/L.
Avobenzone – 500 mL stock solution – was diluted with water containing TCCA. Final concentration trichloroisocyanuric acid in water corresponded to 4 mg/L. This solution was irradiated in the Suntest apparatus for different time intervals.

b.) Lower concentrations solution

Stock solution of avobenzone was prepared by dissolving of 1.25 mg in acetonitrile. and diluted by deionised water. The final concentration was 1.25 mg/L avobenzone in water solution.

Avobenzone – 500 mL stock solution – was diluted with water containing TCCA. Final concentration trichloroisocyanuric acid in water corresponded to 4 mg/L. This solution was irradiated in the Suntest apparatus for different time intervals.

3.2.2 Sample preparation for LUMIStoX

First of all I measured the pH of all irradiated samples. If the pH was between the values 6.8 and 7.2 additional adjustment was not needed. If it was not, acid or base, depending on the pH of the sample, was added.

Bacteria are living in marine environment so to the samples sodium chloride was also added, in order that the concentration suit 2% (w/v) NaCl in water. For the test termoblock was used at 15 ± 0.1° C (DR LANGE LUMIStherm Thermostat).
Luminescence was measured in three parallels, after *V. fisheri* was added into each vial. The first vial represented the control, where 2 % of sodium chloride solution was standard. Luminescence was measured in the luminometer (LUMIStox 300). After

measuring the samples they were placed back on the thermostat at temperature 15 ± 1 ˚C for 30 minutes. After 30 minutes of incubation, luminescence of bacteria was again measured and results were presented as luminescence inhibition. It was also measured EC_{50} values for aqueous solution of avobenzone and trichloroisocyanuric acid (TCCA) were also measured.

3.2.3 Sample preparation for HPLC – DAD method development

For the HPLC method developments I used the avobenzone aqueous solution at a concentration of 25 mg/L.

3.2.4 Sample preparation for LC – MS analysis of chlorinated products

Firstly it was prepared avobenzone in water solution, with concentration 50 mg/L.
Furthermore, it was also prepared solution of TCCA, diluted with water, with final concentration 10 g/L.
Prior to the addition of TCCA, the aqueous solution of avobenzone was divided into five 100 mL flask and supplemented by the addition of 185 µL, 370 µL, 555 µL and 925 µL of TCCA solution that correspond to 0.5 equivalent; 1 equivalent; 1.5 equivalent and 2.5 equivalents respectively.

3.3 Suntest apparatus

Samples of avobenzone in aqueous/ chlorinated solution were irradiated in the chamber (apparatus Suntest XLS +), which via SUNSENSIV ™ sensor mimics solar radiation. With the Suntest apparatus I wanted to determine the stability of avobenzone under certain conditions; temperature and intensity.

Solar radiation in Suntest was managed through a SUNSENSIV ™ sensor in a radiation area 300 - 400 nm/340 nm or 300 – 800 nm/LUX. The device installed was a 1500 W xenon arc lamp. The built-in sensor allowed direct identification and control of body temperature radiation, covering the temperature range 45 °C BST. The entire surface intended for exposure or irradiation in the Suntest device comprises 1.100 m².

500 mL of the tested formulations was spread onto a 79 cm² glass plate, made of borosilicate glass. After about 30 minutes the solution was exposed to UV radiation in Suntest. Meanwhile the solution was stirred with a magnetic stirrer and warmed, in order to increase the solubility. Suntest for my samples simulated a sunny summer day at an intensity of 750, 500 and 250 W/m² of sunlight and the actual temperature was 20.25 °C. Wavelength range of the lamp used in all experiments was 300 – 800 nm. Samples in Suntest were exposed to sunlight at intervals; 30 min, 60 min, 90 min, 120 min and 180 min. The samples were protected from UV light before and after irradiation by aluminium foil.

Samples of avobenzone in aqueous and chlorinated solution were also prepared for photodegradation experiments running on the laboratory desk under ambient conditions and in the dark within aluminium foil.

After irradiation all samples were analysed with a UV-Vis spectrophotometer, HPLC-DAD and toxicity was measured with LUMIStox.

Figure 7: Prepared samples in the Suntest apparatus

3.4 UV–Vis measurements

Immediately after I had taken the samples for the Suntest apparatus, the sample absorbance was measured. The absorption spectra of avobenzone in aqueous solution were measured with the UV-Vis Hewlett Packard 8453 spectrophotometer from 220 to 600 nm.

3.5 HPLC- DAD analysis

For the determination of avobenzone in aqueous/chlorinated solution I used the HPLC-DAD instrument Agilent 1100. Separation of different conmponents was done on a Luna C18 column, 150 mm length and 4.6 mm diameter. Pore size was 3 µm and the detection wavelength was 350 nm. The calibration curve for quantification purposes in the range from 10 to 1000 mg/L was conducted.

Table 4: Chromatographic conditions for HPLC–DAD analysis.

column	Luna C18
volume injection	50 µL
detector	diode array (350 and 272 nm)
flow rate	1 ml/min
mobile phase	A: acetonitrile 85 % B: deionised water 15 %
time analysis	20 min
temperature analysis	24.4 ℃

3.6 LC - MS

The HPLC-UV-MS analyses were performed using a Waters Alliance 2695 (Waters SA, St-Quentin en Yvelines, France) photodiode array detector (DAD) chromatograph. A reversed-phase column distributed by Phenomenex (Kinetex MS C18, 2.6 µm, 100 mm × 2.1 mm) was used at a flow rate of 0.2 mL/min. The mobile phase was composed of acetonitrile (solvent B) and acidified water (formic acid, 0.1% v/v; pH 2.6) (solvent A). To ensure a better separation, a gradient program was used:

Table 5: The gradient program for the liquid chromatography separation.

Time [min]	Water %	CH_3CN	Flow	Curve
0.00	40	60	0.2	1
6.00	10	90	0.2	6
15.00	10	90	0.2	6
20.00	40	60	0.2	6
25.00	40	60	0.2	6

Avobenzone solutions were injected without any further treatment and the injection volumes were 10 and 40 µL for LC/ESI-MS and MS/MS experiments respectively, according to Grbović et al. (2013).

3.7 pH measurements

The pH was monitored using a pH meter, Hanna Instruments HI 8417.

3.8 Toxicity testing with *V. fisheri*

For toxicity testing of the avobenzone liquid dried luminescent bacteria *V. Fisheri (12261)* was used. Through LUMIStox 3000, Dr. LANGR or luminometer light emission/luminescence could be measured, by using the technical requirements of ISO 11348. LUMIStox 3000 has the ability to recognize colour affects in the luminescent bacteria test (Dindal et al., 2010) (http://www.epa.gov/etv/pubs/600etv10026.pdf).

ISO 11348-3 determines the EC value, where it was used solution concentrations with inhibition values between 10% and 90%, by following a dilution scheme. This includes a series of nine dilutions ranging from 1:2 to 1:32. The results are EC_{20}- and EC_{50}-values.

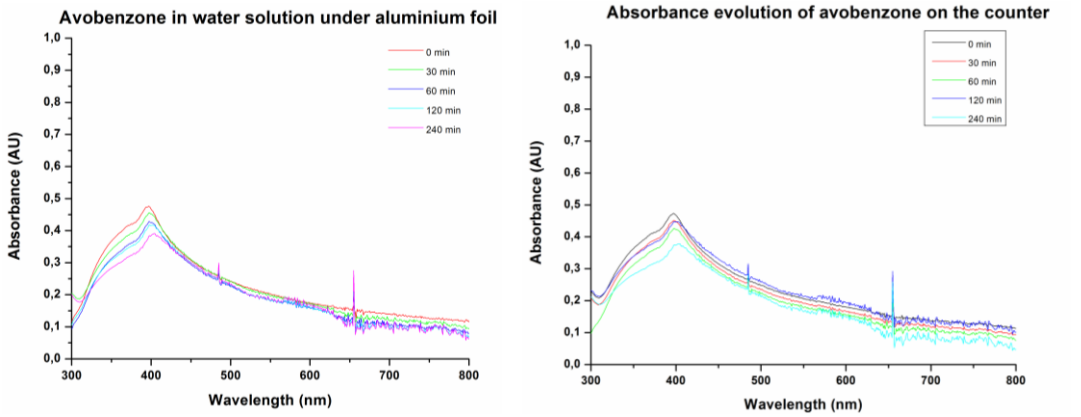
4. RESULTS AND DISCUSSION

4.1 Preliminary UV–Vis spectrophotometer measurements

Samples containing avobenzone in deonised water/chlorinated water, using solvent methanol, were irradiated at various time intervals 30, 60, 120 and 240 minutes in Suntest apparatus. While these samples were irradiated in the apparatus the same prepared samples were left on the counter under the influence of wall lamps, under aluminium foil and the third was left in the dark. Finally, the absorbance of each sample was measured in the UV-Vis spectrophotometer.

4.1.1 Background experiments

8A 8B



8C 8D

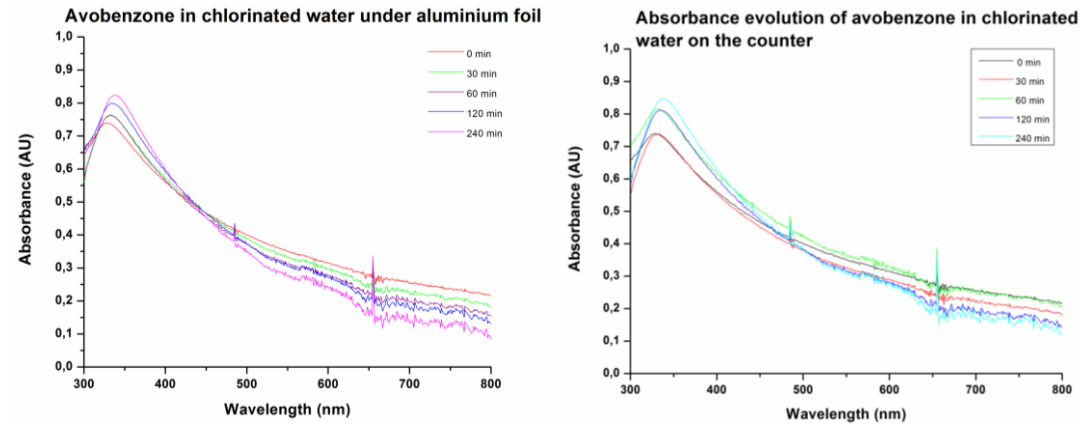


Figure 8: Evolution of UV-Vis absorbance spectra of avobenzone in aqueous/chlorinated solution; 8A: UV-Vis absorbance spectra of avobenzone in deionised water under aluminium foil, 8B: UV-Vis absorbance spectra of avobenzone in deionised water on the counter, 8C: UV-Vis absorbance spectra of avobenzone in

chlorinated water under aluminium foil, 8D: UV-Vis absorbance spectra of avobenzone in chlorinated water on the counter.

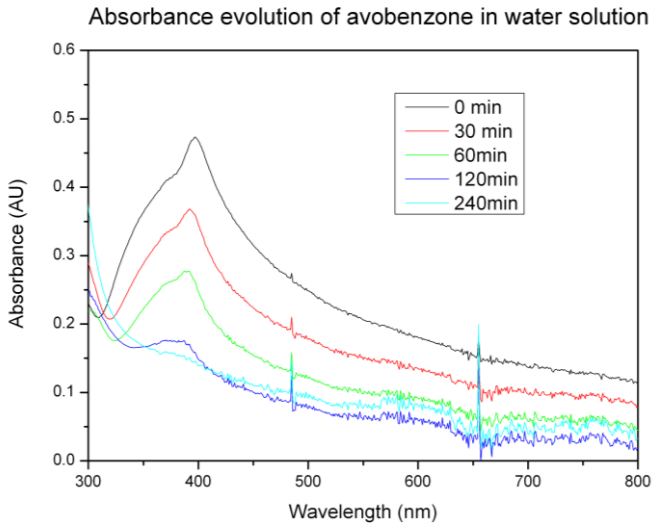
It can be seen that there were minimal changes in the photodegradation of avobenzone under experimental conditions. Avobenzone has its absorption maximum at 397 nm, with chlorination process the absorption maximum was shifted to 329 nm.

4.1.2 SUNTEST experiments

Figure 9A shows the results of UV–Vis spectra of lowest concentrations of avobenzone in deionised and chlorinated water exposed in the Suntest apparatus for three hours. When I irradiate the samples in Suntest, avobenzone shows great sensitivity toward the sunlight.

From the figure 9B, it can be observed that the addition of TCCA changed the absorption spectra (shift toward lower wavelengths) followed by the decrease of absorbance spectra with irradiation time. New compounds formed by chlorination, absorb light at the wavelength 329 nm and not at 397 nm like avobenzone.

9A



9B

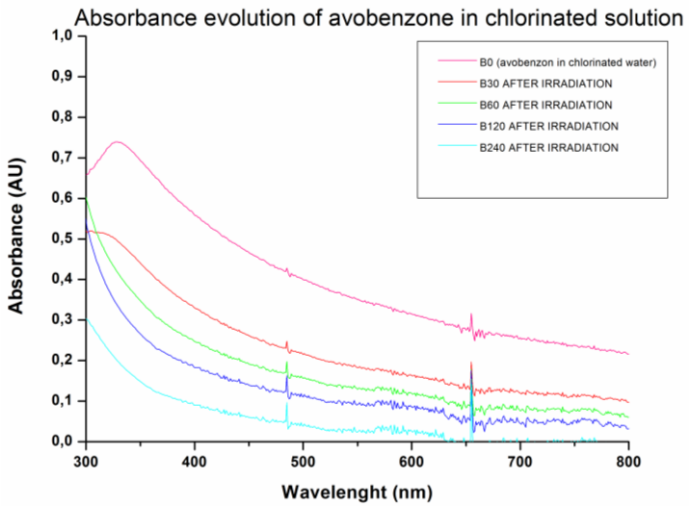


Figure 9: Evolution of UV-Vis absorbance spectra of avobenzone in aqueous solution after Suntest irradiation; 9A: Evolution of UV-Vis absorbance spectra of avobenzone in chlorinated solution after Suntest irradiation; 9B.

Firstly I prepared avobenzone samples in methanol. After irradiation experiments I had to face problems connected with the HPLC-DAD measurements, since avobenzone products were trapped in the guard column and did not reach the detector. From that reason I decided to change the solvent. All experiments were repeated then in acetonitrile and then diluted with deionised water. All results are collected together in section 4.2.

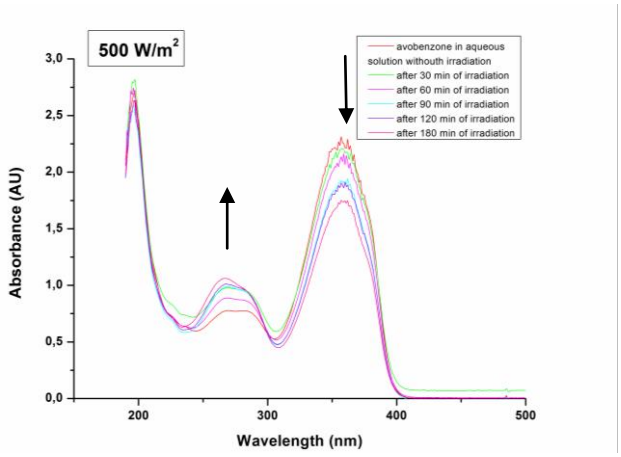


Figure 10: Evolution of UV-Vis absorbance spectra of avobenzone in aqueous solution after three hour in SUNTEST apparatus at light intensities 500 W/m^2.

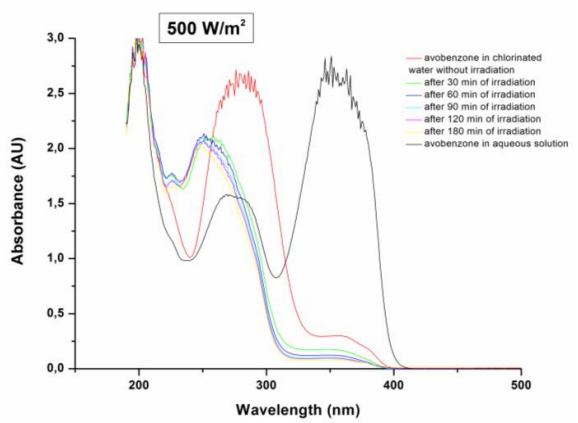


Figure 11: Evolution of UV-Vis absorbance spectra of avobenzone in chlorinated solution after three hour in the Suntest apparatus at light intensity; 500 W/m^2.

Avobenzone in acetonitrile has maximum in absorption spectra at 350 nm. It may exist in two tautomeric form; enol and keto form. Under irradiation, the enol form is photoisomerised to the keto form, which absorb in the UVC region from 260 to 280 nm (Georges et al., 2008). From my study it was observed that keto form under irradiation was increased (peak at 272 nm) and the enol form was decreasing (peak at 350 nm).

4.1.3 LUMIStox results

All samples that were exposed in the Suntest apparatus were also tested for toxicity with the luminescence bacteria *V. fisheri.* LUMIStox Dr. LANGE was used for detection and it was calibrated using ISO 11348 – 3 standards.

4.1.3.1 Toxicity of avobenzone (125 mg/L) in water after Suntest irradiation

First preliminary experiments were conducted where I tested a higher concentration of the sample (125 mg avobenzone/L). As a solvent has been used acetonitrile because of non-toxic affects on bacteria. These samples were first irradiated in the Suntest apparatus at different time intervals.

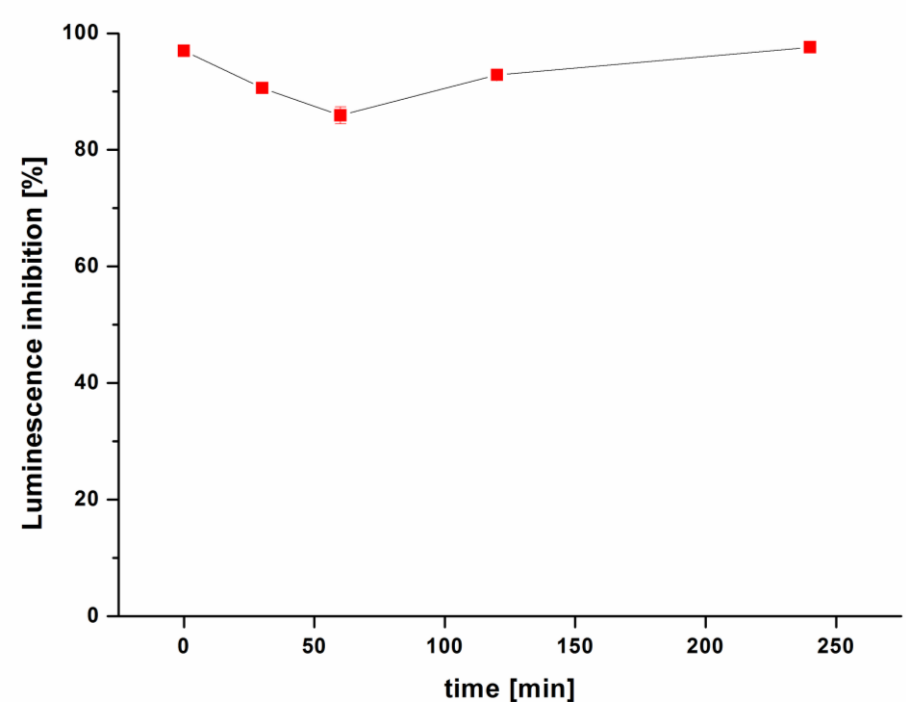


Figure 12: Luminescence inhibition for V. fisheri of 125 mg avobenzone/L.

From figure 12, it can be seen that toxicity of avobenzone in water decreases rapidly with increasing time of irradiation, until it reached one hour of irradiation. After that time, the toxicity again started to increased and reached 97.6 % of luminescence inhibition occurred. I can conclude that after prolonged irradiation, the sample formed toxic by-products.

4.1.3.2 Toxicity of Avobenzone (125 mg/L) in chlorinated water after Suntest irradiation

Secondly I also tested avobenzone (125 mg/L) in chlorinated water (4 mg/L).

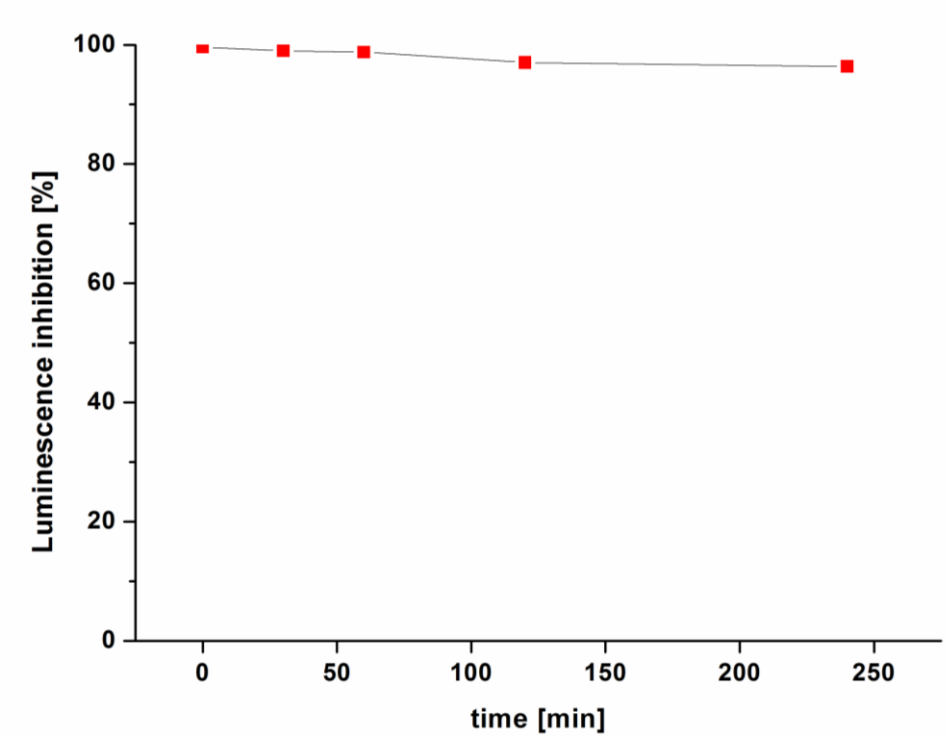


Figure 13: Luminescence inhibition for V. fisheri of 125 mg/L avobenzone in chlorinated water.

From figure 13 it can be observed, that avobenzone in chlorinated water shows much higher toxicity than in an aqueous solution (99.61 %). The compound slowly decomposes; although after four hours of irradiation it still has high toxicity 96.4 %.

4.1.3.3 Toxicity of avobenzone (1.25 mg/L) in water after Suntest irradiation

Secondly I also tested lower concentrations of 1.25 mg avobenzone/L water. The samples were again irradiated in the Suntest apparatus for different time intervals, where I observe how they behaved under the influence of light.

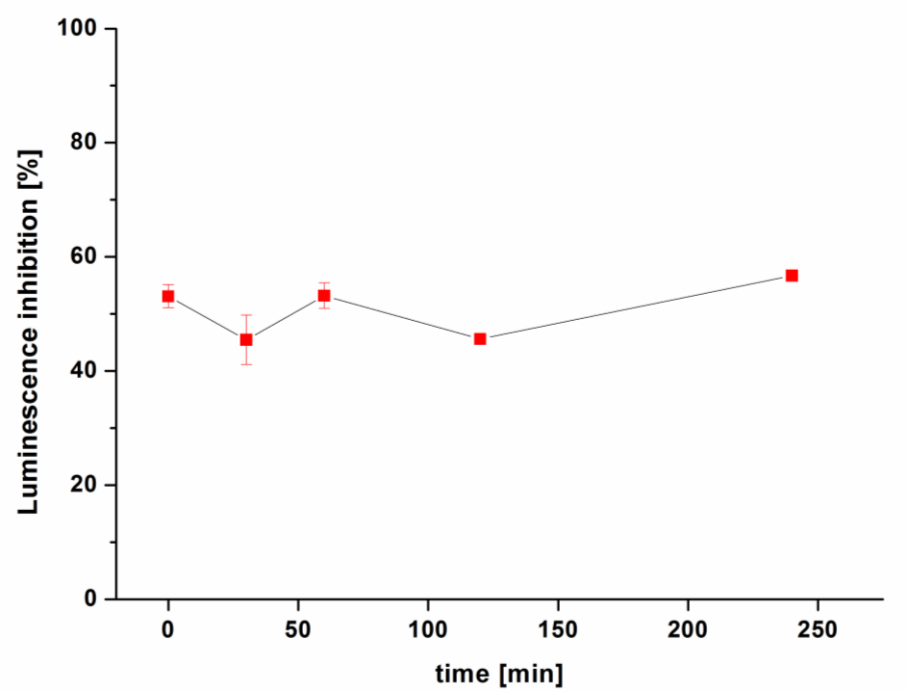


Figure 14: Luminescence inhibition for *V. fisheri* of 1.25 mg/L avobenzone in water.

All samples were prepared in three parallels. Concentration (1.25 mg/L) is considerably lower than in the case of high concentrations (125 mg/L). It can be seen from the figure 14, that the samples had a toxicity of 53.1 % at the beginning. During exposure, the luminescence inhibition varied over the first two hours in the range from 45.4 to 54.1 %. After two hours the luminescence inhibition stared to increase to inhibition 57.1 %. It can be observed that when I irradiated small amount of UV-filter the sample was less toxic, which was to be expected.

4.1.3.4 Toxicity of avobenzone (1.25 mg/L) in chlorinated water (4 mg/L) after Suntest irradiation

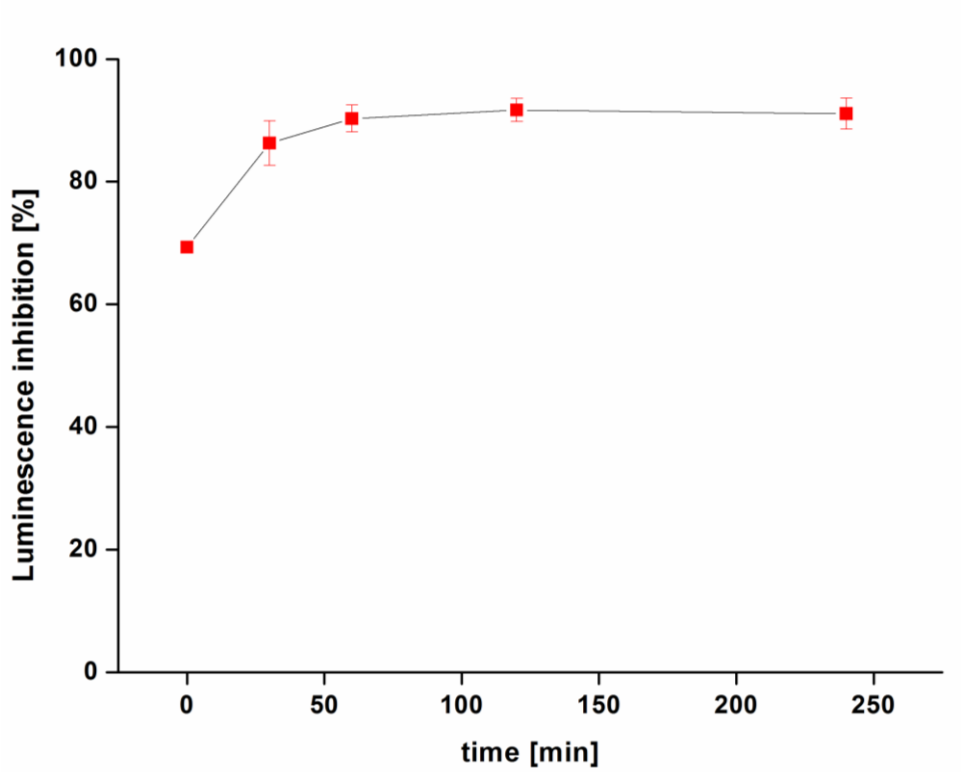


Figure 15: Luminescence inhibition for *V.fisheri* of 1.25 mg/L avobenzone in chlorinated water

The results of luminescence inhibition measurements in the presence of avobenzone in chlorinated water are presented in figure 15. The initial toxicity of the sample was 69.3 %, which is higher that the sample of avobenzone in water. After irradiation, the sample showed great luminescence inhibition which, with time, started to increase. After four hours the sample reached 91.1 % toxicity. It could be concluded that, under the influence of radiation toxic chlorinated by-products may be formed.

4.1.3.5 EC50 value of 125 mg avobezone/L water

After toxicity testing of samples irradiated in Suntest, I also determined EC_{50} value for avobenzone. The EC_{50} value represents the concentration of avobenzone in water, where 50 % of the population dies, after being exposed to the chemical. From EU legislation, the toxicity values are based on EC_{50} values.

Figure 16 presents the dose response curve for the avobenzone aqueous solution. EC_{50} value was determined as 28 mg/L.

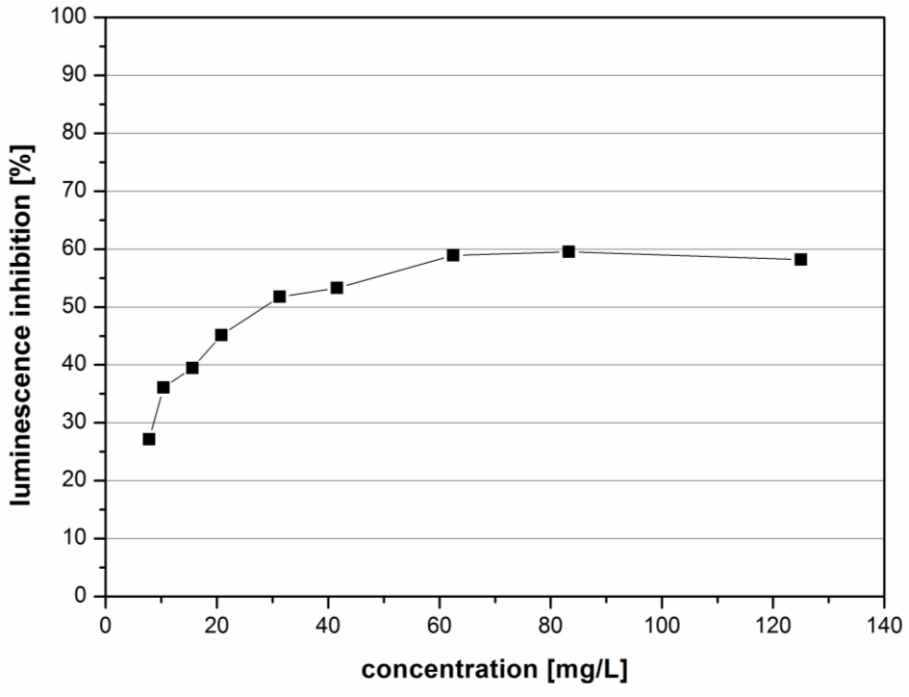


Figure 16: Dose response curve for aqueous solution of 125 mg/L avobenzone in aqueous solution for V. fisheri luminescent bacteria within 30 minutes of exposure.

4.1.3.6 EC_{50} value of chlorinated water

From the figure 17 it can be seen that the concentration of 4 mg/L of TCCA in water take 64.04 % of luminescence inhibition. EC_{50} value was 1.95 mg/L.

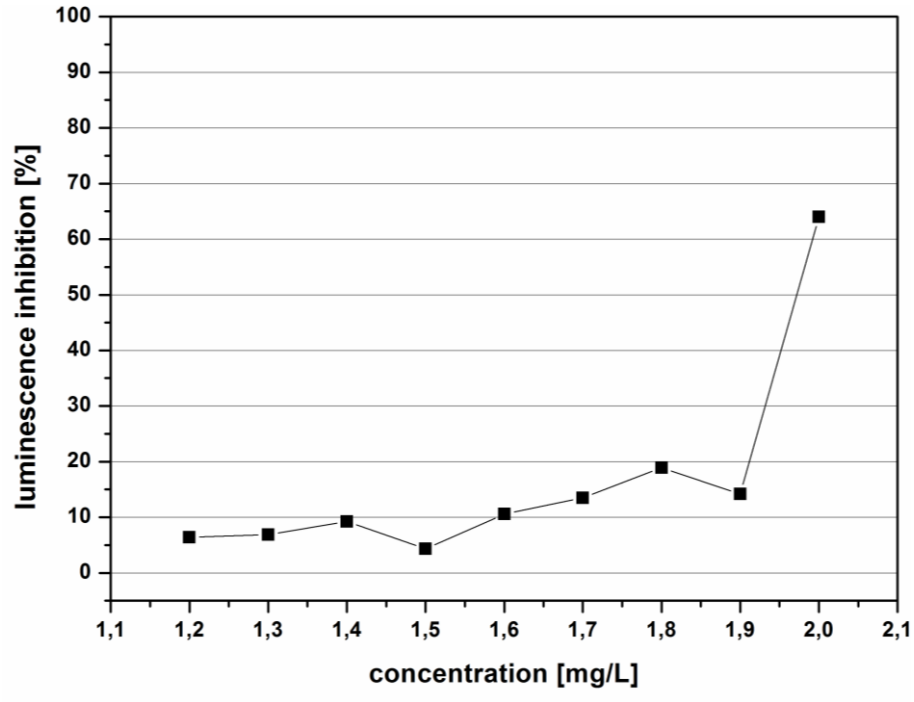


Figure 17: Dose response curve for aqueous solution of 4 mg/L TCCA in aqueous solution for V. fisheri luminescent bacteria within 30 minutes of exposure.

4.1.4 HPLC measurements

4.1.4.1 Selection of an appropriate mobile phase and solvent for HPLC analysis

According to the literature data I prepared my samples in methanol (Yang, 2008; Georges, 2008). Avobenzone was dissolved in methanol and water at the ratio 20:80 and placed on a HPLC instrument under the Column Supelco C18, 250 x 4.6, with the mobile phase methanol:water at a ratio of 85:15 and 92:8 as described in articles by Yang, (2008) and Georges, (2008).

By using the HPLC instrument I could not determine avobenzone. It appears that avobenzone was eluted within the first two minutes with the mobile phase. The problem was also excessive high pressure. The pressure depends on the column (particle size, length, diameter…), through which a solvent of the mobile phase and the flow is published. At a flow rate of 0.5 ml/min, the pressure reached a value of 250 bars. Detection wavelength was 397 nm for avobenzone in aqueous solution and 329 for avobenzone in the chlorinated solution.

Finally I tried another solvent and this was acetonitrile. Avobenzone was dissolved in a mixture of acetonitrile:water at a ratio 50:50. On HPLC I placed a column Luna C18, 150 x 4.6 and pore size 3 µm. Mobile phase which was used in this case was acetonitrile:water at a ratio of 85:15 and the pressure reach a value was around 153 bars, with flow rate 1 mL/min. After 15 minutes avobenzone cames out from the column after retention time 7.44 minutes. In this case the detection wavelength was 350 nm for the solution of avobenzone in aqueous solution and 272 nm for the solution of avobenzone in the chlorinated solution.

4.1.4.2 Selection of appropriate quantity of avobenzone for irradiation

On the basis of the calibration curve, conducted in the range from 10 to 1000 mg/L I decided to perform degradation experiments with the starting concentration of 50 mg/L. I irradiated samples for three hours with intermediate intervals and with the application of different light intensities. The results of degradation varied and degradation did not take place gradually.

For that reasons, I decided to repeat the experiments with a lower concentration of 25 mg/L.

In figure 15 chromatogram of avobenzone in aqueous solution with a retention time of avobenzone at 7.447 minutes is shown. The chromatogram was monitored at the wavelength 350 nm.

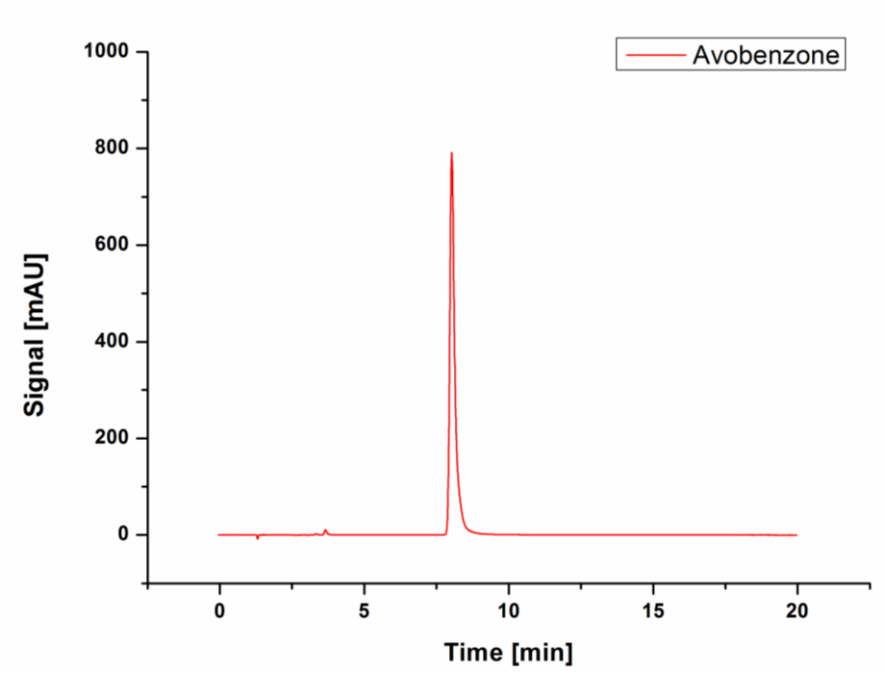


Figure 18: Chromatogram of avobenzone in aqueous solution.

The calibration curve for avobenzone is presented in figure 19. The r^2 value for avobenzone in aqueous solution was 0.999.

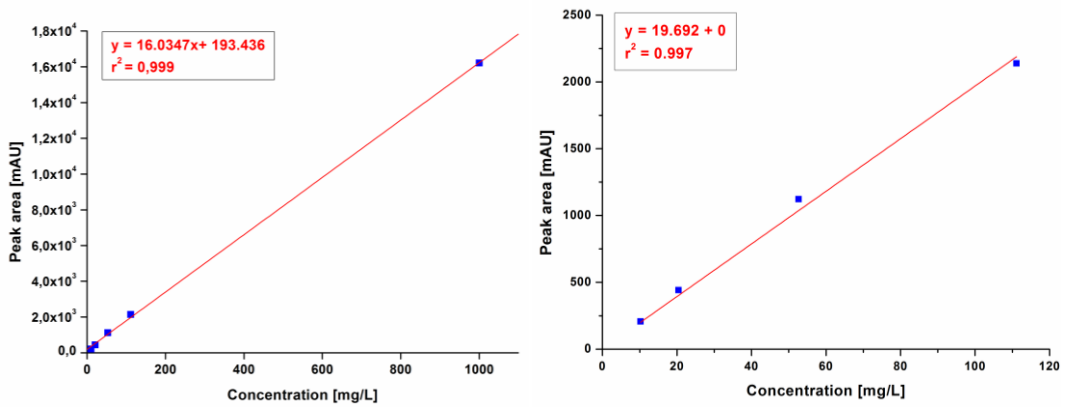


Figure 19: Calibration curve for avobenzone standard solution.

4.2 Main experiments

4.2.1 UV-Vis results

During the experiments absorbance of the avobenzone solutions using UV-Vis spectrophotometer was also measured. Using these results, the results obtained by the HPLC-DAD analysis were confirmed.

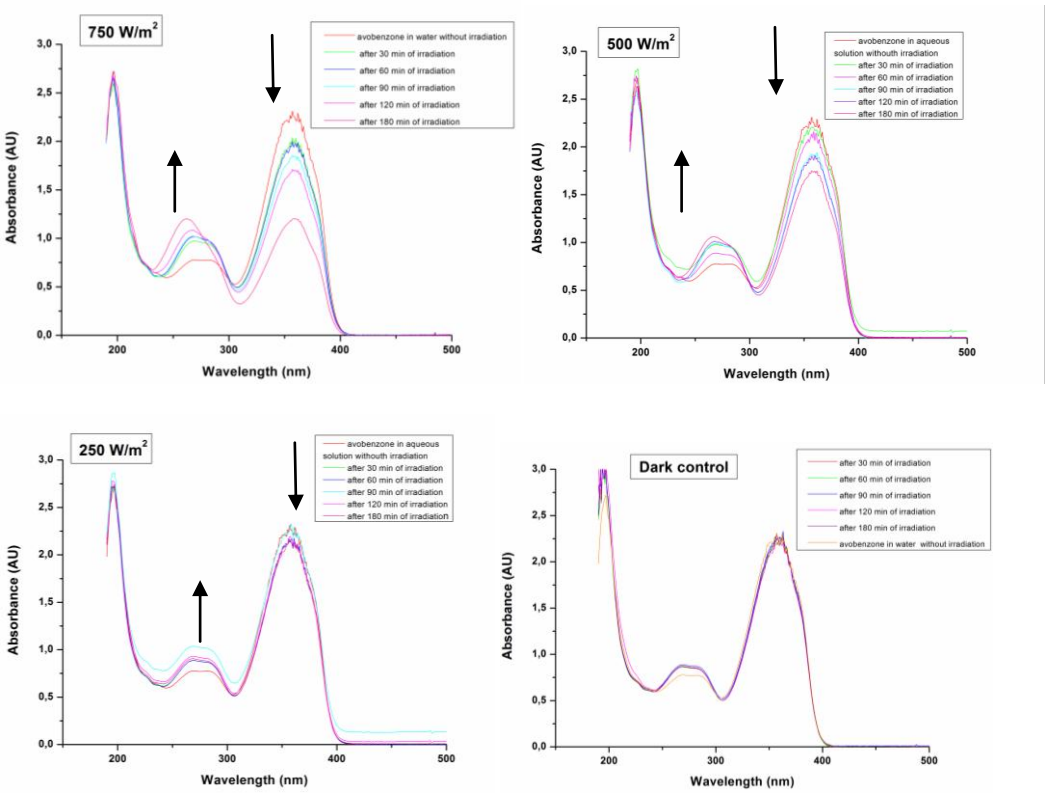


Figure 20: Evolution of the UV-Vis absorbance spectra for avobenzone in aqueous solution after three hour in the Suntest apparatus at different light intensities; 750, 500 and 250 W/m^2.

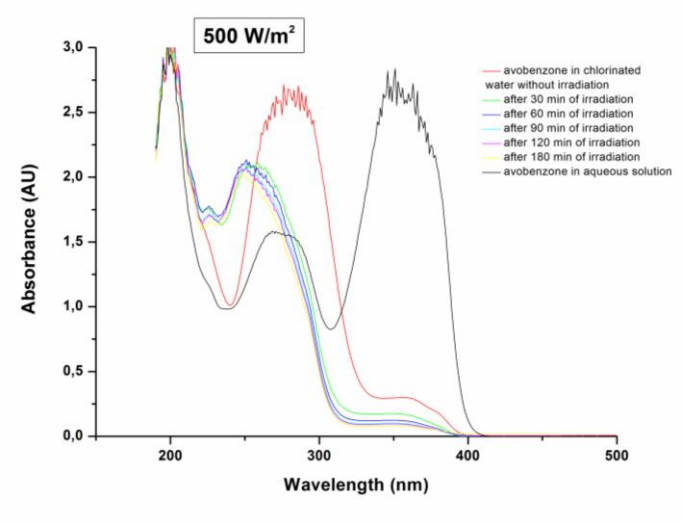


Figure 21: Evolution of the UV-Vis absorbance spectra for avobenzone in the chlorinated solution after three hour in the Suntest apparatus at light intensity; 500 W/m^2.

Avobenzone has maximum absorption at 350 nm dependant on solvent used. It consists in two tautomerics forms; enol and keto form. Under irradiation the enol form is photoisomerised to the keto form, which is absorbed in the UVC region from 260 to 280 nm (Georges et al., 2008). From my study it was observed that keto form under irradiation was increased (peak at 272 nm) and the enol form was decreasing (peak at 350 nm).

4.2.2 Suntest results

During the dark experiment any degradation of the sample was not noticed. Also it could be found that temperature had no affect on the sample degradation as well.
Samples of avobenzone in aqueous solutions were irradiated in the Suntest apparatus using different light intensities; 750, 500 and 250 W/m². Maximum irradiation time was three hours and every half-hour the sample was taken out. The temperature in the chamber ranged from 27.4 °C to 29.1 °C, only at the intensity of 750 W/m² was 10.9 °C. (Table 6)

Table 6: Parameters in Suntest apparatus at different intensities (750, 500, 250 W/m^2)

Parameters in Suntest apparatus	
Irradiance	250 W/m^2
Chamber temperature	27.4 °C
Black standard T	36.1 °C
Irradiance	500 W/m^2
Chamber temperature	29.1 °C
Black standard T	42.8 °C
Irradiance	750 W/m^2
Chamber temperature	10.9 °C
Black standard T	36.8 °C

Figure 22 shows degradation of avobenzone in aqueous solution at different light intensities. Avobenzone slight degradation was observed at 250 W/m². After three hours in Suntest the degradation was 3.33 ± 3.25 % of the compound. The next experiment was followed by irradiation with light intensity at 500 W/m². Here a gradual degradation of the compound was observed. After 90 minutes avobenzone decreased to 4.81 %. After three hours of exposure the substance stabilized at 4.84 ± 1.9 %. Finally, the light intensity of 750 W/m² was used and the observed substantial degradation that developed was quite differently than the other two intensities. Immediately after 30 minutes, the concentration increased quite rapidly. After three hours of irradiation 38.7 ± 9.9 % of degradation was observed.

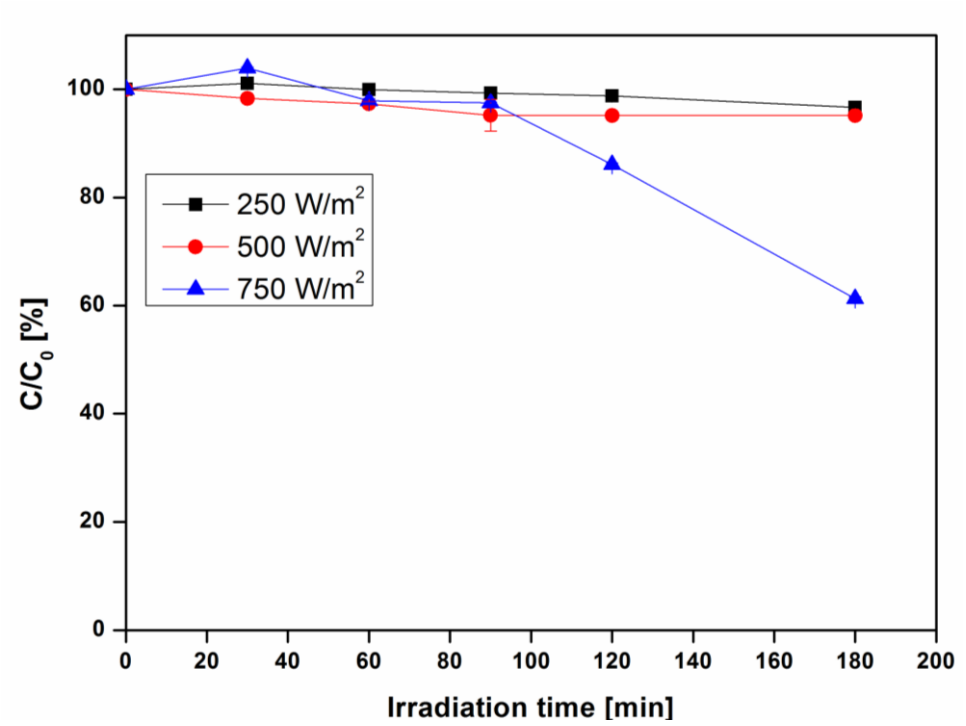


Figure 22: Evolution of avobenzone in aqueous solution under simulated sunlight intensities for 750 W/m^2, 500 W/m^2 and 250 W/m^2.

4.2.3 Toxicity testing

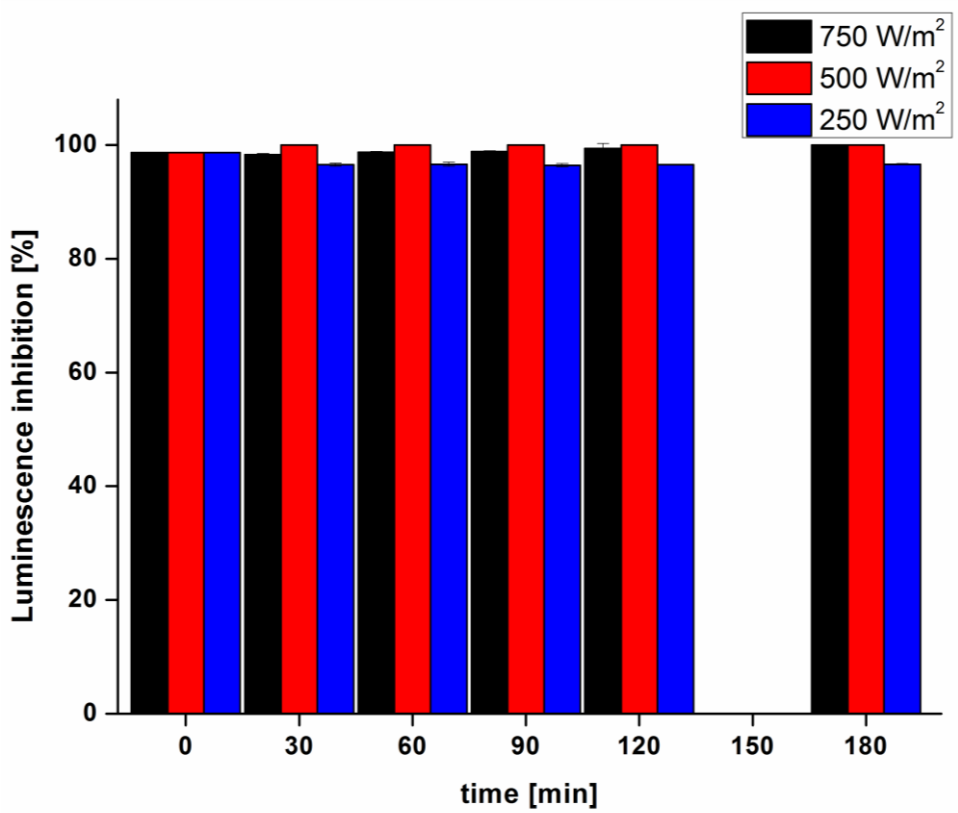


Figure 23: Evolution of luminescence inhibition for V. fisheri bacteria for avobenzone in aqueous solution under Suntest irradiation at different intensities; 750, 500 and 250 W/m2.

In order to understand if the light intensity can influence on the toxicity of the samples, we performed toxicity measurement of samples, irradiated at three different light intensities; 750, 500 and 250 W/m^2. At intervals; 30, 60, 90, 120 and 180 minutes the samples were taken out and analysed for toxicity with *V. fisheri* bacteria. From figure 23 it can be seen that at all three intensities (750, 500 and 250 W/m^2) the luminescence inhibition was very high for all samples. The samples which were irradiated with the intensity of 750 W/m^2 expressed the highest toxicity started with inhibition 98.6 % to 100 % after three hours of exposure. All samples that were exposed to 500 W/m^2 have shown 100 % luminescence inhibition which did not change during irradiation. However, the samples, which were exposed to 250 W/m^2 expressed a little bit lower inhibition from 98.6 to 96.54 %.

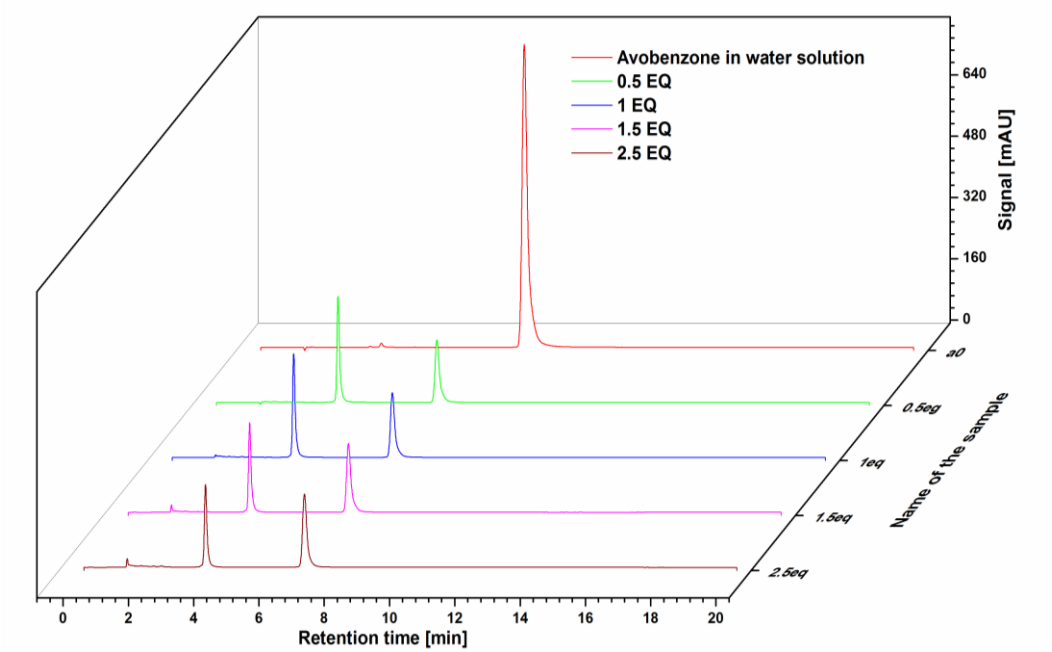
It could be concluded that light intensity does not influence the toxicity of the sample significantly. The initial luminescence inhibition was very high and remained such even after almost three hours of irradiation, irrespective of the intensity of light.

4.3 Stability study of avobenzone in chlorinated water

4.3.1. Equivalents of TCCA

The mixture of avobenzone and TCCA was monitored by HPLC-DAD analysis. We observed the formation of two new compounds eluting at retention times 3.6 min and 6.7 min, at all concentration of TCCA, added in the range 0.5-2.5 eq.

24a.)



24b.)

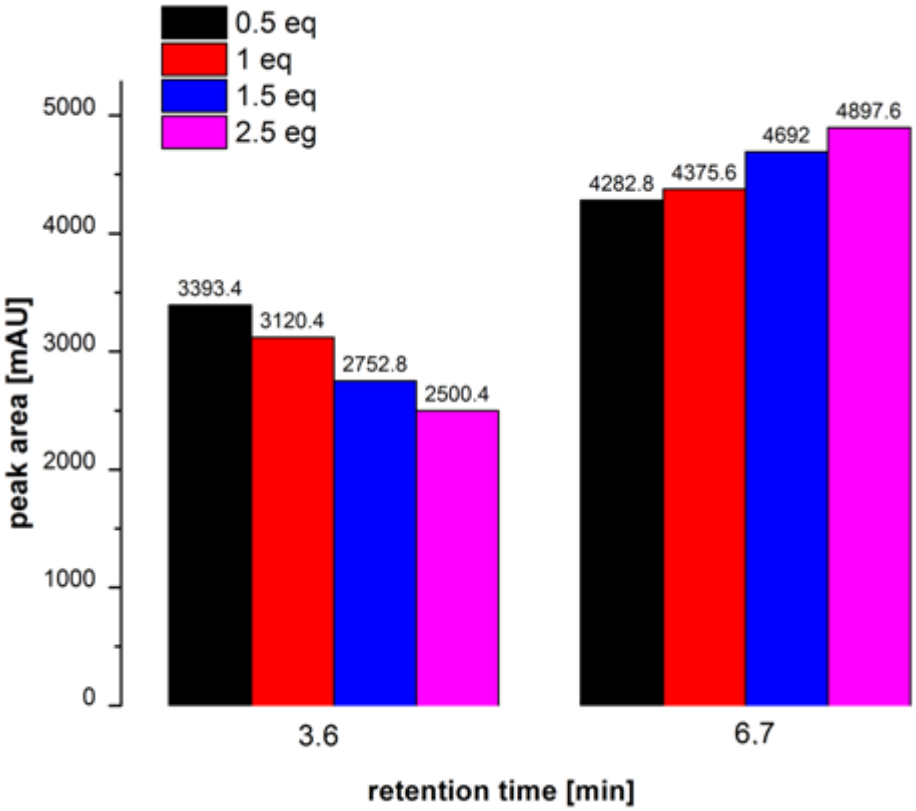


Figure 24: Peak areas of two products formed, monitored by HPLC-DAD regarding the addition of various amount of TCCA into the solution (0.5; 1; 1.5; 2.5 equivalent) (figure 24 a) and presented in columns (figure 24 b).

After the addition of TCCA into the solution two peaks were detected with the retention time of 3.6 and 6.7 min, while avobenzone was eluted at the retention time of 7.44 minutes. From the figure 24 it can be seen, that the product with the retention time of 3.6 minutes slowly decreased with addition of greater amount of TCCA. The other chlorinated product with the retention time of 6.7 minutes increased after additing chlorinating agent.

4.3.2 Evolution of avobenzone in chlorinated water after irradiation in Suntest apparatus

The sample of avobenzone in chlorinated water was also irradiated in the Suntest apparatus. Figure 25 shows the evolution of degradation of the UV filter and chlorinated products. Immediately after addition of TCCA addition two new chlorinated byproducts were detected, as presented in the previous section. At wavelength 272 nm two peaks were detected at the retention times of 3.6 and 6.7 minutes (figure 25).

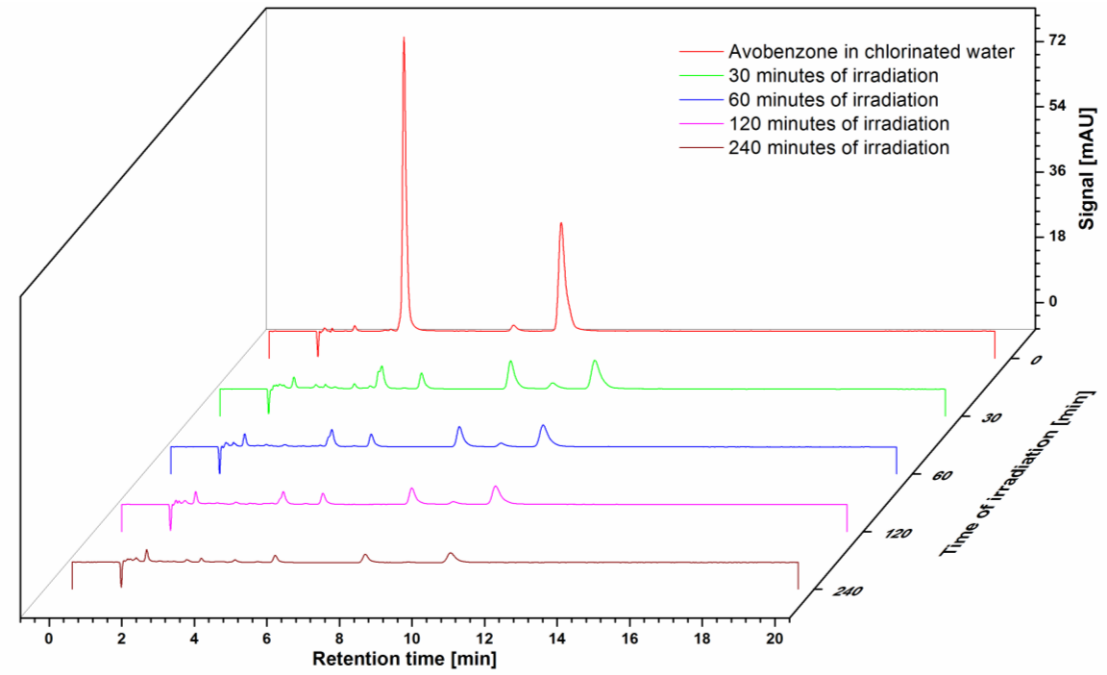


Figure 25: HPLC-DAD evolution of avobenzone in chlorinated water after four hours of exposure in the Suntest apparatus at an intensity 500 W/m^2.

This solution was immediately put into the Suntest apparatus and irradiated for four hours; samples were taken at different time intervals. Just after 30 minutes of irradiation many photoproducts were observed, formed with increasing irradiation time. The identification of photoproducts is still under investigation.

4.3.3 LC – MS results

In order to identify the main chlorinated products, it was used LC-MS. The final concentrations of avobenzone in chlorinated water that were injected was 50 mg/L, and the amount of added TCCA was in the range 0.5-2.5 equilibrium. After an hour Na_2SO_4 was added into the samples in order to stop chlorination procedure (Grbović et al., 2013). Sample preparation is described in Experimental work.

From figure 26 can be seen that LC-MS analysis revealed the formation of three avobenzone byproducts in chlorinated water. Two mono-chlorinated products were detected with m/z 345 and 185 and one di-chlorinated products with m/z 379.

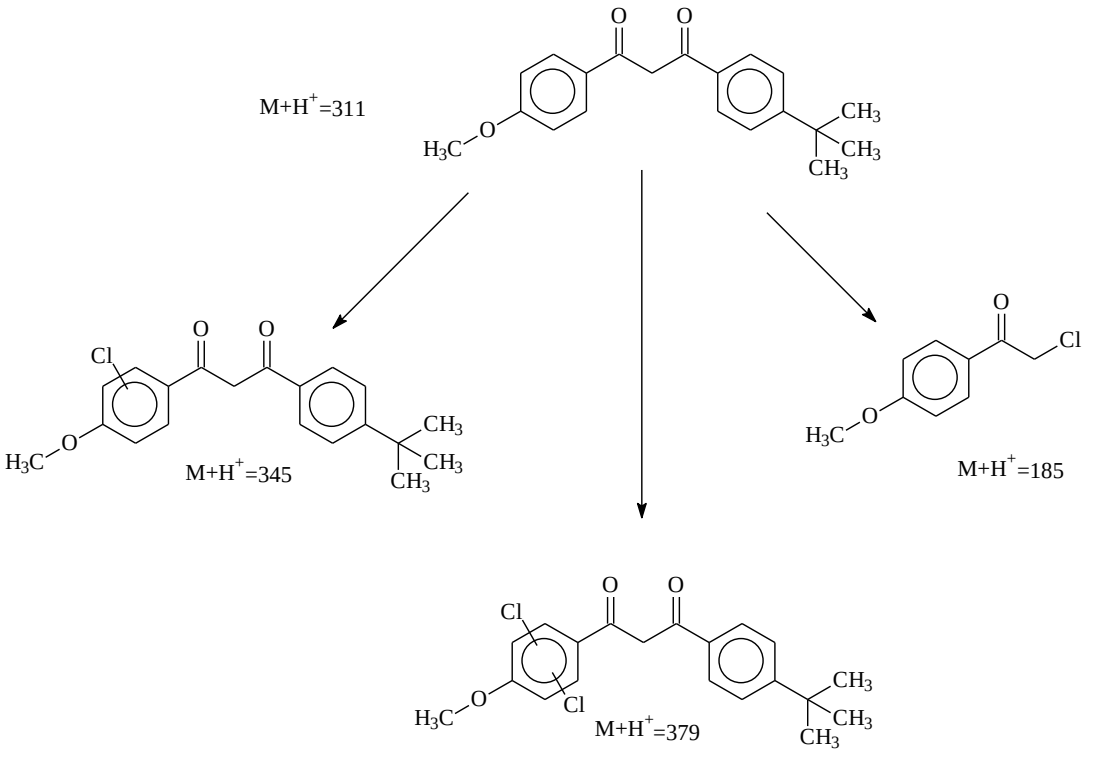
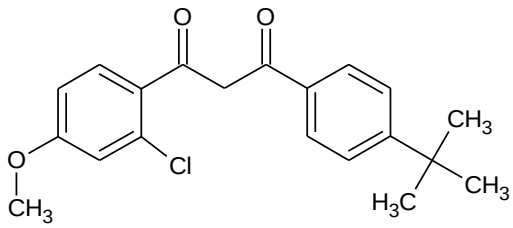


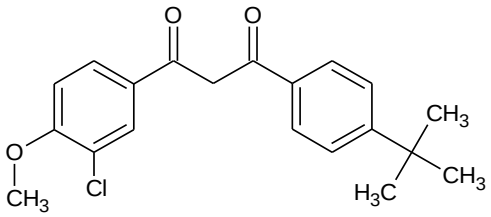
Figure 26: By-products of avobenzone analysed by LC–MS.

Possible structures of chlorinated products (positions of chloro atom in the molecule) are presented in the figure 28.

1.1

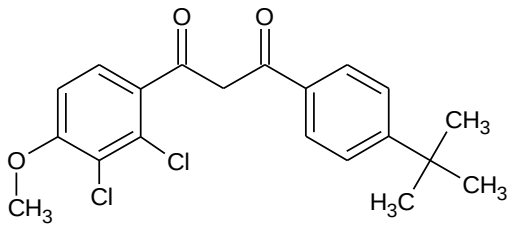


1-chloro-butylmethoxydibenzoylmethane;

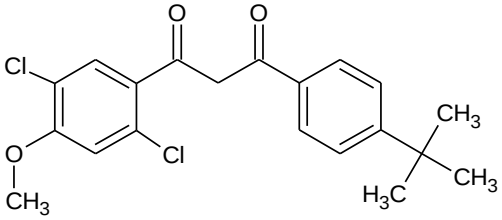


2-chloro-butylmethoxydibenzoylmethane;

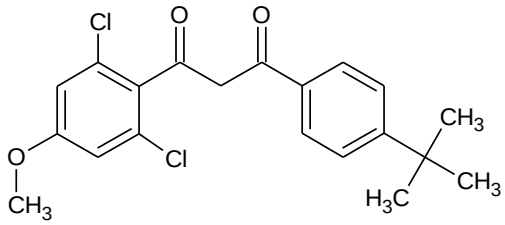
1.2



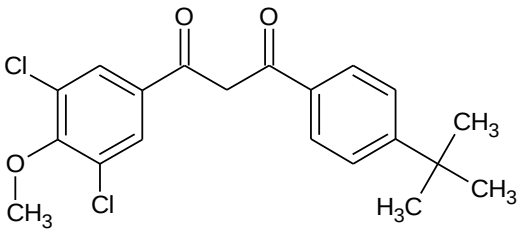
1.2-dichloro-butylmethoxydibenzoylmethane;



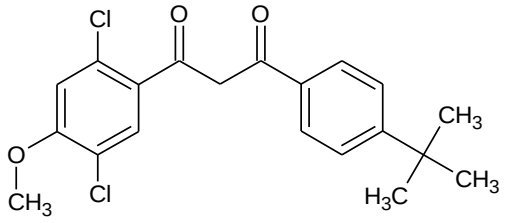
1.4-dichloro-butylmethoxydibenzoylmethane



1.5-dichloro-butylmethoxydibenzoylmethane;



2.4-dichloro-butylmethoxydibenzoylmethane



2.5-dichloro-butylmethoxydibenzoylmethane

Figure 27: Possible structures for avobenzone in chlorinated water; 1-chloro-butylmethoxydibenzoylmethane; 2-chloro-butylmethoxydibenzoylmethane; 1,2-dichloro-butylmethoxydibenzoylmethane;1,4-dichloro-butylmethoxydibenzoylmethane;1,5-dichloro-butylmethoxydibenzoy methane; 2,4-dichloro-butylmethoxydibenzoylmethane and 2,5-dichloro-butylmethoxydibenzoylmethane.

This chlorinated by product could be: 1-chloro-butylmethoxydibenzoylmethane or 2-chloro-butylmethoxydibenzoylmethane (Figure 27 (1.1)), which correspond to 345 m/z. The other di-chlorinated products with two chlorine ions and 379 m/z could be; 1.2-dichloro-butylmethoxydibenzoylmethane, 1.4-dichloro-butylmethoxydibenzoylmethane, 1.5-dichloro-butylmethoxydibenzoylmethane, 2.4-dichloro-butylmethoxydibenzoylmethane and 2.5-dichloro-butylmethoxydibenzoylmethane. A new compound has 68 amu greater that the compound of avobenzone (311 m/z), figure 27 (1.2).

5. CONCLUSION

Avobenzone was found to be a non stable product and its degradation was rapid when it was exposed to sunlight. After three hours in the Suntest apparatus 38.7 ± 9.9 % was degraded at the light intensity of 750 W/m^2. The samples were also irradiated in Suntest with two other light intensities; 250 and 500 W/m^2. At those two intensities I could observe lower degradation. After three hours of irradiation with the light intensity of 250 W/m^2 avobenzone was degraded for 3.33 ± 3.25 %, and with the light intensity 500 W/m^2, for 4.81 ± 118.05 %. During the irradiation the same sample was also placed on the counter and in the dark, under aluminium foil. Their absorbance was measured by a UV-Vis spectrophotometer, where it was observed near-zero changes in decomposition.

Toxicity of avobenzone in water is not declining over time with irradiation time. For the larger amount of avobenzone in aqueous solution (125 mg/L) it could be seen that the main portion of degradation occured in the first one hour. In the fourth hour toxicity of the sample increaseed to 97.6 % of luminescence inhibition. When chlorine was added to the sample its toxicity increased to 99.61 %. EC50 value for avobenzone was 28 mg/L.

In case of lower quantities of avobenzon in irradiated sample (1.25 mg/L avobenzone in aqueous solution) toxicity reached a value of 53.1 %. During exposure luminescence inhibition varied in the first two hours from 45.4 to 54.1 %. After that time, it gradually increased until it reached a steady state at 57.1 % of inhibition. In the presence of chlorine the luminescence inhibition was relatively higher (69.3 %), but after irradiation in the Suntest apparatus, the sample exhibited greater luminescence inhibition, 91.1 %.

Avobenzone in chlorinated water was also analysed by the HPLC-DAD instrument and LC-MS. Immediately after the addition of TCCA new chlorinated byproducts were detected. At wavelength 272 nm peaks at the retention times 3.6 and 6.7 minutes were detected. With LC-MS two mono-chlorinated products were detected with m/z 345 and 185, as well as one di-chlorinated product with m/z 379. The real structure (position of chlorination) is still under investigation. Longer irradiation time (four hour) lead to the decomposition of chlorinated products to unknown products.

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ANNEX

Table 1: Luminescence inhibition of the samples 1.25 mg/L avobenzone in chlorinated water (4 mg/L)

SAMPLE	LUMINESCENCE INHIBITION (1)	LUMINECSENCE INHIBITION (2)	LUMINESCENCE INHIBITION (%) (3)	AVERAGE	STDEV
H0	68.62	70.03	/	69.325	0.997021
H30	88.60	88.11	82.11	86.27333	3.613867
H60	92.25	90.67	87.93	90.28333	2.185803
H120	90.35	93.01	/	91.68	1.880904
H240	92.42	92.70	88.17	91.09667	2.538431

Table 2: Luminescence inhibition of the samples 125 mg/L avobenzone in aqueous solution

Sample	Luminescence inhibition (%) 1	Luminescence inhibition (%) 2	Luminescence inhibition (%) 3	AVERAGE	STDEV
1% acetonitrile	**0**	**0**	**0**	**0**	**0**
0,1% acetonitrile	**0**	**0**	**0**	**0**	**0**
A0	97.2	96.7	97.02	96.97333	0.253246
A30	90.56	90.44	90.87	90.62333	0.221886
A60	84.51	85.81	87.43	85.91667	1.462919
A120	92.9	93.1	92.54	92.84667	0.283784
A240	97.56	97.7	97.43	97.563333	0.135031

Table 3: Luminescence inhibition of the samples 125 mg/L avobenzone in chlorinated water (4 mg/L)

Sample	Luminescence inhibition (%) 1	Luminescence inhibition (%) 2	Luminescence inhibition (%) 3	AVERAGE	STDEV
B0	99.61	99.6	99.62	99.61	0.01
B30	98.73	98.99	99.27	98.99667	0.270062
B60	98.67	98.78	98.83	98.76	0.081854
B120	98.21	96.32	96.56	97.03	1.028931
B240	97.24	96.31	95.52	96.35667	0.860949

Table 4: Luminescence inhibition of the samples 1.25 mg avobenzone/L water

SAMPLE	LUMINESCENCE INHIBITION (%) (1)	LUMINESCENCE INHIBITION (%) (2)	LUMINESCENCE INHIBITION (%) (3)	AVERAGE	STDEV
G0	52.43	53.74	52.90	53.085	2.02233
G30	44.02	46.88	52.55	45.45	4.34146
G60	52.42	51.44	55.68	53.18	2.21982
G120	45.82	45.41	57.80	45.615	0.28991
G240	57.81	56.37	55.96	56.71333	0.97161

Table 5: Inhibition of luminescence in V. Fisheri bacteria for 125 mg avobenzone/L water.

Concentration (mg/L)	Luminescence inhibition (%)
125	58.22
83.3	59.56
62.5	58.94
41.6	53.29
31.3	51.79
20.8	45.16
15.6	39.44
10.4	36.11
7.80	27.14

Table 6: Inhibition of luminescence in V. Fisheri bacteria for 4 mg/L chlorinated water.

CONCENTRATION (mg/L)	Luminecsence inhibition [%]
4	64.04
2.67	14.19
1.33	18.9
1	13.46
0.66	10.57
0.50	4.31
0.33	9.23
0.25	6.88
0.17	6.38

Table 7: Sample A- AVOBENZONE IN WATER (125 mg/L) – SUNTEST

Sample	Start pH	Final pH
A0	6.30	6.81
A30	6.31	6.80
A60	4.96	7.02
A120	4.85	6.96
A240	4.88	7.01

Table 8: Sample B- AVOBENZONE IN CHLORINATED WATER (125 mg/L avobenzone + 4 mg/L TCCA) – SUNTEST

Sample	Start PH	Final PH
B0	6.25	7.01
B30	5.35	7.03
B60	4.79	6.91
B120	4.14	7.00
B240	4.11	7.19

Table 9: Sample E- AVOBENZONE IN CHLORINATED WATER (125 mg/L avobenzone + 4 mg/L TCCA) – on the counter

Sample	Start PH	Final PH
E120	4.50	6.92

Table 10: Sample G - AVOBENZONE IN WATER (1.25 mg/L avobenzone) - SUNTEST

Sample	Start PH	Final PH
G0	7.19	6.85
G30	7.30	6.81
G60	6.70	6.89
G120	6.13	7.03
G240	6.54	7.01

Table 11: Sample H – AVOBENZONE IN CHLORINATED WATER (1.25 mg/L avobenzone + 4 mg/L TCCA) - SUNTEST

Sample	Start PH	Final PH
H0	6.80	7.14
H30	5.70	6.85
H60	4.96	7.14
H120	5.49	7.04
H240	5.59	6.97

Table 12: Results of equivalents

Sample	329 nm		250 nm	
	3.6 min	**6.7 min**	**3.6**	**6.7 min**
2.5 eq	186.5	1284.2	2500.4	4897.6
1.5 eq	205.8	1229.1	2752.8	4692.0
1 eq	233.6	1145.9	3120.4	4375.6
0.5 eq	235.6	1125.6	3393.4	4282.6

Table 13: Spectrophotometer results – Avobenzone in aqueous solution after irradiation in Suntest

Intensity 250 W/m^2		
Sample	**Absorbance**	
	358 nm	**272 nm**
Avobenzone in agueous solution withouth irradiation	2.25990	0.77432
30 min	2.24930	0.79236
60 min	2.10310	0.88514
90 min	2.00400	0.91564
120 min	2.02950	0.97973
180 min	1.99780	0.98769
Intensity 500 W/m^2		
30 min	1.99650	0.95462
60 min	1.92050	0.96559
90 min	1.90710	0.98138
120 min	1.8865	1.00399
180 min	1.74080	1.03990
Intensity 750 W/m^2		
30 min	1.99420	0.96697
60 min	1.93840	1.01140
90 min	1.83530	1.01050
120 min	1.69240	1.05760
180 min	1.19970	1.09580
BLANK		
30 min	2.25680	0.78160
60 min	2.15610	0.81147
90 min	2.08080	0.86866
120 min	2.12130	0.94251
240 min	2.09580	0.92929

Table 14: HPLC results – Avobenzone in aqueous solution after irradiation in Suntest

Intensity 250 W/m^2					
Sample	**Peak area 1**	**Peak area 2**	**average**	**STDEV**	**Loss of absorbtion**
Without irradiation	5912.1	5903.1	5907.6	6.4	
30 min	5979.7	5963.2	5971.45	11.7	
60 min	5942.7	5867.4	5905.5	53.2	0.03 %
90 min	5867.3	5867.4	5867.35	0.1	0.68 %
120 min	5834.2	5833.7	5833.95	0.3	1.24 %
180 min	5708.8	5713.4	5711.1	3.3	3.33 %
Intensity 500 W/m^2					
30 min	5800.2	5816.9	5808.55	8.35	1.68 %
60 min	5739.9	5760.1	5750	10.1	2.67 %
90 min	5505.5	5741.6	5623.5	118.05	4.81 %
120 min	5620	5623.8	5621.9	1.9	4.84 %
180 min	5620	5623.8	5621.9	1.9	4.84 %
Intensity 750 W/m^2					
30 min	6134.1	6144.4	6139.25	5.15	
60 min	5772.5	5793	5782.75	10.5	2.11 %
90 min	5756.9	5764.9	5760.9	4	2.48 %
120 min	5080	5091.8	5085.9	5.9	13.9 %
180 min	3612.2	3632	3622.1	9.9	38.69 %

Table 15: pH results – Avobenzone in aqueous solution after irradiation in Suntest

AFTER IRRADIATION IN SUNTEST APPARATUS – PH RESULTS		
Intensity 250 W/m^2	pH $_{initial}$	pH $_{final}$
30 minutes	6.05	7.02
60 minutes	5.95	6.99
90 minutes	4.81	7.01
120 minutes	5.17	6.88
180 minutes	4.80	6.81
Intensity 500 W/m^2		
30 minutes	4.64	6.88
60 minutes	4.66	7.10
90 minutes	4.65	7.13
120 minutes	4.44	6.85
180 minutes	4.50	7.01
Intensity 750 W/m^2		
30 minutes	6.49	7.13
60 minutes	5.01	7.01
90 minutes	4.52	6.89
120 minutes	4.68	6.82
180 minutes	4.47	6.80
BLANK		

30 minutes	7.17	7.17
60 minutes	6.77	7.13
90 minutes	7.20	7.13
120 minutes	7.17	7.17
180 minutes	6.65	6.91

Table 16: Spectrophotometer results – Avobenzone in aqueous solution after irradiation in Suntes

AFTER IRRADIATION IN SUNTEST APPARATUS – LUMISTOX RESULTS Intensity 750 W/m^2				
Sample	**Luminescence inhibition (%)**	**Luminescence inhibition (%)**	**AVERAGE**	**STDEV**
0	98.67	98.68	98.675	0.0071
30	98.17	98.46	98.315	0.205061
60	98.62	98.8	98.71	0.127279
90	98.78	98.92	98.85	0.098995
120	98.8	100.01	99.405	0.855599
180	100.02	99.99	100.005	0.021213
Intensity 500 W/m^2				
30	100.01	100	100.005	0.007071
60	100.01	100	100.005	0.007071
90	100	100.01	100.005	0.007071
120	100.02	100.01	100.015	0.007071
240	100.02	100	100.01	0.014142
Intensity 250 W/m^2				
30	96.73	96.35	96.54	0.268701
60	96.34	96.89	96.615	0.388909
90	96.67	96.24	96.455	0.304056
120	96.6	96.51	96.555	0.06364
240	96.7	96.55	96.625	0.106066